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A Yeast Mutation That Stabilizes a Plasmid Bearing a Mutated *ARS1* Element

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To identify the *trans*-acting factors involved in autonomously replicating sequence (ARS) function, we initiated a screen for *Saccharomyces cerevisiae* mutants capable of stabilizing a plasmid that contains a defective ARS element. The *amm* (altered minichromosome maintenance) mutations recovered in this screen defined at least four complementation groups. *amm1*, a mutation that has been studied in detail, gave rise to a 17-fold stabilization of one defective *ARS1* plasmid over the level seen in wild-type cells. The mutation also affected the stability of at least one plasmid bearing a wild-type ARS element. *amm1* is an allele of the previously identified *TUP1* gene and exhibited the same pleiotropic phenotypes as other *tup1* mutants. Plasmid maintenance was also affected in strains bearing a *TUP1* gene disruption. Like the *amm1* mutant, the *tup1* disruption mutant exhibited ARS-specific plasmid stabilization; however, the ARS specificities of these two mutants differed. The recovery of second-site mutations that suppressed many of the *tup1* phenotypes but not the increased plasmid maintenance demonstrates that the plasmid stability phenotype of *tup1* mutants is not a consequence of the other defects caused by *tup1*.

DNA replication in the yeast *Saccharomyces cerevisiae*, like that in higher eucaryotes, occurs by initiation at many origins along each chromosome (21, 24). Specific chromosomal sequences have been identified that have many properties expected of an origin of replication (for a review, see reference 12). These autonomously replicating sequence (ARS) elements act in *cis* and allow plasmids containing them to be maintained extrachromosomally in the yeast nucleus. Recent experiments using two-dimensional gel electrophoresis have shown that replication initiates at or near the plasmid ARS (1, 10). It seems likely, therefore, that ARS elements act as replication origins in yeast chromosomes.

cis-acting sequences required for proper ARS function on plasmids have been the subject of many studies (12, 23). However, except for the identification of a few proteins that bind to conserved sequences within ARS elements (4, 31), little is known about the *trans*-acting factors involved in the initiation and control of DNA replication in yeast cells. Several laboratories have screened for yeast mutants that affect plasmid maintenance in the hope of identifying proteins that interact with ARS elements (13, 17, 30). Such mutants have been identified, but it is not known whether the mutations are in genes that code for proteins that bind to the ARS sequences on the plasmids.

Plasmids that contain both a yeast centromere and an ARS element have high mitotic stability in yeast cells (5). Insertion mutations within *ARS1* have been constructed such that plasmids containing these mutated *ARS1* elements are mitotically unstable, exhibiting up to 20-fold increases in loss rate compared with control plasmids that have not been mutated (14). The elevated loss rates, as determined by colony sectoring, are due to an increase in 1:0 plasmid segregation events. This pattern of plasmid loss is consistent with a defect in plasmid replication (14). As a means of identifying gene products involved in the initiation of replication, we used these mitotically unstable plasmids to screen for yeast mutants that suppress the ARS defect and stabilize the plasmids. This approach is similar to one used to identify simian virus 40 gene products that interact with the virus origin (29). The simian virus 40 study showed that origin defects can be suppressed by mutations in the T antigen, a known origin-binding protein. Experiments using bacteriophage f1 have shown that second-site mutations that resulted in either quantitative or qualitative changes in proteins suppressed insertions of 800 base pairs (bp) of DNA into the essential f1 origin (6, 7). These *trans*-acting mutations also suppressed deletions of 100 bp of the 140-bp phage origin. Screens for yeast mutants that affect the maintenance of yeast minichromosomes have been undertaken in other laboratories (13, 17, 30), and although mutants have been identified, it is not known whether the affected proteins interact directly with yeast ARS elements. In this paper, we summarize the results of our screen for mutants and describe in detail the characterization of one of the mutations that was identified.

MATERIALS AND METHODS

Bacterial and yeast strains. *Escherichia coli* JA194 was used for the propagation of plasmids. The yeast strains used in this study are listed in Table 1. Haploid strain 4795-408 and diploid strain CTY1 are congenic and were used to generate plasmid-stabilizing mutants. Strain 40-4c is a haploid segregant from the third backcross between the original *amm1* mutant isolate and strain 4795-303. Strain 40-4c was used for quantitative plasmid stability determinations. Strain *TUP1*::Tn*3-URA3*, which contains a *TUP1* gene disruption, was constructed by transplacement of a linear fragment containing the gene disruption into wild-type strain 4795-408 (described below). Standard methods were used for the growth and manipulation of yeast strains (28). Plasmids were introduced into yeast strains via transformation (11, 28) or crosses to *kar1* strains that contain the plasmid (14).

Plasmids. Plasmids pDK217, pDK221, pDK223, pDK243, and pDK282 (14) and plasmid pDK239 were provided by D. Koshland. pDK217, pDK221, and pDK223 contain mutationally debilitated *ARS1* elements and were used in the screen to identify yeast mutants with increased plasmid maintenance. pDK243 contains the intact *ARS1* element,

