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Reversion of Autonomously Replicating Sequence Mutations in *Saccharomyces cerevisiae*: Creation of a Eucaryotic Replication Origin within Procaryotic Vector DNA

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To investigate how a defective replicon might acquire replication competence, we have studied the reversion of autonomously replicating sequence (ARS) mutations. By mutagenesis of a *Saccharomyces cerevisiae* plasmid lacking a functional origin of replication, we have obtained a series of *cis*-acting mutations which confer ARS activity on the plasmid. The original plasmid contained an ARS element inactivated by point mutation, but surprisingly only 1 of the 10 independent Ars$^+$ revertants obtained shows a back mutation in this element. In the remainder of the revertants, sequence changes in the M13 vector DNA generate new ARSs. In two cases, a single nucleotide change results in an improved match to the ARS consensus, while six other cases show small duplications of vector sequence creating additional matches to the ARS consensus. These results suggest that changes in replication origin distribution may arise de novo by point mutation rather than by transposition of preexisting origin sequences.

Eucaryotic DNA replication initiates at multiple sites in chromosomes, an adaptation allowing the replication of a large genome in a short S phase without the need for fast fork movement. The activation of multiple replication units in the S phase is regulated both temporally and spatially (for a review, see reference 13); furthermore, the cell can ensure that its genome is replicated only once in any cell cycle by preventing initiation at any origin which has already been replicated. Replication must also be coordinated with other activities in the cell cycle, and the length of the S phase must be compatible with the overall length of the cell cycle. The requirement to complete replication before the onset of mitosis is highlighted in budding yeast cells, in which mitosis occurs almost immediately upon completion of DNA synthesis, but the mechanism allowing this coordination is not understood.

In the yeast *Saccharomyces cerevisiae*, the replication of plasmid molecules as autonomous minichromosomes requires the presence of DNA sequences termed autonomously replicating sequence (ARS) elements (for a review, see reference 22). These elements provide an origin of replication for plasmid replication (4, 14) and appear to have a similar role in *S. cerevisiae* chromosomes (15, 19). Comparison of the DNA sequence of ARS elements has led to the identification of a conserved 11-base-pair (bp) consensus sequence, 5′-(A/T)TTTATPuTTT(A/T)-3′. Detailed mutational analysis of four yeast ARS elements (3, 8, 17, 23, 26) has demonstrated an essential role for this core sequence; small mutations in this region can eliminate ARS function, and it is assumed that this sequence constitutes the binding site for an initiator protein. An AT-rich region situated 3′ to the T-rich strand of the consensus sequence is important for origin function but shows little primary sequence similarity between ARSs. The deletion of this region can abolish or at least reduce ARS function, although small deletions or insertions in this region have little effect. It is not clear how the flanking region contributes to origin function. Palzkill and Newlon (23), in their analysis of the C2G1 ARS, note that the flanking region contains multiple near matches to the ARS consensus and present evidence that these motifs stimulate ARS activity. Some, but not all, ARSs have flanking regions which assume bent conformations, and a functional significance for this has been suggested by analogy to the role of bent DNA in procaryotic origin function (28, 30). In contrast, Umek and Kowalski (27) show that the ability of the flanking region to allow initiation of replication correlates with its hypersensitivity to nucleases, implying that a necessary property of neighboring DNA is to facilitate unwinding.

To gain further insight into the sequence requirements for autonomous replication, we have constructed a number of *S. cerevisiae* minichromosomes carrying mutated ARS elements. These replication-deficient plasmids have been used to isolate *cis*-acting mutations which restore the replication competence of the plasmid in *S. cerevisiae*. Surprisingly, the majority of the replication-competent plasmids obtained do not contain mutations simply reverting the ARS to its wild-type sequence but contain new ARSs generated by mutation of the procaryotic vector sequence. We have analyzed the sequences and structures of these novel ARSs and show that in each case the acquisition of ARS function can be explained by the generation of a sequence that shows a close match to the ARS consensus sequence. This suggests that the sequence requirements for replication origin function in *S. cerevisiae* are rather simple.

MATERIALS AND METHODS

Strains and plasmids used. For standard growth, M13 bacteriophage were propagated in *Escherichia coli* JM101. The *mutD5* strain DKE7 [(F′ *lac* Tn*10* Tet^r) *thr-1 ara-14 leuB6 mutD5 lacY1 tsx-33 supE44 galK2* λ$^-$ Rac$^-$ *hisG4*(Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3*(Oc) *thi-1*] was used for mutagenesis of phage M13 DNA (derived from strain CGSC 6485 [ES1578], from the *E. coli* Genetic Stock Center). The *S. cerevisiae* strain used was W303-1A (**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*). *S. cerevisiae* media and the transformation of spheroplasts were as described elsewhere (25).

