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Isolation and Characterization of an Autonomously Replicating Sequence from *Ustilago maydis*

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DNA fragments that function as autonomously replicating sequences (ARSs) have been isolated from *Ustilago maydis*. When inserted into an integrative transforming vector, the fragments increased the frequency of *U. maydis* transformation several-thousandfold. ARS-containing plasmids were transmitted in *U. maydis* as extrachromosomal elements through replication. They were maintained at a level of about 25 copies per cell but were mitotically unstable. One ARS characterized in detail, which we called UARS1, was localized to a 1.7-kilobase fragment. UARS1 contained a cluster of active sequences. This element could be reduced further into three separate subfragments, each of which retained ARS activity. The smallest one was 383 base pairs (bp) long. Although not active itself in yeast, this small fragment contained seven 8-bp direct repeats, two contiguous 30-bp direct repeats, and five 11-bp units in both orientations with sequences similar but not identical to the consensus sequence found to be crucial for ARS activity in *Saccharomyces cerevisiae*.

Fragments have been isolated from yeast genomic DNA that supply nonreplicating plasmids with the ability to replicate autonomously in *Saccharomyces cerevisiae* (3, 9, 31). The frequency of occurrence of these genetic elements, or autonomously replicating sequences (ARSs), in the genome is in line with the number of origins of replication estimated from electron microscopy and DNA fiber autoradiography (see reference 35 for review). For several years these observations were used to support the notion that the ARS is equivalent to an origin of replication (29). Any uncertainty over this question was eliminated recently by the direct demonstration by two-dimensional gel electrophoretic analysis that the ARS element functions as a replication origin (6, 20). ARSs were first identified as sequences linked to genetic markers that transformed yeast cells at high frequencies (18, 31). Plasmid DNA lacking an ARS can transform yeast cells only by rare integrative recombination into the cellular genome (15). In contrast, the transformation frequency may be 10^3 to 10^4 times higher when the plasmid is carrying an ARS. This differential transformation frequency has been used as a diagnostic measure for defining ARS sequences operationally.

Our interest in ARS elements stems from our efforts to establish a high-frequency transformation system in the basidiomycete fungus *Ustilago maydis* as a means for isolating and cloning genes. A few years ago, Banks (1) reported transformation of *U. maydis* to antibiotic G418 resistance by using a plasmid containing a yeast 2μm DNA origin and the bacterial resistance factor aminoglycoside phosphotransferase gene. Preliminary evidence suggested that the plasmid was maintained in transformants by autonomous replication, but the transformation frequency was low, around 10 per microgram of DNA. More recently, Leong and colleagues (34) developed a pUC12 derivative plasmid containing a bacterial phosphotransferase gene conferring resistance to the antibiotic hygromycin B (13) behind a *U. maydis hsp70*-like (heat shock protein) promoter. Again transformants to hygromycin resistance arose at a low frequency, but through integrative recombination of the plasmid into the *U. maydis* genome. To accomplish our goal of transforming *U. maydis* at high frequency, we isolated genomic DNA fragments from *U. maydis* capable of conferring high transformability on the integrative plasmid developed by Leong's group (34). We analyzed one such fragment in detail and found that it directed autonomous replication of the plasmid on which it was carried.

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MATERIALS AND METHODS

Strains and plasmids. *U. maydis* 518 (a_2b_2) is a wild-type haploid strain obtained from Robin Holliday (National Institute for Medical Research, London). Cells were cultured in YEPS (1% yeast extract, 2% Bacto-peptone [Difco], 2% sucrose) at 32°C. *Escherichia coli* DH5 (Bethesda Research Laboratories), used in transformation, was made competent by the procedure of Hanahan (14). Plasmid pHL1 (34) was kindly provided by Sally Leong (University of Wisconsin, Madison).