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
4795-303	*MATα leu2 trp1 his7 ade2 ade3 can1*	L. Hartwell
4795-408	*MATa leu2 ura3 his7 ade2 ade3 can1*	L. Hartwell
CTY1	*MATa/α leu2/leu2 trp1/+ ura3/+ his7/his7 ade2/ade2 ade3/ade3 can1/can1*	This work
40-4c	*MATa leu2 ura3 his7 ade2 ade3 can1 amm1*	This work
TUP1::Tn*3-URA3*	*MATa leu2 ura3 his7 ade2 ade3 can1 tup1*$^-$	This work
BS212	*MATα kar1 ade1 ade2 trp1 ura3 leu2*	L. Hartwell
BS213	*MATa kar1 his7 ura3 leu2*	L. Hartwell
CTY8	*MATa/α leu2/leu2 ura3/ura3 his7/his7 ade2/ade2 ade3/ade3 can1/can1 TUP1*::Tn*3-URA3/+*	This work
CTY9	*MATa/α leu2/leu2 trp1/+ ura3/+ his7/his7 ade2/ade2 ade3/ade3 can1/can1 TUP1*::Tn*3-URA3/amm1*	This work
CTY10	*MATa/α leu2/leu2 trp1/+ his7/his7 ade2/ade2 ade3/ade3 can1/can1 amm1/+*	This work
CTY11	*MATa/α leu2/leu2 ura3/+ his7/his7 ade2/ade2 ade3/ade3 can1/can1 amm1/amm1*	This work

and pDK282 contains the *ARS2* element. pDK239 was derived from YCp41 (9) by the removal of the 0.9-kilobase (kb) *Hin*dIII-*Eco*RI *ARS1* fragment, followed by filling in and ligating the free ends. pDK239 plasmids containing the chromosome III ARS elements C2G, J11D, and A6C (23) were made by ligating *Sal*I linkers onto the ARS fragments of interest and then inserting these fragments into a single *Sal*I site in pDK239. The ARS fragments are as follows: for A6C, a 1.2-kb *Bam*HI-*Cla*I fragment; for J11D, a 1.0-kb *Sal*I-*Eco*RI fragment; and for C2G, a 3.4-kb *Eco*RI fragment. These fragments were excised from plasmids provided by C. Newlon. pDK239-*ARS1* was constructed as described above by using a 1.4-kb *Eco*RI fragment containing *ARS1-TRP1* (33). YEp24 and CV13 have been described (2, 3). Plasmid YIp5-51 was made by ligating *Eco*RI linkers onto the 1.1-kb *Bam*HI-*Eco*RI *CEN5* fragment (18) and inserting it into the *Eco*RI site of YIp5 (33). *Sal*I linkers were added onto the 1.4-kb *Eco*RI *TRP1-ARS1* fragment, which was subsequently ligated into the *Sal*I site of the plasmid. Plasmid pFW10, provided by V. MacKay, F. E. Williams, and R. Trumbly, contains the *TUP1*::Tn*3-URA3* gene disruption.

Screen for plasmid-stabilizing mutants. The haploid yeast strain 4795-408 (*leu2 ade2 ade3*) was mutagenized with ethyl methanesulfonate to 40% viability, as described previously (28). Mutagenized cells diluted into YEPD medium were grown to the log phase and transformed with the *LEU2 ade3-2*p *ARS1*-defective plasmids pDK217, pDK221, and pDK223. Leu$^+$ transformants were purified and then streaked on nonselective YEPD plates at 23, 30, and 36°C. The plates were incubated until the pink-white colony sectoring pattern that arises as cells lose plasmids was clearly visible under a stereo microscope. Mutagenized cells that gave rise to colonies with fewer white sectors than the unmutagenized parent strain were examined further.

Scoring of phenotypic properties. The *amm1* mutant was crossed to strain 4795-303, and the resulting diploids were sporulated and dissected by standard procedures (19). The plasmid-stabilizing mutation was scored by visual examination of spore colonies. Whenever possible, diploid cells from red colonies were sporulated, since cells from such colonies contained two or more copies of the defective *ARS1* plasmid. When sporulated, these diploids could give rise to tetrads in which all four spores contained the plasmid. However, most of the tetrads arising from such diploids contained haploid segregants that did not receive the plasmid. To introduce a plasmid into these haploids, cells were either transformed with the plasmid or mated to *kar1-1* strains containing the plasmid of interest. When the latter method was used, cytoductants containing the desired haploid nucleus harboring the plasmid acquired from the *kar1-1* strain were selected by using appropriate synthetic media supplemented with canavanine to select against rare diploids and haploid cytoductants containing *kar1* nuclei. Tetrads were then streaked on YEPD plates and grown at 30°C to analyze plasmid stabilization.

The ability of strains to grow on nonfermentable carbon sources was determined by streaking cells on YPG plates that contained glycerol as a sole carbon source (28), followed by incubation at 34°C. After a 3-day incubation, it was easy to distinguish between the wild-type strains and the poorly growing mutant strains by comparing colony sizes.

The presence of *tup1* mutant phenotypes was scored as follows. Clumping of mutant cells was determined by growth in liquid culture; the presence of large clumps of yeast cells in dilute cultures is easily observable by eye. Production of shmoos (8) was scored by microscopic examination of cells. Uptake of dTMP was scored by the ability of strains to grow on plates containing 100 μg of dTMP per ml, 50 μg of aminopterin per ml, and 6 mg of sulfanilamide per ml (35). Mating ability was determined by a patch test. Cells to be tested were patched with a wild-type strain on YEPD plates. The plates were incubated at 30°C overnight and then replica plated to select for diploids. Confluent growth of the replica-plated cells was scored as mating positive, no growth was scored as mating negative, and the appearance of a few isolated colonies growing in a mating patch was scored as poor mating.

Isolation of revertants. Independent *MATα amm1* and *tup1* mutant cultures were enriched for cells with reduced clumping by passaging cells through liquid medium until cultures became less flocculent (16). The cultures were then streaked for single colonies, and plates were screened for cells that produced colonies with normal morphologies, indicative of reversion of the shmoo phenotype. These colonies were scored for the presence of other mutant phenotypes. The majority of *tup1* mutant cultures did not yield nonshmoo revertants. Therefore, plates were screened for colonies that did not clump when resuspended in liquid medium, and these were chosen for further analysis.