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TABLE 1. Core sequences of the alleles of the *HO* ARS used (17)

Clone	*HO* ARS allele	Sequence	Ars phenotype
g65	Wild type	ATTTAATATTTTGGA	+
i56	956	ATTTAATATTTTaGA	+
k9	1048	ATTTAATATTcTaGA	−
k8	887	AccaAATATTTTGGA	−
j80	870	ATcgAATATTTTGGA	−
k1	918	ATaaAATATTTTGGA	−

Plasmid M13se102 is M13mp9 containing the *S. cerevisiae URA3* gene (16). Plasmids k1, k8, k9, and j80 are M13mp93 (a *supE*-independent version of M13mp9) containing *URA3* and inactive derivatives of the *HO* ARS created by making point mutations in the consensus sequence (Table 1) (17).

pSE295 is a derivative of YCp50 containing the *SUP4-o* gene (18). pSE296 is an Ars$^-$ derivative of pSE295; the *ARS1* element was removed from pSE295 by digestion with *Xho*I and *Bgl*II, followed by removal of the single-stranded ends with mung bean nuclease and reclosure. A 98-bp *Sal*I fragment containing a functional *HO* ARS was obtained from plasmid pSE354, a derivative M13-h12 (17) possessing an extra *Sal*I site created by *Sma*I cleavage and subsequent addition of *Sal*I linkers (5′-GGTCGACC-3′). A single copy of the *HO* ARS was cloned into the *Sal*I site of pSE296 to give pDK296-HO (orientation, *SUP4-o Ssp*I-*Fok*I *URA3*, where *Ssp*I-*Fok*I indicates the disposition of restriction sites in the *HO* ARS fragment). A 104-bp *Sal*I fragment containing the i56 allele of the *HO* ARS was obtained from pDKG227, a derivative of M13-i56 (17) possessing an extra *Sal*I site created by *Eco*RI cleavage, filling in with Klenow polymerase, and addition of *Sal*I linkers. A single copy of the i56 *HO* ARS was cloned into the *Sal*I site of pSE296 to give pDK296-i56HO (orientation of ARS fragment is opposite that of pDK296-HO). *Hae*III fragments of approximately 340 bp carrying the A17 and B12 mutations were cloned into the *Sma*I site of M13mp93; the resulting construct was then digested with *Eco*RI and *Hin*dIII, and the ends were filled in with Klenow polymerase before ligation of *Hin*dIII linkers (5′-CCAAGCTTGG-3′). After digestion with *Hin*dIII, the fragments were cloned into the *Hin*dIII site of pSE296 to yield plasmids pDK296-A17 and pDK296-B12 (orientation, *Hae*III [6663] to *Dde*I-*Hae*III [7004] *SUP4-o URA3*).

Isolation of *cis*-acting suppressor mutations. A stock of each plasmid was made by inoculating phage into a 1/100 dilution, in 2× TY (16 g of tryptone per liter, 10 g of yeast extract per liter, 5 g of NaCl per liter), of a saturated JM101 culture, followed by growth at 37°C for 5 h. This stock has a titer of 10^{11} PFU per ml. For each experiment, 1.5 ml of 2× TY medium was inoculated with 15 μl of a saturated culture of *E. coli* DKE7 grown in minimal medium and 10 μl of a 10^{-6} dilution of the phage stock. After growth at 37°C for 6 h, single-stranded DNA was prepared (1). The entire DNA sample was then transformed into *S. cerevisiae* W303-1A spheroplasts selecting for Ura$^+$. M13 phage DNA gives a background of abortive microcolonies upon transformation, and it was necessary to transfer the transformants to fresh selective plates. This was achieved by macerating the top agar, plating out onto selective plates lacking uracil, and allowing colonies to grow. The majority of colonies appeared to be integrants, easily distinguished by their even colony morphology. Transformants unstable for the Ura$^+$ phenotype give a more crinkly colony morphology, and we were able to screen for such transformants. These were colony purified and checked to be unstable for the Ura$^+$ phenotype before we attempted to rescue phage into *E. coli* by transforming JM101 with *S. cerevisiae* DNA. In those cases in which we could rescue phage, we checked that they were indeed Ars$^+$ by transforming purified phage DNA into yeast cells. All Ars$^+$ revertants were obtained from separate initial phage preparations; single-stranded DNA was used to facilitate easy preparation of DNA of sufficient quality for *S. cerevisiae* transformation from a large number of independent cultures.

Determination of location of suppressor mutations. The locations of the A17, B12, and D10 mutations were first determined by marker rescue experiments. The revertants were digested with *Fok*I, and the three largest fragments were isolated (Fig. 1). These are *Fok*I-A (3.3 kilobases, coordinates 226 to 3561), *Fok*I-B (2.8 kilobases, 3561 to within the *URA3* gene), and *Fok*I-C (0.9 kilobase, 6327 to 8). Coordinates refer to the M13mp9 sequence. Any mutations within these regions were transferred to M13se102 by marker rescue. The isolated fragment was incubated at 100°C for 2 min in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA and then hybridized to single-stranded M13se102 DNA in 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$ for 2 h at 65°C. The heteroduplex was then transformed into W303-1A spheroplasts, selecting for Ura$^+$. After growth at 30°C for 5 days, the transformants were replica plated onto selective plates lacking uracil. DNA was made from transformants (up to 40 per plate) which appeared