Transformation of *U. maydis*. All procedures were performed at room temperature unless otherwise noted. Log-phase cells (50 ml at 10^7/ml) were washed once in SCS buffer (20 mM sodium citrate [pH 5.8]–1 M sorbitol buffer) and suspended in 1 ml of the same buffer containing Novozyme 234 (25 mg/ml) (NovoBiolabs). After incubation for 10 min with gentle mixing, the resulting protoplasts were centrifuged at 1,000 × *g* for 10 min, washed twice with SCS buffer and once with 10 mM Tris hydrochloride (Tris-HCl) (pH 7.5)–0.1 M $CaCl_2$–1 M sorbitol buffer (STC). This protoplasting procedure is 100% efficient, although the yield of protoplasts is 20 to 40% based on the initial number of cells. Approximately 70% of the protoplasts could be regenerated to viable cells. For transformation, protoplasts were suspended in 1 ml ice cold STC buffer. Transforming DNA, 15 μg of heparin, and 50 μl of protoplast suspension (approx. 10^6 protoplasts) were mixed and incubated on ice. After 10 min, 0.5 ml of STC containing 40% (wt/wt) polyethylene glycol 4000 (Sigma Chemical Co.) was added, and the sample was incubated for an additional 15 min. Five milliliters of molten (48°C) regeneration agar (YEPS, 1 M sorbitol, 1.5% agar) was added, and the entire mixture was spread on a plate of regeneration agar (15 ml). After incubation for 4 h at

32°C, plates were overlaid with 0.5 ml of 8-mg/ml hygromycin B (Calbiochem). Transformants were counted after 4 days. Transformation frequencies are expressed as transformants per microgram of input DNA. The response for plasmids bearing an ARS sequence is linear over the range 2 to 80 ng of DNA.

DNA. To prepare genomic DNA from *U. maydis*, protoplasts were generated from a 1-liter cell culture, washed once with SCS and twice with 0.1 M EDTA (pH 8.0)–1 M sorbitol. Protoplasts were suspended in 5 ml of 50 mM Tris-HCl (pH 8.0)–50 mM EDTA and lysed by addition of 50 μl of 10% sodium dodecyl sulfate (SDS) and 50 μl of 10-mg/ml proteinase K. After incubation at 37°C for 1 h, the lysate was extracted twice with a mixture (1:1) of phenol and chloroform and once with chloroform. One-tenth volume of 3 M sodium acetate (pH 7.5) was added, and the DNA was precipitated and spooled onto a glass rod after slow addition of 2.5 volumes of ethanol. DNA was dissolved in 2 ml of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE) mixed with 20 μl of 5-mg/ml RNase A (Worthington) and incubated for 30 min at 37°C. Extraction with phenol-chloroform, precipitation with ethanol, and digestion with RNase A were repeated, followed by a final extraction with phenol-chloroform and precipitation with ethanol. Final product (ca. 1 mg of DNA) was redissolved in TE. *U. maydis* DNA enriched for plasmid was prepared from protoplasts by the method of Hirt (16). A rapid alkaline extraction procedure was used to prepare plasmid DNA from *Escherichia coli* (5).

Isolation of ARSs. *U. maydis* DNA (15 μg) was partially digested with *Sau*3A (15 U) for 1 h at 37°C and fractionated by centrifugation in a 10 to 40% gradient of neutral sucrose for 18 h at 25,000 rpm in the Beckman SW41 rotor. Fragments ranging in size from 2 to 10 kilobases (kb) were pooled, dephosphorylated with calf intestinal phosphatase, and ligated at 14°C for 12 h with pHL1 cut to a linear form with *Bam*HI at an insert-to-vector ratio of 5:1. DNA from the ligation reaction (1.2 μg) was used to transform *U. maydis* protoplasts. A total of 336 hygromycin-resistant transformants were pooled by washing the surface of the agar plate with 1 ml of YEPS containing hygromycin (0.1 mg/ml) diluted with 50 ml of the same medium and then incubated at 32°C until the culture reached a density of 5×10^7 cells per ml. DNA enriched for low-molecular-weight DNA was prepared and used to transform *E. coli* DH5 to ampicillin resistance. From a total of 141 transformants, 30 were chosen for further analysis. Rapid lysates were prepared from these 30 transformants, and the plasmid DNA was digested with *Hin*dIII and *Sst*I. Unique fragments were present in 12 of the 30 plasmid preparations.