Quantitative plasmid stability assays. For plasmid stability determinations, a single selectively grown colony was resuspended in 5 ml of YEPD to give an initial cell density of approximately 10^5 cells per ml. The cells were then grown for 8 to 10 generations at 23°C until they reached the stationary phase. Samples of cells were taken prior to and after nonselective growth. These cells were diluted and plated on YEPD at 23°C to determine the cell densities of the starting and final cultures. No fewer than 200 colonies from each time point were scored for the presence of the plasmid. The presence of *ADE3* plasmids was determined by visual examination of colony color. Strains bearing all other plas-

mids were replica plated to selective-medium plates. The rate of plasmid loss was calculated by using the formula $X = 1 - (f/i)^{1/n}$, where X is the proportion of cells that lost the plasmid in each generation, i is the fraction of plasmid-containing cells in the initial culture, f is the fraction in the final culture after nonselective growth, and n is the number of generations of nonselective growth. Each plasmid loss value presented in Tables 3, 5, and 6 is the mean and standard deviation for four independent transformants. Fold effect values presented in Tables 3 and 6 are for those plasmids for which stability data for the wild-type and mutant strains were found to be significantly different at a 99% confidence limit by single-classification analysis of variance (software provided by F. J. Rohlf).

RESULTS

Identification of mutants. Plasmids with a mutationally debilitated ARS element are mitotically unstable in yeast cells (12). Three such plasmids, pDK217, pDK221, and pDK223, constructed by D. Koshland contain small deletions and *Bam*HI-linker insertions in domain C of the *ARS1* element (14). The *ARS1* mutations in plasmids pDK221 and pDK223 are deletions of less than 30 bp approximately 200 and 250 bp, respectively, from the *Bgl*II site (14). pDK217 contains a deletion of approximately 70 bp in domain C, 240 bp from the *Bgl*II site. These *ARS1* mutations increase plasmid loss from 1% per cell per generation to 5% (pDK217), 19% (pDK221), and 22% (pDK223) per cell per generation presumably because of a failure to initiate replication (14). Plasmid loss can be monitored by a colony color assay that employs properties of mutations in the adenine biosynthetic pathway (25). Wild-type yeast strains form white colonies on YEPD plates. Mutations in the *ADE2* gene (*ade2*) lead to the accumulation of a red pigment. Mutations in the *ADE3* gene (*ade3*) are epistatic to those in *ADE2* such that *ade2 ade3* mutants give rise to white colonies. When *ade2 ade3* strains are transformed with plasmids pDK217, pDK221, and pDK223, which carry the partially functional *ade3-2*p allele, they give rise to pink colonies if they contain one copy of the plasmid and red colonies if they contain two or more copies (14). Loss of the plasmid during nonselective growth of a colony results in the formation of white sectors. pDK217, pDK221, and pDK223 are normally lost at high frequencies and produce colonies with large numbers of white sectors. This visual assay was originally used to identify the defective *ARS1* plasmids used in this study and is sensitive enough to allow plasmids with only twofold differences in their loss rates to be distinguished (14). We used this assay to screen for yeast cells with *trans*-acting mutations that suppress the *ARS1* mutations present in plasmids pDK217, pDK221, and pDK223, as determined by their ability to produce colonies with fewer white sectors.

Haploid yeast strain 4795-408 was treated with the mutagen ethyl methanesulfonate and then transformed with plasmid pDK217, pDK221, or pDK223 (described in Materials and Methods). Transformants were streaked on nonselective YEPD plates to allow plasmid loss and then screened for a decrease in white sectoring, which would signal a possible plasmid-stabilizing mutation. Twenty mutants were identified, and genetic analysis showed that for six of the mutants the increased plasmid maintenance was due to a single nuclear mutation. The majority of mutants were recovered in screens with pDK217 and pDK221. Candidates were not examined further if plasmid stabilization could not be clearly attributed to a single nuclear mutation after three backcrosses. The mutations that altered minichromosome maintenance (*amm*) defined at least four genes as determined from complementation analysis and mapping data (Table 2).

TABLE 2. Complementation groups

Mutation[a]	No. of isolates	Dominant (D) or recessive (R)	Plasmid used in isolation
amm1	1	R	pDK221
amm2	1	D	pDK221
amm3	3	R	2-pDK217 1-pDK221
amm4	1	R	pDK217

[a] All mutations were isolated in haploids.

***amm1* mutant.** The *amm1* mutant was identified in a screen of mutagenized cells transformed with plasmid pDK221. Plasmid pDK221 was lost at a rate of 19.0% per cell per generation in wild-type 4795-408 cells but at a rate of only 1.1% per cell per generation in *amm1* mutant strains. This increase in plasmid stability was clearly evident in the visual colony color assay (Fig. 1). When the *amm1* mutant was cured of the plasmid and retransformed with pDK221, the stable plasmid phenotype was present in the retransformed cells, indicating that the increased stability was not due to an alteration in the original plasmid.

Compared with wild-type cells, the *amm1* mutant grew normally at temperatures ranging from 17 to 36°C on YEPD plates and on a complete synthetic medium. However, *amm1* mutant strains displayed temperature-sensitive growth on medium containing glycerol as the sole carbon source (Gly^{ts}). While the growth of mutant strains in YPG at 23°C was comparable to that of the wild type growth at 34°C was 33% slower than that of the wild type. This reduced growth in glycerol medium at 34°C, however, was not due to an increased production of [rho^-] cells in the *amm1* strains at elevated temperatures (data not shown).