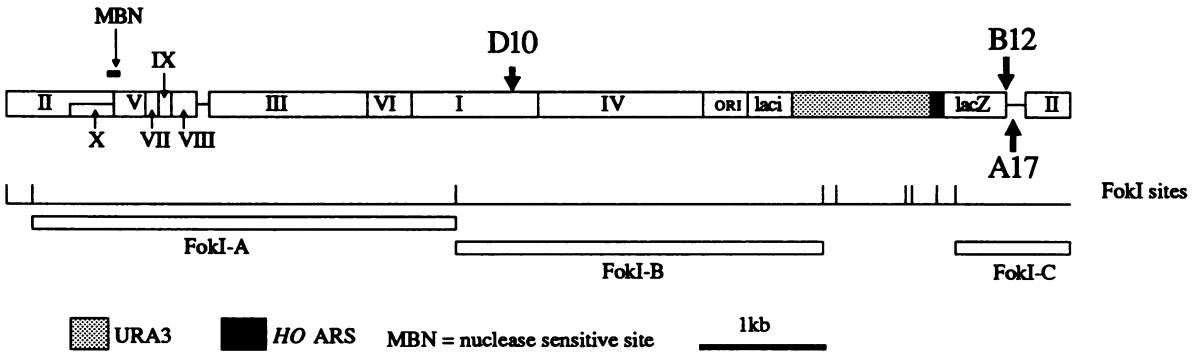

FIG. 1. Location of the A17, B12, and D10 mutations within the M13 genome. Coding regions for M13 genes (indicated by roman numerals) and the location of the *URA3* and dead *HO* ARS in a typical construct are shown. Also indicated are the positions of the three *Fok*I fragments used in marker rescue experiments as well as the single nuclease hypersensitive site. kb, Kilobases.

to be unstable for the Ura$^+$ phenotype when streaked onto fresh selective plates, and phage were rescued into *E. coli*. These phage were then tested for their Ars phenotype by transformation into *S. cerevisiae*. This allows the identification of restriction fragments having ARS activity. The mutations lie within those *Fok*I fragments indicated in Fig. 1.

Random restriction subfragments of the *Fok*I fragments were cloned into the *Sma*I site of M13mp93 (1). Clones were tested for ARS activity by cotransformation with M13se102 into *S. cerevisiae* and, if positive, sequenced. Sequencing was performed by the dideoxy procedure (1), using [α-^{35}S]dATP (Dupont, NEN Research Products) and Klenow polymerase (Pharmacia, Inc.); sequencing reactions were performed at 50°C. In the case of the D10 mutation, we have sequenced it as both *Dde*I (3362 to 4014) and *Hpa*II-*Dde*I (3843 to 4014) fragments. The sequence of the minimal functional fragment is identical to the published M13mp9 sequence, except for the single nucleotide change (Fig. 2). The A17 and B12 mutations have been sequenced as both *Hae*III (6663 to 7004) and *Hae*III-*Dde*I (6663 to 6888) fragments cloned into the *Sma*I site of M13mp93, although in all cases, when we cloned fragments of the M13 genome into the M13mp93 polylinker site, we could obtain sequence data only when the fragment was in a direct repeat orientation with respect to its homologous sequence; in the opposite orientation, the single-stranded DNA forms a stem-loop structure preventing sequencing. The A17 and B12 mutations have also been sequenced in the original revertant isolates, using a synthetic oligonucleotide (5′-AACAGGAA GATTGTATA-3′) which hybridizes about 30 bp away from the site of the A17 mutation. We also sequenced the Ars$^-$ parental clones and M13se102, using this primer. The changes shown in Fig. 2 are the only differences between the revertants and the parental clones for the minimal functional fragment. Except for these changes, the sequences of all clones are identical to the published M13mp9 sequence, except for a C-to-T change at position 6731 present in all clones (both Ars$^-$ and Ars$^+$).

Mapping nuclease hypersensitive sites. We mapped the nuclease hypersensitive sites by the method of Umek and Kowalski (27). Double-stranded phage DNA was banded twice in CsCl. It was incubated in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA at 37°C for 15 min before the addition of sufficient mung bean nuclease (New England BioLabs, Inc.) to relax all the supercoiled form in 30 min at 37°C, as monitored by agarose gel electrophoresis. The DNA was then restricted with *Hin*dIII or *Ava*II, labeled by filling in the overhangs with Klenow polymerase in the presence of [α-^{32}P]dCTP, and then denatured with glyoxal and run on a 0.9% agarose gel (20). The gel was dried and autoradiographed.