Southern hybridization. DNA was transferred from agarose gels to a Zeta Probe membrane (BioRad Laboratories) by the alkaline blotting method described by Reed and Mann (25). Hybridization was performed at 68°C in 1.5 × SSPE (0.27 M NaCl, 0.015 M sodium phosphate [pH 7.5], 0.015 M EDTA) containing 1% SDS, 0.5% nonfat dry milk (Carnation), and 0.5 mg of denatured salmon sperm DNA per ml. DNA probes were labeled with random primers according to Feinberg and Vogelstein (12).

DNA sequencing. The 383-base-pair (bp) *Ssp*I-*Ssp*I subfragment of the 1.7-kb UARS1 region was subcloned by ligation into the *Hin*cII site of pUC18. The DNA was sequenced with ^{35}S-labeled nucleotide (4) by the dideoxy chain termination method (26) with Sequenase (United States Biochemical Corp.).

CHEF gels. Chromosome-sized DNA molecules were separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis by the method of Chu et al. (11) at 200 V with 80-s pulse cycle for 27 h at 10°C. *U. maydis* protoplasts were prepared from 100 ml of cell culture (2×10^7 cells per ml), suspended in 1 ml of 0.1 M EDTA (pH 8.0)–1 M sorbitol, and mixed with 2 ml of 1% low-melting-point agarose (37°C) in 0.125 M EDTA, pH 8.0. The mixture was poured into a mold and solidified on ice for 30 min. Slices from the molded plugs were placed in 4 ml of lysis buffer containing 10 mM Trizma base, 0.5 M EDTA, pH 9.0, 1% lauroyl sarcosine, and 2 mg of proteinase K per ml and incubated at 50°C for 48 h. Prior to use, agarose plugs were washed with 0.5 M EDTA (pH 9.5) for 2 h at room temperature. Electrophoresis was carried out in 45 mM Trizma base–45 mM boric acid–1 mM EDTA.

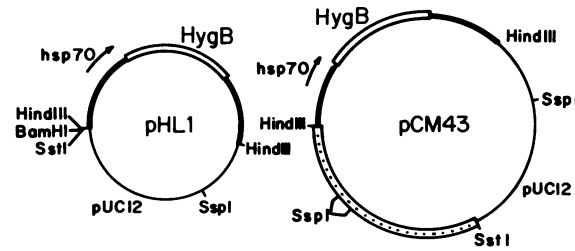

FIG. 1. Transforming plasmids. Plasmid pHL1 is a pUC12 derivative (33) containing the aminoglycoside phosphotransferase gene from *Streptomyces hygroscopicus*, conferring hygromycin resistance (13), behind a *U. maydis* hsp70-like (heat shock protein) promoter. Plasmid pCM43 has a 3.4-kb fragment of *U. maydis* DNA containing UARS1. It was constructed as follows. Plasmid pHL1 DNA (5 μg) was partially cut with *Hin*dIII (12 U) for 1 h at 37°C in the presence of ethidium bromide (75 μg/ml), and the digestion products were separated by agarose gel electrophoresis. The band containing full-length linear pHL1 DNA was excised, and the DNA was extracted from the piece of agarose gel. The DNA was then digested with *Sst*I, dephosphorylated with calf intestinal phosphatase, and repurified after deproteinization by agarose gel electrophoresis. A 3.4-kb restriction fragment obtained by digesting the 7.4-kb insert in plasmid 18 (Table 1) with *Hin*dIII and *Sst*I was ligated into the vector DNA to yield pCM43.

RESULTS

Isolation of ARSs. In *S. cerevisiae*, plasmids containing chromosomal DNA elements conferring autonomous replication transform yeast spheroplasts at much higher frequencies than plasmids capable of being maintained only by integration into the yeast genome (2, 9). We used a similar selection scheme exploiting the differential transformation frequency to isolate DNA segments from *U. maydis* DNA capable of autonomous replication. High-molecular-weight DNA prepared from *U. maydis* was partially digested with *Sau*3A restriction endonuclease. Digestion products were fractionated by centrifugation in a sucrose gradient, and DNA fragments from 2 to 10 kb in size were isolated. These fragments were ligated into the single *Bam*HI site of the pUC12 derivative plasmid pHL1 (Fig. 1), and the mixture was used to transform *Ustilago* protoplasts. From a ligation mixture containing 1 μg of pooled fragments and 0.2 μg of linear pHL1 as input DNA, about 300 hygromycin-resistant transformants were obtained. DNA enriched in plasmid was extracted from the pooled transformants, and the resulting preparation was then used to transform competent *E. coli* cells. A total of 141 *E. coli* transformants were obtained, and 30 of these were picked for further study. Analysis of rapid lysates revealed that all 30 clones contained plasmids, that