ARS specificity of an *amm1* mutant. To determine whether the *amm1* mutation was specific for particular ARS elements, we measured the loss rates of plasmids containing various ARS elements. The first plasmids examined were three *ARS1*-defective plasmids, pΔK217, pDK221 and pDK223, which contain lesions in domain C of the ARS, and pDK217, and the $ARS1^+$ parent plasmid, pDK243, from which the mutant plasmids were derived. The loss rates of these plasmids in wild-type and *amm1* mutant cells are shown in Table 3. All three of the mutant *ARS1* plasmids were stabilized in the *amm1* mutant, the greatest effect being a 17-fold stabilization of plasmid pDK221 to a loss rate of only 1.1% per cell per generation. This loss rate was comparable to that of the $ARS1^+$ parent plasmid pDK243 in the wild-type yeast strain. However, plasmids pDK217 and pDK223 were stabilized only four and sixfold, respectively, and neither plasmid was as stable as the wild-type control plasmid in wild-type cells. This suggests a possible DNA sequence specificity in the stabilization of the mutant *ARS1* plasmids. Increased plasmid stability was not due to an increase in plasmid copy number. Cells containing one copy of plasmid pDK217, pDK221, or pDK223 form pink colonies due to the presence of the *ade3-2*p allele. Two or more copies of the plasmid in a cell is evidenced by the formation of a red colony. Visual analysis of *amm1* mutant colonies containing these plasmids showed no increase in the number of red colonies compared with colonies from wild-type cells.

The $ARS1^+$ plasmid pDK243 may have been slightly less stable in the *amm1* mutant strain than in the wild-type

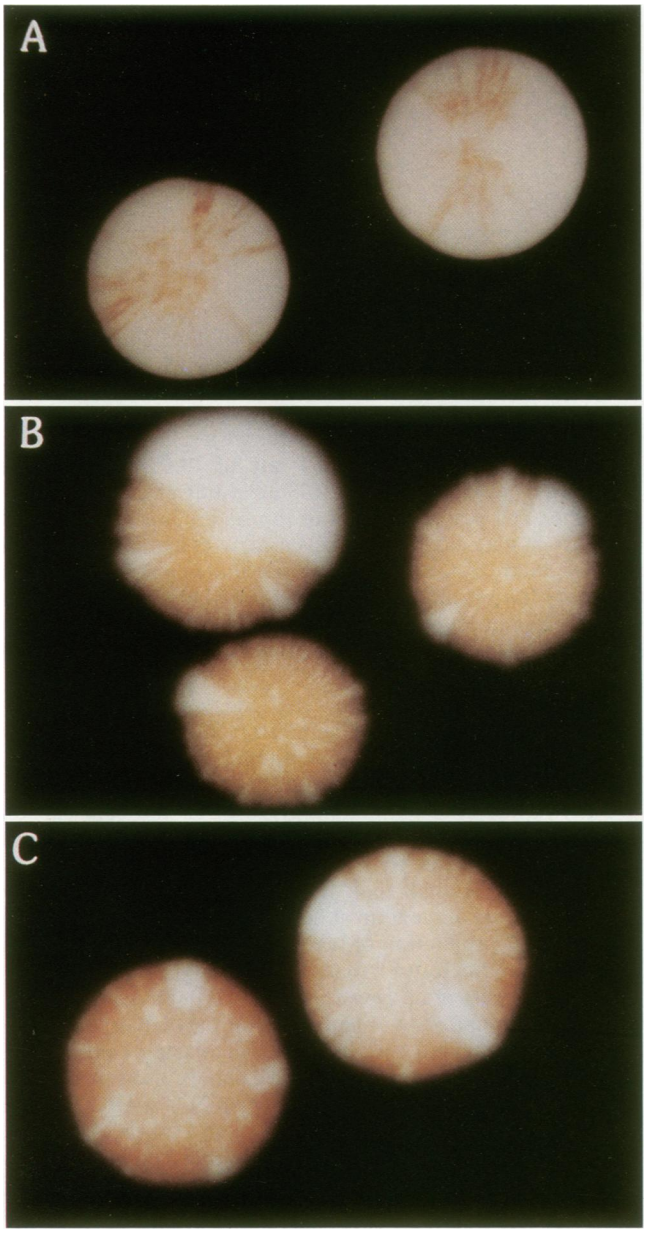

FIG. 1. Stabilization of *ARS1*$^-$ plasmid pDK221 in the *amm1* mutant. Wild-type strain 4795-408 and *amm1* mutant strain 40-4c were transformed with the plasmids of interest. Transformants were streaked on YEPD plates and incubated at 30°C for 3 days. The plates were then placed at room temperature until the red pigment developed and the sectoring pattern which arises as cells lose the plasmid was clearly visible. The data shown are for 4795-408 (a) and 40-4c (b) transformed with pDK221. The rates of loss of the plasmid in these strains were 19.0% per cell per generation and 1.1% per generation, respectively. Shown for comparison is 4795-408 transformed with the *ARS1*$^+$ parent plasmid pDK243 (c), which was lost at a rate of 1.2% per generation.

control strain (Table 3). However, comparison of the loss rates of two unrelated *ARS1*$^+$ plasmids, YIp5-51 and pDK239-*ARS1*, showed that there was no significant difference in plasmid stabilities between the wild-type and *amm1* mutant strains. We therefore conclude that the *amm1* mutation had little or no effect on plasmids containing an unmutagenized *ARS1* element. The small difference in the stability of pDK243 in wild-type and *amm1* mutant strains might have reflected a plasmid context effect that altered the chromatin conformation of the ARS sequences in the plasmid.

The stabilities of several plasmids containing other ARS elements were also examined (Table 3). The loss rates of YEp24 and CV13, plasmids that contain the 2μm ARS element, were not significantly affected by the *amm1* mutation. A plasmid with the *ARS2* element, pDK282, may have been slightly more stable in the *amm1* mutant strain than in the wild-type control strain; however, the effect was small.