Determination of plasmid stability. Plasmid stabilities were quantitated by measuring plasmid loss rates during growth in rich media. This was done by inoculating approximately 10^4 cells, which had been grown on plates selecting for the plasmid, into 10 ml of YEPD medium (20 g of Bacto-Peptone per liter, 10 g of yeast extract per liter, 20 g of glucose per liter). A portion was removed and plated on appropriate plates to measure the cell density and the initial percentage of cells that contained the plasmid. The culture was then allowed to grow to saturation, and total cell density and the percentage of plasmid-containing cells were again measured. Plasmid loss rate per generation was calculated from the following formula: loss rate = $1 - (F/I)^{1/N}$, where F is the final percentage of plasmid-containing cells, I is the initial percentage of plasmid-containing cells, and N is the number of generations.

RESULTS

Strategy for isolation of *cis*-acting suppressors. The plasmids used were replication-incompetent derivatives of an M13mp vector containing the *S. cerevisiae URA3* gene and one of a series of mutant ARS derivatives, created by making point mutations in the consensus sequence of the *HO* ARS (17). These plasmids can only transform *S. cerevisiae* by integration into a chromosome and so by this criterion are designated Ars$^-$. Details of the sequences of the dead ARSs used are given in Table 1; the sequences differ from functional ARS elements by 1 to 3 base changes.

Single-stranded DNA was prepared from the Ars$^-$ phage after propagation in a *mutD5* mutator strain of *E. coli*. To select for plasmids capable of autonomous replication (Ars$^+$), we used the DNA to transform a *ura3 S. cerevisiae* strain, and Ura$^+$ transformants were screened for those that were unstable for the Ura$^+$ phenotype. M13 phage were recovered into *E. coli* by transforming with *S. cerevisiae* DNA, and the Ars phenotype of the plasmid was then rechecked by yeast transformation. From a total of 146 mutagenesis and transformation experiments, we obtained 39 independent transformants unstable for the Ura$^+$ phenotype (a frequency of $<0.1/\mu$g of DNA). Of these, M13 phage could be recovered in *E. coli* from 29 transformants, and on rechecking by transformation into *S. cerevisiae*, it was found that 10 of these phage were indeed Ars$^+$. Southern analysis of DNA from a representative set of tranformants unstable for the Ura$^+$ phenotype is shown in Fig. 3. Strains from which Ars$^+$ plasmid can be rescued into *E. coli* (i.e., the desired class of revertant) contained autonomously replicating plasmid of the original size. However, strains from which plasmid cannot be rescued often showed hybridization to high-molecular-weight material or the presence of free plasmid of altered mobility. These have not been investigated further, but large structural changes in the vector M13 sequence which prevent the phage from being viable in *E. coli* have presumably occurred. Finally, strains from which only Ars$^-$ phage could be rescued again showed a hybridization pattern not characteristic of free monomer plasmid. In some cases, the presence of multiple bands suggest that a complex rearrangement has occurred, perhaps involving the release by recombination of viable but Ars$^-$ phage from a larger replicating molecule.

Location of mutations conferring autonomous replication. The *HO* ARS region in each of the 10 independent Ars$^+$ revertants was sequenced; surprisingly, only 1 of the revertants showed a sequence change in this element. This mutation was a C-to-T transition in a k9 clone resulting in a reversion to the i56 sequence. This element has a closer match to the ARS consensus and the wild-type *HO* sequence and is known to have ARS activity (Table 1) (17). In the remaining nine revertants, sequence changes had presumably occurred elsewhere in the plasmid to create new ARS elements. The location and nature of these changes have been determined for eight of these revertants by a combination of marker rescue experiments, subcloning, and sequencing. We initially isolated three large *Fok*I restriction fragments from revertants (Fig. 1) and performed marker rescue experiments (see Materials and Methods) to determine whether a particular fragment could confer autonomous replication when hybridized to an Ars$^-$ plasmid, M13se102. The mutation to a *Fok*I fragment having been located,