TABLE 1. Transformation frequencies of plasmids containing random DNA inserts[a]

Plasmid	Insert size (kb)	No. of transformants per 0.2 μg of DNA
5	5.6	1,390
7	2.8	1,030
9	4.0	1,320
11	7.4	1,872
12	7.1	1,375
13	8.2	1,888
14	2.7	2,331
18	7.4	2,694
19	6.8	592
20	5.2	2,219
22	5.2	692
30	11.7	2,016
pHL1	None	0

[a] Plasmids containing unique *U. maydis* genomic DNA inserts were tested for transformation efficiency; 0.2 μg of each plasmid DNA was used to transform protoplasts. At this level of DNA, the response in number of transformants is no longer linear with the dose of DNA.

these plasmids were all larger in size than pHL1, and that in this collection 12 different unique DNA inserts were represented. DNA from each of the 12 different plasmids was used to transform *U. maydis* protoplasts. The transformation frequency in each case was several orders of magnitude higher than the transformation frequency of the plasmid containing no insert (Table 1).

Plasmid 18, which gave the highest frequency of transformation, was chosen for further study. The cloned DNA segment contained in this plasmid was 7.4 kb. When the plasmid was digested with *Sst*I and *Hin*dIII, the 7.4-kb sequence was excised and cut into four fragments, two terminating in *Hin*dIII-*Sst*I ends and two terminating in *Sst*I ends. These were ligated separately back into pHL1 to identify the fragment with ARS activity. A 3.4-kb fragment contained the ARS element. We have observed that the plasmid pCM43 (Fig. 1) harboring this 3.4-kb fragment had a transformation frequency of up to 9×10^4 per microgram of DNA and used it in the analysis described below. Transformation frequencies by plasmids containing the other three fragments were no higher than pHL1 alone (ca. 10 to 20 transformants per microgram).

In an operational sense, two properties are expected of a plasmid containing an ARS (35). These are high frequency of transformation and instability under nonselective conditions. When we examined cells transformed to hygromycin resistance with pCM43 but grown for 15 generations in the absence of hygromycin, less than 20% remained resistant to hygromycin when replated on hygromycin medium. After 100 generations, less than 0.1% remained resistant to hygromycin. In contrast, cells transformed to hygromycin resistance with the integrating plasmid pHL1 and grown for 100 generations without selection showed no loss in viability when plated on medium containing hygromycin. Direct measurement indicated that the plasmid was lost. When DNA extracted from transformed cells grown for 20 generations in the absence of hygromycin was examined by Southern analysis, the level of plasmid present was less than one-fourth of that from transformed cells grown in the presence of hygromycin (Fig. 2). These experiments demonstrate that the plasmid lacks a functional segregating element and that it is not integrated.

Transmission of the ARS plasmid in transformed cells. The ARS-containing plasmid appears to be maintained as an