A comparison of the stabilities of plasmids bearing three different chromosome III ARS elements indicated that ARS elements isolated from a single chromosome responded differently to the *amm1* mutation, demonstrating that the *amm1* mutation was ARS specific. Plasmids pDK239-J11D, containing ARS J11D, and pDK239-A6C, containing ARS A6C (23), were lost at approximately the same rate in the wild-type and *amm1* mutant strains (Table 3). In contrast, plasmid pDK239-C2G, which contains a third chromosome III ARS element, C2G, was 3.2-fold less stable in the *amm1* strain than in the control strain. Since pDK239-C2G was the only plasmid that showed a large decrease in plasmid stability in the mutant strain, we sought to confirm this finding by an independent method. Therefore, we performed a pedigree analysis on wild-type and mutant strains containing plasmid pDK239-C2G. In wild-type strain 4795-408 we observed seven divisions in which either the mother or daughter cell did not receive the plasmid in a total of 536 cell divisions, yielding a segregation frequency of 0.013. In *amm1* mutant strain 40-4c we observed 15 divisions with plasmid loss in a total of 310 cell divisions, yielding a segregation frequency of 0.048. The segregation frequency of plasmid pDK239-C2G in the mutant strain was 3.7-fold greater than in the wild-type strain, very similiar to the 3.2-fold difference in stability found in the plasmid loss experiments. Therefore, using two different procedures, we found that a plasmid containing the C2G ARS was less stable in the *amm1* mutant. Since plasmid contexts are the same for all three chromosome III ARS-containing plasmids and for pDK239-*ARS1* and none of these showed a difference in plasmid stability between the wild type and mutant, we conclude that the C2G ARS was affected by the *amm1* mutation.

Analysis of the data presented in Table 3 leads to the following conclusions. First, the *amm1* mutation exhibited some specificity in its stabilization of *ARS1*-defective plasmids. Second, plasmids bearing other ARS elements were specifically affected by the *amm1* mutation. This was clearest for plasmid pDK239-C2G, which was destabilized by the mutation.

Mapping the *amm1* mutation. Genetic analysis of *amm1* indicated that the increased stability of plasmid pDK221 was due to a single recessive nuclear mutation that is loosely linked to the mating type locus on the right arm of chromosome III (Table 4). Only three nonparental ditype asci were observed among 53 tetrads examined for the segregation of mating type and increased plasmid stability. Since the *amm1* mutation is not centromere linked, as determined from segregation analysis of *trp1* and *amm1* in tetrads, we conclude that the *amm1* map position is distal to *MAT* on chromosome III.

During routine growth of tetrads on YEPD plates, it was observed that *MAT*α *amm1* mutants, but not *MAT***a** *amm1* mutants, had an abnormal colony morphology. Microscopic examination of cells from these *MAT*α colonies showed that *MAT*α *amm1* cells were elongated and had cellular projections characteristic of shmoos (8). While production of

TABLE 3. Stability of plasmids in *amm1* mutants

Plasmid	Plasmid elements	% Plasmid loss per cell per generation[a]		Fold effect[b]
		4795-408 (WT) ($\bar{x}$ ± SD)	40-4c (*amm1*) ($\bar{x}$ ± SD)	
pDK217	*CEN3 LEU2 ADE3 ARS1-1194*	5.0 ± 0.5	1.3 ± 0.3	+3.8
pDK221	*CEN3 LEU2 ADE3 ARS1-12728*	19.0 ± 2.6	1.1 ± 0.2	+17.3
pDK223	*CEN3 LEU2 ADE3 ARS1-12829*	21.9 ± 2.9	3.6 ± 0.4	+6.1
pDK243	*CEN3 LEU2 ADE3 ARS1*	1.2 ± 0.3	2.5 ± 0.5	−2.1
YIp5-51	*CEN5 URA3 ARS1*	3.7 ± 0.8	2.7 ± 0.5	NS
pDK239-*ARS1*	*CEN3 LEU2 ARS1*	3.1 ± 0.9	2.0 ± 0.5	NS
pDK282	*CEN3 LEU2 ADE3 ARS2*	4.5 ± 0.2	2.8 ± 0.3	+1.6
YEp24	*URA3* 2μm	3.2 ± 0.6	2.2 ± 0.1	NS
CV13	*LEU2* 2μm	2.5 ± 0.5	2.7 ± 0.9	NS
pDK239-A6C	*CEN3 LEU2* A6C	3.0 ± 0.3	1.7 ± 0.4	NS
pDK239-C2G	*CEN3 LEU2* C2G	1.8 ± 0.2	5.7 ± 0.6	−3.2
pDK239-J11D	*CEN3 LEU2* J11D	5.6 ± 0.7	4.7 ± 0.2	NS

[a] Frequencies of plasmid loss are presented as the means ($\bar{x}$) and standard deviations (SD) of loss values obtained for four independent transformants. WT, Wild type.

[b] Fold stabilization (+) or destabilization (−) of plasmids by the *amm1* mutant strain is given for those plasmids for which the data sets for the wild type and mutant were found to be significantly different at a 99% confidence limit by a single-classification analysis of variance. NS, Loss values for the wild type and mutant were not significantly different by this analysis.

shmoos is usually a cellular response elicited by the presence of mating pheromone, *MAT*α *amm1* cells produced shmoos under nonmating conditions. In addition, both *MAT***a** *amm1* and *MAT*α *amm1* mutants grown in liquid culture exhibited a high degree of clumping.

Clumping and α-specific production of shmoos are among the pleiotropic phenotypes reported for strains bearing mutations in the *TUP1* gene (15, 16, 26, 32), a gene that maps 36 centimorgans distal to *MAT* on the right arm of chromosome III (16, 20, 35). Some of the other phenotypes attributed to mutations at the *TUP1* locus, including uptake and incorporation of dTMP, aberrant mating of α mutants, and poor sporulation of homozygous mutant diploids, were also seen in *amm1* strains. In crosses between *amm1* and *AMM1* strains, the *tup1*-related phenotypes (clumping and growth on dTMP) were recessive, as were the increased plasmid stability phenotype and the temperature-sensitive growth on glycerol. All four of these phenotypes cosegregated in the 53 tetrads that were examined (Table 4). In three additional tetrads, in which gene conversion events occurred, all of the *tup1*-related phenotypes, as well as the Gly^{ts} and plasmid stability phenotypes, coconverted. These results suggest that either the *amm1* mutation is allelic to *tup1* or that there are two mutations, one at the *tup1* locus and a second (*amm1*) that is closely linked to the *tup1* locus.