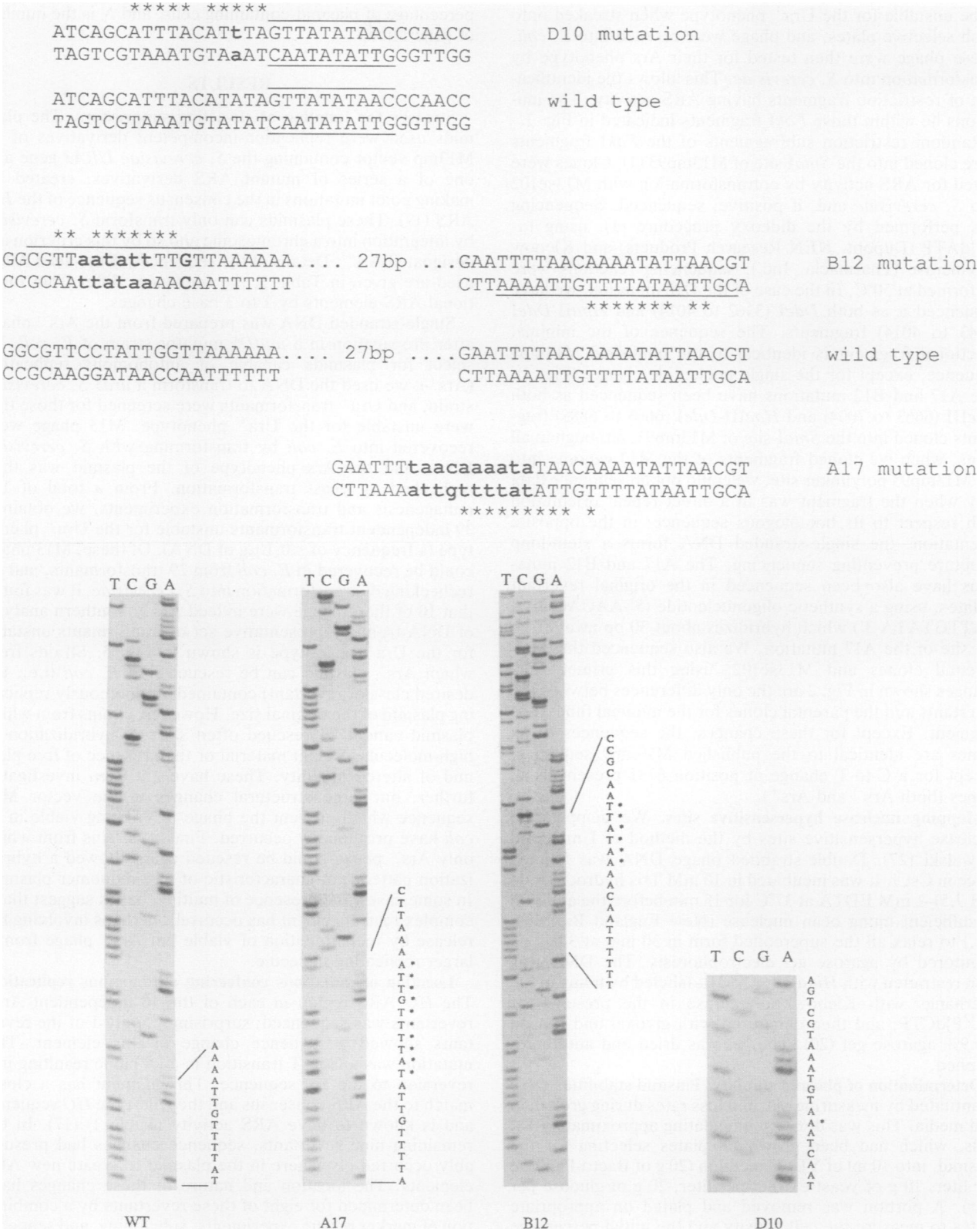

FIG. 2. Sequences changes in Ars$^+$ revertants. Sequence changes in A17, B12, and D10 ARSs are shown in bold lowercase letters. Asterisks indicate matches to the consensus sequence. The 10-bp palindrome adjacent to the D10 mutation and the inverted repeat created by the B12 mutation are also indicated. The presence of palindromes has been noted in a number of ARSs (17). Below are shown sequencing gels for M13se102 and original A17 and B12 revertants, sequenced directly by using a special sequencing primer (see Materials and Methods). Also shown is the sequence of the minimal *Hpa*II-*Dde*I D10 ARS cloned into the *Sma*I site of M13mp93 and sequenced by using the standard sequencing primer. WT, Wild type.

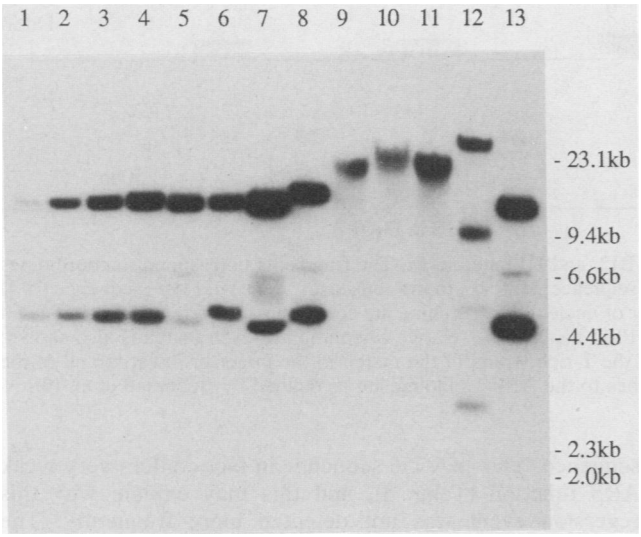

FIG. 3. Southern analysis of DNA from a series of transformants unstable for the Ura$^+$ phenotype. Uncut *S. cerevisiae* DNA (25) was run on a 0.8% TAE (Tris acetate-EDTA) gel, transferred to nitrocellulose, and probed with labeled M13mp93 DNA. Lanes 1 to 4, Strains from which Ars$^+$ phage can be rescued into *E. coli* (lane 1 is the k9-to-i56 revertant, lane 2 is uncharacterized, lane 3 is an A17 revertant, and lane 4 is a B12 revertant); lanes 5 to 10, strains from which phage cannot be rescued; lanes 11 to 13, strains from which only Ars$^-$ phage can be rescued in *E. coli*. D10 also showed the presence of autonomously replicating plasmid identical in size to A17 and B12 (data not shown). The molecular weight marker used was phage λ DNA digested with *Hin*dIII. kb, Kilobases.

smaller restriction fragments were subcloned into the *Sma*I site of M13mp93, and clones were tested for replication competence by cotransforming with M13se102. Sequencing of the Ars$^+$ fragments revealed that the revertants contained one of three types of mutation, each isolated more than once (Table 2). These mutations occur in M13 vector sequences, and they have occurred by either point mutation or minor rearrangement of vector sequences and not by insertion of *S. cerevisiae* DNA.