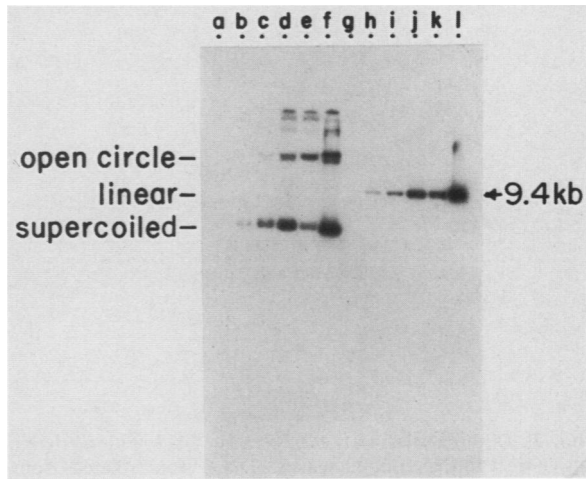

FIG. 2. Transmission of plasmid DNA in transformants. DNA enriched for plasmid was isolated from transformants grown in 50 ml of culture propagated through 20 generations in YEPS with or without hygromycin. Approximately equal amounts of DNA, as determined by ethidium bromide fluorescence enhancement, were loaded on each lane of a 0.7% agarose gel containing 40 mM Tris-acetate (pH 8.0)–2 mM EDTA. After electrophoresis and blot transfer to a Zeta probe membrane, Southern hybridization was carried out with ^{32}P-labeled pUC12 DNA as the probe. Lanes a to f, DNA isolated from cells was run directly; lanes g to l, DNA was first digested with *Sst*I. Lanes: a and g, untransformed control; b and c, transformants grown for 20 generations, minus hygromycin; d, transformant plus hygromycin (0.1 mg/ml); e, transformant plus hygromycin (0.2 mg/ml); f, uncut pCM43; g, untransformed control; h and i, transformants grown for 20 generations minus hygromycin, *Sst*I cut; j, transformant plus hygromycin (0.1 mg/ml), *Sst*I cut; k, transformant plus hygromycin (0.2 mg/ml), *Sst*I cut; l, control pCM43 DNA, *Sst*I cut.

extrachromosomal element through replication. When DNA from cells transformed by the plasmid lacking an ARS was analyzed by Southern hybridization, it was apparent that the plasmid had become integrated into the cellular genome (data not shown) as previously reported by Wang et al. (34). On the contrary, the electrophoretic mobility of the plasmid DNA isolated from cells transformed by the ARS-containing plasmid pCM43 was suggestive of circular topology (Fig. 2). When undigested DNA was electrophoresed in an agarose gel, two major bands with mobilities identical to those of control form I and form II plasmid DNA were observed. Some minor bands with slower mobilities were also observed and were presumed to be higher-order forms of plasmid DNA. After the DNA was cut with a restriction enzyme which cleaves the input plasmid once, a single band with mobility corresponding to that of full-length linear plasmid DNA was seen. Furthermore, no gross structural rearrangement of the input transforming DNA was apparent.

Evidence for replication of the ARS plasmid pCM43 was obtained by monitoring the sensitivity of methylated plasmid DNA to restriction endonuclease digestion after transformation (24). ARS-containing plasmid pCM43 prepared from the *dam*$^+$ *E. coli* strain DH5, which methylates adenine in the sequence GATC, was cut by *Dpn*I but not by the isoschizomer *Mbo*I (Fig. 3). However, after transformation of *U. maydis* and 20 generations of growth of transformants under antibiotic selection, plasmid DNA extracted from the transformants had become resistant to digestion by *Dpn*I but sensitive to *Mbo*I, indicating a loss of methylation. This loss

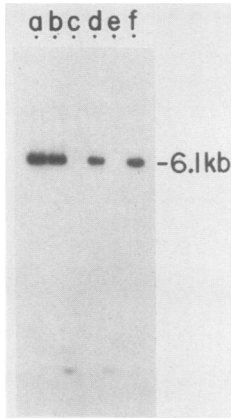

FIG. 3. Differential digestion of plasmid DNA with methylation-sensitive restriction endonucleases. DNA was isolated from *U. maydis* transformed with pCM43 and grown for 20 generations under hygromycin (0.2 mg/ml) selection. DNA was digested with the indicated restriction endonucleases, electrophoresed in a 0.7% agarose gel, and analyzed by Southern hybridization. To avoid complications in interpretation arising from homology of plasmid pCM43 with genomic DNA sequences, the probe used was pUC12 DNA, which only hybridizes to sequences on the plasmid. All DNA samples were cut with *Hin*dIII. *Hin*dIII digestion of pCM43 yields fragments of 3.3 and 6.1 kb. The 6.1-kb fragment contains pUC12 and the ARS sequence and hybridizes with the pUC12 DNA probe. The 3.3-kb fragment contains the *U. maydis hsp70* gene and the hygromycin phosphotransferase gene and does not hybridize with the probe. Lanes a to c, Control plasmid DNA isolated from *dam*$^+$ *E. coli* DH5 and cut with the indicated enzymes. Lanes d to f, DNA isolated from *U. maydis* transformed with pCM43 and cut with the indicated enzymes. Lanes a and d, *Hin*dIII; lanes b and e, *Hin*dIII, *Mbo*I; lanes c and f, *Hin*dIII and *Dpn*I. The fast-moving fragment in lanes c and e is about 0.8 kb in length. This is the largest product left after *Mbo*I or *Dpn*I digestion of the 6.1-kb fragment and must contain a junction between the ARS and the pUC12 sequences. The largest fragment expected from digestion of pUC12 is 341 bp. Fragments smaller than about 500 bp ran off the gel.