TABLE 4. Mapping the *amm1* mutation[a]

Phenotypes analyzed[b]	Tetrads observed[c]			Map distance (cM[d])
	P	NPD	T	
Stb:MAT	16	3	34	49.1
Stb:Trp	4	7	31	>50[e]
Stb:Gly^{ts}	50	0	0	<1.0
Stb:Flk	53	0	0	<1.0
Stb:Tup	53	0	0	<1.0

[a] The original *amm1* mutant isolate was crossed to strain 4795-303. An *amm1* segregant was then backcrossed two more times to strain 4795-408. Tetrad data from these three crosses are presented.

[b] Segregation of the following phenotypes was analyzed: Stb, increased stability of plasmid pDK221; MAT, mating type locus; Trp, centromere-linked *trp1* marker; Gly^{ts}, growth on glycerol at 34°C; Flk, cell clumping; Tup, growth on dTMP.

[c] Tetrad segregation data are given as the number of parental ditype (P), nonparental ditype (NPD), and tetrad type (T) asci observed.

[d] cM, Centimorgans.

[e] Value estimated from the frequency of second division segregations.

An *amm1* mutant strain was mated to a *tup1* mutant, and plasmid maintenance, cell clumping, and dTMP uptake were analyzed in the resultant diploid to determine whether *amm1* is allelic to *tup1*. The *tup1* mutant used in this study was generated by disrupting the *TUP1* gene in strain 4795-408 by transplacement (22) with a linear DNA fragment bearing the *TUP1*::Tn*3-URA3* allele, provided by V. MacKay, F. E. Williams, and R. Trumbly. The resulting Ura$^+$ transformants were phenotypically Tup1$^-$, and *URA3* mapped to the *tup1* locus. A *TUP1*::Tn*3-URA3* transformant was mated to a wild-type control strain to generate the +/*tup1* diploid CTY8 and to an *amm1* mutant to yield the *amm1*/*tup1* diploid CTY9 (Table 5). Strain CTY8 was phenotypically Tup1$^+$; it did not grow on TMP-containing medium and did not clump in liquid medium. Diploid CTY9 was phenotypically Tup1$^-$. Therefore, the *amm1* mutant contains a *tup1* mutation.

If the plasmid stabilization phenotype of the *amm1* mutant is due to the *tup1* mutation it harbors, then the *tup1* disruption mutant should fail to complement the increased plasmid maintenance phenotype of an *amm1* mutant. In addition, the *tup1* mutation might itself affect plasmid maintenance. We examined plasmid maintenance in the *amm1*/*tup1* mutant diploid CTY9 and found that this diploid lost pDK221 at a rate of 3.9% per cell per generation, a value that is significantly lower than the approximately 8.0% per cell

TABLE 5. Stability of plasmid pDK221 in *tup1*/*amm1* mutants

Strain[a]	% Plasmid loss per cell per generation[b] ($\bar{x}$ ± SD)
4795-408 (+)[c]	19.0 ± 2.6
40-4c (*amm1*)[c]	1.1 ± 0.2
TUP1::Tn*3-URA3*[d]	5.0 ± 0.6
CTY1 (+/+)	7.7 ± 1.2
CTY8 (+/*TUP1*::Tn*3-URA3*)	7.6 ± 0.9
CTY10(+/*amm1*)	8.5 ± 0.4
CTY11(*amm1*/*amm1*)	1.9 ± 0.6
CTY9(*amm1*/*TUP1*::Tn*3-URA3*)	3.9 ± 1.0

[a] The relevant *amm1* and *tup1* genotypes of the strains are given in parentheses. +, Wild type.

[b] Frequencies of plasmid loss in the haploid and diploid strains used for complementation analysis are presented as described for Table 3. *ARS1*$^-$ plasmid pDK221 was used for the stability measurements for all strains listed.

[c] Data for haploid strains 4795-408 and 40-4c are the same as in Table 3.

[d] Data for haploid strain *TUP1*::Tn*3-URA3* are the same as in Table 6.

TABLE 6. Plasmid stability in the *tup1* disruption strain

Plasmid	% Plasmid loss per cell per generation[a]			Fold effect vs[b]	
	TUP1::Tn*3*-*URA3* (*tup1*) ($\bar{x}$ ± SD)	4795-408 (WT) ($\bar{x}$)	40-4c (*amm1*) ($\bar{x}$)	+/*tup1*	*amm1*/*tup1*
pDK217	2.5 ± 0.8	5.0	1.3	+2.0	−1.9
pDK221	5.0 ± 0.6	19.0	1.1	+3.8	−4.5
pDK223	3.6 ± 0.5	21.9	3.6	+6.0	NS
pDK243	1.8 ± 0.5	1.2	2.5	NS	NS
pDK282	9.6 ± 0.7	4.5	2.8	−2.1	−3.4

[a] Frequencies of plasmid loss are presented as described for Table 3. The mean loss values for plasmids in wild-type (WT) strain 4795-408 and in *amm1* mutant strain 40-4c were taken from data presented in Table 3.

[b] Relative stabilization (+) or destabilization (−) of the plasmid by *TUP1*::Tn*3*-*URA3* when compared with the wild-type strain or the *amm1* mutant. NS, Plasmid stabilities were not significantly different.