Sequence of mutations conferring Ars$^+$ phenotype. The three mutations creating new ARSs are designated A17, B12, and D10. The A17 and B12 mutations map to within 50 bp of each other in a noncoding region of the M13 genome between the end of the *lacZ* gene and the start of the M13 gene II open reading frame (positions 6749 and 6691 for A17 and B12, respectively) (Fig. 1). Unlike most of the plasmid, this region is not required for selection in *S. cerevisiae* and is apparently not necessary for M13 growth in *E. coli*; thus, this region is presumably free to mutate. Perhaps coincidentally, the A17 and B12 mutations are in a region of the M13 genome which, in wild-type phage, serves to enhance the function of the essential core of the phage replication origin, although this function is not required in the M13mp cloning vector derivatives (10).

TABLE 2. Summary of mutations isolated

Parental vector	No. of expts	No. in which *HO* ARS reversion occurred	No. of mutations isolated[a]			No. of other mutations
			A17	B12	D10	
j80	18	0	2	0	0	1
k1	18	0	0	0	0	0
k8	20	0	0	2	0	0
k9	90	1	0	2	2	0

[a] No obvious alteration in the ARS activity of A17, B12, or D10 was seen when transformants were grown at 25, 30, or 37°C; similarly, the parental Ars$^-$ plasmids showed no evidence of ARS activity at any of these temperatures. None of the revertants showed altered phage plaque morphology.

TABLE 3. ARS activities of novel M13 ARSs

Plasmid	Genotype	Loss rate per generation[a]
pSE296	*CEN4 URA3*	Ars$^-$
pSE295	*CEN4 URA3 ARS1*	0.12 ± 0.01
pDK296-HO	*CEN4 URA3 HO* ARS	0.22 ± 0.01
pDK296-i56HO	*CEN4 URA3* i56 *HO* ARS	0.42 ± 0.02
pDK296-A17	*CEN4 URA3* A17 ARS	0.20 ± 0.01
pDK296-B12	*CEN4 URA3* B12 ARS	0.20 ± 0.01

[a] Values are means plus or minus standard deviations for three independent transformants.

The A17 mutation (Fig. 2), is a duplication of a 10-bp sequence and creates a good match (10/11) to the ARS consensus sequence. The B12 mutation appears to have been generated by the correction of an imperfect inverted repeat to a perfect one and duplicates a plausible match (9/11) to the ARS consensus sequence. This correction may have been facilitated by base pairing in single-stranded M13 DNA and then repair, either in *E. coli* or following transformation of the single-stranded DNA into *S. cerevisiae*. The B12 mutation may function as an ARS because of a synergistic interaction between the duplicated sequences, or the new match to the consensus may be in a better environment for ARS function. The D10 mutation is an A-T transversion in the M13 gene I coding region (position 3995) resulting in a conservative tyrosine-to-phenylalanine change in the gene I protein, which is apparently not detrimental to M13 growth. This mutation results in an improved match (10/11) to the ARS consensus sequence.

All three revertant ARSs show a G immediately 3′ to the ARS consensus (Fig. 3); this feature is frequent in ARSs (17), and the mutation of this G in the *HO* ARS, yielding the i56 allele, greatly decreases ARS function (Table 3). A 10-bp palindromic sequence immediately 3′ to the D10 mutation may also be relevant to ARS function (Fig. 2).

ARS function in other sequence environments. The A17, B12, and D10 mutations can confer ARS function when transferred to the genome of M13se102 by marker rescue. Not only can these mutations function in the absence of the dead *HO* ARS, but they can also act as ARS elements in other sequence environments. When cloned as small restriction fragments (Fig. 4) into the *Sma*I site of M13mp93, the resulting plasmids are Ars$^+$ as determined by cotransformation with M13se102. Furthermore, we have cloned the 340-bp *Hae*III fragment of the M13 genome, carrying either A17 or B12 mutations, into an Ars$^-$ centric *S. cerevisiae* plasmid, pSE296, to yield pDK296-A17 and pDK296-B12. The resulting plasmids are capable of autonomous replication upon transformation into *S. cerevisiae* and show a mitotic stability at least as good as a similar construct carrying the wild-type yeast *HO* ARS (Table 3). Thus, these mutations have created functional ARS sequences entirely from procaryotic sequences.