TABLE 7. Analysis of revertants of *amm1* and *tup1* mutants[a]

Strain	α-Shmoo	Mate × **a**	Mate × α	Flk	Tup	Stb	Gly[ts]
α-WT[b]	−	+	−	−	−	−	+
α-*amm1*[b]	+	Poor	Poor	+	+	+	−
Rev1–Rev4	−	−	−	−	−	+	−
α-*tup1*[b]	+	Poor	Poor	+	+	+	ND
Rev5	−	−	−	−	+	+	
Rev6	+	Poor	−	+/−	+/−	+	ND
Rev7–Rev9	+	Poor	Poor	+/−	+−	+	ND

[a] Independent *MAT*α *amm1* mutants, Rev1 to Rev4, that failed to shmoo were checked for the phenotypes indicated. A *TUP1*::Tn*3*-*URA3* transformant was crossed to wild-type strain 4795-303. Five different α-*tup1* segregants from tetrads of this cross were used to isolate revertants, Rev5 to Rev9, with reduced clumping. These revertants were screened for the indicated phenotypes. Mate ×, Ability to mate with cells of the indicated mating type (see Materials and Methods); ND, not analyzed. Other designations are defined in Table 4, footnote *b*.

[b] Shown for comparison are properties of a *MAT*α wild-type strain, a *MAT*α *amm1* mutant, and a *MAT*α *tup1* strain.

per generation loss rate seen in both the wild-type diploid strain CTY1 and in the heterozygous mutant diploids CTY8 (+/*tup1*) and CTY10 (+/*amm1*). The reductions were modest because of a possible effect of ploidy: plasmid stability was approximately 2.5-fold higher in the +/+ diploid strain CTY1 than in the congenic haploid strain 4795-408. We have not examined the nature of the increased plasmid maintenance in the diploid strain. Since the *tup1* mutant did not complement the increased plasmid stability mutation of the *amm1* mutant, we conclude that *amm1* is allelic to *tup1*.

Plasmid maintenance in the *tup1* mutant. The stabilities of various plasmids were measured to determine whether plasmid maintenance is affected in the *tup1* disruption mutant and, if so, whether the *tup1* mutant shows the same ARS specificity as the *amm1* mutant. The results of this study are shown in Table 6. The three defective *ARS1* plasmids, pDK217, pDK221, and pDK223, were two-, four-, and sixfold more stable in the *tup1* mutant strain than in the isogenic wild-type strain 4795-408. However, plasmid stability analysis and visual observation of colony sectoring patterns showed that pDK217 and pDK221 were more stable in *amm1* mutant strain 40-4c than in the *TUP1*::Tn*3*-*URA3* disruption strain. pDK223 was lost at the same rate in both the *amm1* and *tup1* mutant strains. These results demonstrate that although the two mutants showed similar specificities for the mutant *ARS1* plasmids, the degree of plasmid stabilization differed.

We also compared the stability of plasmids pDK243 (*ARS1*$^+$) and pDK282 (*ARS2*) in the wild type and in the *amm1* and *tup1* mutant strains. As described above, we found that *ARS1* plasmids were not affected by the *amm1* mutation. Analysis of stability data for *ARS1*$^+$ plasmid pDK243 leads us to conclude that the *tup1* disruption mutation had no effect on the maintenance of this plasmid. As mentioned above, pDK282 may have been slightly more stable in *amm1* strain 40-4c than in wild-type strain 4795-408 (Table 2). In contrast, plasmid stability analysis and colony sectoring showed that this plasmid was less stable in the *tup1* disruption strain than in the wild-type strain (Table 6). These results indicate that, like the *amm1* mutant, the *tup1* mutant exhibited ARS-specific plasmid stabilization. However, the ARS specificity of the disruption mutant differs from that of the *amm1* mutant. Yeast strains bearing the the *tup1* disruption allele used in these experiments have the same phenotypic properties as yeast strains in which the *TUP1* gene has been deleted (F. E. Williams and R. Trumbly personal communication). Therefore, the data suggest that elimination of *TUP1* function can give rise to ARS-specific effects, and since the *amm1* mutant differs in its plasmid stabilization properties it is likely that the *amm1* mutant is not a null mutant but has partial or altered protein function.

Suppression of phenotypes in *amm1* and *tup1* mutants. Given the pleiotropic nature of *tup1* mutations, their plasmid maintenance effects could be caused by one of the other defects, for example altered dTMP metabolism. Strains with partial reversion of the *amm1* and *TUP1*::Tn*3*-*URA3* alleles were isolated to examine this possibility. Partial reversion of phenotypes in *tup1* mutants has been reported and was used to show that one of the *tup1* mutant phenotypes, resistance to UV-induced *CAN1* mutations, is a consequence of increased cell clumping (16). By analogy, this approach would reveal whether plasmid maintenance depends on one of the other *tup1* phenotypes. Analysis of clumping revertants, one obtained from each of four independent α-*amm1* and five independent α-*TUP1*::Tn*3*-*URA3* cultures, is shown in Table 7. All nine revertants showed partial suppression of the mutant phenotypes, and in no instance was plasmid maintenance affected. The four independently isolated *amm1* revertants (Rev1 to Rev4) were identical in their phenotypes. They no longer clumped or produced shmoos, and they failed to grow on medium that requires dTMP utilization. These revertants continued to stabilize plasmid pDK221 and were Gly^{ts}. In addition, they had mating defects that were more severe than those of the strains from which they were isolated. The revertants isolated from *TUP1*::Tn*3*-*URA3* strains (Rev5 to Rev9, Table 7) showed a similar range of phenotypic suppression.

The revertant analysis demonstrated that increased plasmid stability cannot be a consequence of altered dTMP metabolism or cell clumping, since plasmid pDK221 was still stable in strains that no longer exhibited these mutant phenotypes. We can rule out a relationship between plasmid maintenance and *MAT*α mating defects or production of shmoos based on the fact that *MAT***a** *amm1* mutants that failed to exhibit these phenotypes still stabilized the plasmid. However, it is possible that one of the *tup1* phenotypes we have not monitored or a yet undiscovered *tup1* mutant phenotype correlates with plasmid stability in *tup1* strains. Additionally, since none of the partial revertants we examined affected plasmid stability, perhaps the defect leading to this phenotype cannot be suppressed.