Structural requirements for autonomous replication. The acquisition of ARS function of plasmids containing A17, B12, and D10 mutations can be explained in terms of creating an improved match to the ARS consensus sequence. However, we have examined the structure of the

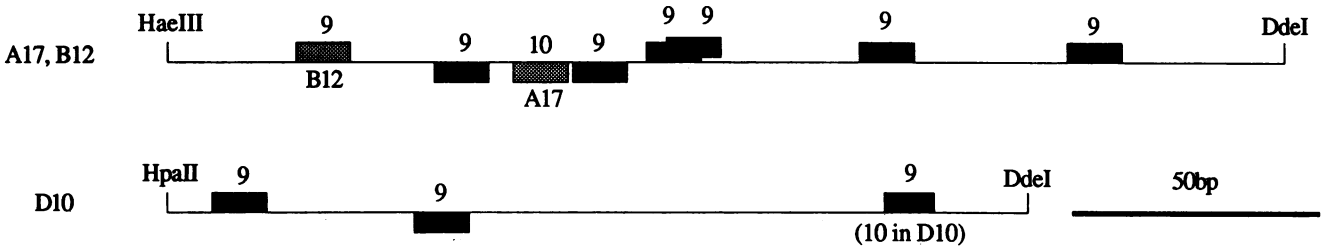

FIG. 4. Structure of the minimal Ars$^+$ fragments containing the A17, B12, and D10 mutations. The fragments correspond to coordinates 6663 to 6888 (*Hae*III-*Dde*I) and 3843 to 4014 (*Hpa*II-*Dde*I) of the M13mp9 sequence. Matches to the consensus in the wild-type sequence (■) and newly created matches in A17 and B12 (▩) are shown. The number of nucleotides matching the consensus for each match is indicated above each box. Note that the D10 mutation changes a 9/11 match to a 10/11 match. The relative orientation of each match is also shown; the location of the box above or below the line indicates the location of the T-rich strand of the match in the upper or lower strand of the sequence, respectively. These Ars$^+$ sequences contain no obvious matches to the ABF-1 binding site as defined by Buchman et al. (6).

DNA in the region of the mutations to see whether it has any of the properties reported for ARS flanking regions. Umek and Kowalski (27) have shown a correlation between hypersensitivity to single-strand specific nucleases at neutral pH and the ability to function as flanking DNA in an ARS. We have mapped the nuclease hypersensitive sites in M13mp93 and our revertants. There appears to be a single, major sensitive site at position 850 in the M13mp93 genome (Fig. 1); we could not detect any minor bands in M13mp93 or any of our revertants (data not shown). As Umek and Kowalski (27) note, unwinding at one site may suppress unwinding at another, so it is possible that potential unwinding at the ARSs is masked by the major site in the M13 vector.

We also examined the reversion sites to see whether the DNA is bent, since a number of ARSs appear to have bent flanking regions. The 340-bp *Hae*III fragment containing the A17 or B12 mutations runs anomalously slowly on cold, nondenaturing gels, implying that the DNA is bent (data not shown). Also, computer calculations imply that this region is bent; using the BEN program of Eckdahl and Anderson (11), we note that the A17 and B12 mutations lie within a region with an ENDS ratio (an index of curvature) of 1.12; the mutations increase this to 1.18. With the same program, the D10 mutation does not appear to be near a region of bent DNA; there is a weak, bending locus (ENDS ratio, 1.12) some 400 bp 3′ to the 10/11 consensus match, but this is not included in the minimum fragment necessary for ARS function.

DISCUSSION

Investigations of the sequence requirements for autonomous replication in *S. cerevisiae* have largely been confined to mutating wild-type sequences and determining the effect on ARS activity. Here we describe a different approach in which *cis*-acting mutations that generate ARS function in plasmids that were previously replication incompetent are obtained. The Ars$^-$ plasmids that we used contained derivatives of the *HO* ARS that, in one case, differs from a functional ARS element by only one nucleotide change, so it was surprising that most of the mutations that we obtained created ARSs de novo from procaryotic vector sequences. This result is perhaps even more remarkable given that most of the vector sequence is required either for replication in *E. coli* or for selection in *S. cerevisiae* and thus many mutations that might generate ARSs in the vector would not be recovered. The one example of reversion within the *HO* ARS was obtained from the k9 plasmid, and the single transition generates a closer match to the wild-type *HO* ARS sequence. This new i56 sequence in fact confers very weak ARS function (Table 3), and this may explain why this reversion event was not detected more frequently. The *mutD5* mutator allele in the *E. coli* strain used for M13 growth increases nucleotide substitution mutations and frameshifts (12, 24), but the A17 and B12 mutations are typical neither of the *mutD5* mutator spectrum nor of spontaneous mutations in phage M13 (9). We cannot rule out the possibility that these mutations occurred in *S. cerevisiae*, perhaps during conversion of the single-stranded DNA used for transformation to the double-stranded form, but it is clear that the Ars$^+$ revertants recovered from *S. cerevisiae* have not acquired yeast genomic sequences. This compares with the situation in the fission yeast *Schizosaccharomyces pombe* in which, following transformation, Ars$^-$ plasmids can acquire *Schizosaccharomyces pombe* chromosomal DNA that enables them to replicate autonomously (29).