Conceivably, both intragenic and extragenic mutations could lead to the partial suppression of phenotypes that was observed. For example, if there is more than one functional

domain in the *TUP1*-encoded protein, one might expect to recover partial revertants with a second mutation in the *TUP1* gene. If the *TUP1*-encoded protein normally functions as part of a complex, one might recover extragenic suppressors. Genetic analysis of Rev9 showed that partial suppression of phenotypes in this revertant was due to a second-site mutation unlinked to the *tup1* locus. Therefore, extragenic mutations can suppress *tup1* mutant phenotypes. This result raises two possibilities, that the *TUP1* gene product interacts with other proteins or that the requirement for *TUP1* function can be bypassed.

DISCUSSION

To identify and study the *trans*-acting factors necessary for ARS function, we screened for yeast mutants that could rescue the mitotic instability of plasmids containing a mutationally debilitated *ARS1* element. The mutations we identified defined at least four complementation groups. Screens for mutants undertaken in other laboratories have led to the identification of 16 complementation groups that result in decreased minichromosome stability (17, 30) and six complementation groups that result in improved maintenance of plasmids containing a mutated *HO* ARS (13). Thus, it is clear that we have identified mutations in only a few of the many genes that code for products that are likely to affect ARS function. We do not know whether any of the mutations we have identified belong to the previously identified complementation groups.

Analysis of the *amm1* mutant is described in this paper. *amm1* mutations resulted in increased stability of *ARS1*-defective plasmids and temperature-sensitive growth on glycerol-containing medium. The *amm1* mutation is linked to the mating-type locus on chromosome III, and complementation analysis showed that *amm1* is an allele of the previously identified, but poorly understood, *TUP1* locus. We have designated this new *tup1* mutation as *tup1-20*.

Yeast strains bearing the *tup1-20* mutation stabilized defective *ARS1* plasmids pDK217, pDK221, and pDK223. These plasmids have small deletions and *Bam*HI-linker insertions in domain C of the *ARS1* element (14). The stabilities of pDK221 and pDK223 were greatly enhanced by the *tup1-20* mutation. Plasmid pDK217 was stabilized but to a lesser extent. The stabilization of plasmids by the *tup1-20* mutation was ARS specific, perhaps reflecting a DNA sequence specificity. For example, the *ARS1* deletions in the plasmids used in this study may have removed a DNA sequence element, or changed a crucial spacing between sequence elements important for normal *ARS1* function. The *tup1-20* mutation may code for a protein that can recognize and interact with the sequence created by the *Bam*HI-linker mutagenesis. A plasmid containing another ARS element might be stabilized, destabilized, or unaffected by the mutation, depending on its sequences. Our findings are consistent with this model.

The *tup1-20* mutation, like other *tup1* alleles, is highly pleiotropic. Some of the many phenotypes reported for *tup1* mutants include the uptake and incorporation of dTMP into DNA, insensitivity to glucose repression, overproduction of iso-2-cytochrome *c*, Ca^{2+}-dependent clumping, derepression of the invertase gene, α-specific mating defects, α-specific shmoo production in non-mating conditions, and absence of sporulation in homozygous mutant diploids (15, 16, 26, 32–34). We looked at plasmid maintenance in a number of *tup1-20* and *TUP1*::Tn*3-URA3* strains that exhibit reduced clumping. None of the revertants that were isolated was wild type for all the *tup1* phenotypes being monitored, and none of these partial revertants showed reduced plasmid maintenance. In at least one instance, the partial reversion of phenotypes was due to an extragenic mutation.

Both increases and decreases in plasmid stability were observed in a strain bearing a *TUP1*::Tn*3-URA3* mutation. We had expected to identify mutations in genes for *trans*-acting factors that interact with ARS elements and are involved in initiation of replication. However, since it is likely that the Tn*3-URA3* insertion into the *TUP1* gene generated a null mutation, the observed ARS specificity in the *tup1* mutant cannot have resulted from a direct interaction of *tup1* protein with the ARS elements. It remains a possibility that the *TUP1*-encoded protein is part of a replication complex and that it imparts specificity to the complex. However, it is difficult to account for all of the pleiotropic phenotypes of *tup1* mutants by assuming that the only defect is in DNA replication.

We propose two models that could account for the observed *tup1* mutant phenotypes. The first model is that *TUP1* encodes a transcription factor. Several of the phenotypes, including derepression of glucose-repressible enzymes and elevated levels of iso-2-cytochrome *c*, suggest that transcriptional regulation may be affected in *tup1* mutant strains. Support for this possibility comes from studies of mutations in an unlinked gene, *SSN6*. *ssn6* mutants display the same range of pleiotropic effects as do *tup1* mutants (27), suggesting that these two gene products may have closely related functions, and it has been shown that derepression of invertase in *ssn6* mutants occurs at the transcriptional level (27). A defect in transcription would account for the altered plasmid maintenance seen in *tup1* mutants if transcription is normally involved in replication or if the *TUP1* gene product is required for transcription of a replication protein. Partial phenotypic suppression in the mutant would result from compensating mutations in other gene-specific transcription factors.

In the second model we propose that *TUP1* encodes a nonspecific DNA-binding protein. Mutations that reduce or eliminate the DNA-binding activity would result in altered chromatin structure. Differential sensitivity of ARS elements to the alteration would lead to ARS-specific plasmid effects. This model also accounts for the observed pleiotropy, since it is likely that the transcription of some genes may be more affected by altered chromatin structure than others. As in the first model, we predict that second-site mutations in specific transcription factors would lead to the partial suppression of the phenotypes that we have observed.

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