These novel M13 ARS sequences can function in the absence of the mutated *HO* ARS to confer autonomous replication in a number of plasmid environments. The A17 and B12 ARSs function at least as well as the yeast *HO* ARS (Table 3) and, from a biochemical point of view, can be considered to be efficient, firing in 80% of the S phases. ARS function in each case can be explained by the acquisition of a sequence that shows an obvious match to the ARS consensus sequence, emphasizing the crucial role of this element (Fig. 5). It is of interest to compare these results with those of Palzkill and Newlon (23), who have reported that the activity of the C2G1 ARS is dependent on an exact match to the consensus and additional near matches in the region 3′ to the T-rich strand of the consensus. The consensus created by the A17 mutation is essentially a duplication of a 9/11 match to create an overlapping 10/11 match, and there is a further match 3′ to these elements. The new consensus of the B12 mutation occurs close to multiple near matches in the correct orientation, and the mutation forms a perfect inverted repeat with respect to one 9/11 match. Thus with both these mutations, the acquisition of ARS activity can be thought to occur as a result of a duplication of an imperfect match to the ARS consensus. However, it is not clear whether near matches to the ARS consensus play any role in the activity of the D10 mutation. In this case, the nearest 9/11 match is over 200 bp 3′ to the T strand of the ARS; this element may contribute to ARS function but is outside the minimal fragment required for ARS activity. Perhaps a single ARS consensus can, in principle, confer autonomous replication, but a replicator can also be created by the proximity of consensus-related sequences that would

	A17	TGTTATATTTT	
	B12	GTTAATATTTT	
	D10	ATTTACATTTA	
Consensus		TTTTATATTTT	
		A G A	
	wt M13	-G---------	(a)
Ars$^+$	wt *HO*	---A-------	(b)
	wt M13	G--A-------	(c)
	wt M13	-----C-----	(d)
	wt M13	G--A-------	(c)
	wt M13	-----C--A--	(e)
Ars$^-$	*HO* 1048	---A----C--	(f)
	HO	-NNA-------	(g)
	HO 917	G-AA-------	(h)

FIG. 5. Comparison of the ARS consensus sequence with revertant ARSs. Sequences created or duplicated by the A17, B12, and D10 ARS reversion mutations (top) are aligned with the ARS consensus sequence (defined by Broach et al. [5]). Deviations from the ARS consensus that still permit (Ars$^+$) or eliminate (Ars$^-$) autonomous replication are shown (bottom). In this analysis, only one A and four T nucleotides in the consensus are completely conserved for ARS function (boldface). The sequences are A17 mutation (a), wild-type *HO* ARS (17) (b), B12 mutation (c) (note that one copy of this sequence in wild-type M13 is inactive but two copies in B12 are Ars$^+$), D10 mutation (d), wild-type M13 corresponding to the site of the D10 mutation (e), and *HO* ARS mutations, NN = CA, CG, or AA (17) (f, g, and h). wt, Wild type.

be inadequate individually. The assay for autonomous replication is a genetic one, detecting only those elements that are biochemically efficient. Weak matches to the ARS consensus may function biochemically as origins but not with an efficiency that allows their genetic detection.

We examined the novel ARSs to see whether they also have structural properties reported for the flanking regions of natural ARSs. The reversion mutations do not occur in the vicinity of sites hypersensitive to single-strand specific nucleases, but we cannot rule out the possibility that such sites were masked by the vector used. Two of the reversion mutations occur in a bent region of the M13 genome, and it is conceivable that this feature contributes to the activity of the generated ARS consensus sequences. However, all of our revertants create improved or additional matches to the core consensus rather than creating one of the other structural features propounded for flanking sequences.

Whatever the exact sequence requirements for origin function in *S. cerevisiae* are, the observation that ARSs can be created de novo in procaryotic DNA by mutation implies that the sequence requirements of the *S. cerevisiae* DNA replication apparatus for initiation are not very demanding. In contrast, procaryotic replication origins with complex structures (such as the *oriC* or ColE1 origins) would be unlikely to arise spontaneously by mutation (21, 31). This difference in replication origin complexity may reflect a requirement to change the distribution of replication origins in eucaryotic chromosomes. Replication origin distribution appears to be the primary determinant of S-phase length, given a fixed rate of replication fork movement (2). If the duration of the S phase is adapted to the duration of the cell cycle so that DNA replication does not impede cell division, then selective pressures may result in changes in the distribution of initiation sites (7). Our results suggest that new origins could be created by mutation rather than by duplication and transposition of existing origins. In addition, global changes in origin spacing could be achieved by modifying the DNA replication apparatus to alter the specificity of sequences from which replication can commence. In *S. cerevisiae*, this specificity may be set at a level that is conducive to the mutational creation of new origins.

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