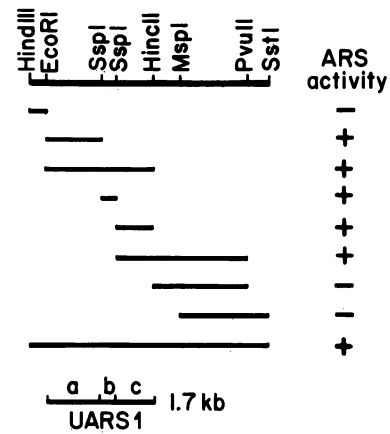

FIG. 4. Localization of UARS1 by restriction analysis. The *Hin*dIII-*Sst*I insert in pCM43 was removed and purified by agarose gel electrophoresis. The indicated enzymes were used to reduce the fragment further. The termini of each fragment were made flush by filling in recessed ends with Klenow DNA polymerase or removing protruding ends with the exonuclease associated with T4 DNA polymerase. Each fragment was cloned separately by blunt-end ligation into the single *Ssp*I site of pHL1, and the resulting plasmid DNA was amplified in *E. coli*. Protoplasts of *U. maydis* were transformed with each recombinant plasmid, and ARS activity was determined. The frequency of transformation (no. of transformants per 0.2 μg of each plasmid) was as follows: *Hin*dIII-*Eco*RI, <10; *Eco*RI-*Ssp*I, 3,880; *Eco*RI-*Hin*cII, 4,400; *Ssp*I-*Ssp*I, 3,440; *Ssp*I-*Hin*cII, 3,680; *Ssp*I-*Pvu*II, 2,150; *Hin*cII-*Pvu*II, <10; *Msp*I-*Sst*I, <10; *Hin*dIII-*Sst*I, 5,360.

in the pattern of methylation is in keeping with a mode of transmission in which the plasmid replicates autonomously.

Copy number. Under the conditions we used for transformation, the copy number of the ARS plasmid was maintained at about 25 per cell. This estimate was arrived at by comparing the signal on a Southern blot of a set of known amounts of plasmid DNA with the signal arising from plasmid DNA extracted from transformed cells. It should be noted, however, that this number is only an approximation, since no internal standard was used as a reference.

The doubling time of transformants was greatly reduced at high concentrations of hygromycin. Over a 10-fold range in the concentration of hygromycin, the growth rate varied almost 3-fold. In contrast, the plasmid copy number appeared to be invariant over the same 10-fold range in hygromycin concentrations.

Subcloning and sequence analysis of the ARS. The sequence conferring ARS activity contained within the 3.4-kb fragment in pCM43 was mapped by subcloning DNA fragments from this region. After excising the 3.4-kb fragment by digesting the plasmid with *Hin*dIII and *Sst*I, a battery of restriction enzymes was used to reduce it to a number of smaller fragments (Fig. 4). The termini of each fragment were made flush by filling in recessed 3′ ends with Klenow DNA polymerase I or removing protruding 3′ ends with the exonuclease activity associated with T4 DNA polymerase. The blunt-ended fragments were separately ligated into the single *Ssp*I site of the nonreplicating vector pHL1, and the product in each case was tested for ARS activity by measuring the transformation frequency in *U. maydis* protoplasts. A 1.7-kb *Eco*RI-*Hin*cII fragment was strongly positive and thus contained the ARS. This sequence is referred to as UARS1, for *Ustilago* ARS 1. Each of the three subfragments contained within this 1.7-kb region was by itself strongly positive in conferring high-frequency transformation, although they were not tested individually for mitotic stability. Thus, UARS1 contains a cluster of sites, each of which is individually active.

The smallest high-frequency transforming fragment was contained within a 383-bp *Ssp*I-*Ssp*I restriction fragment. Its sequence was determined (Fig. 5). By itself this fragment was not particularly A+T rich (56%) and contained no sequence identical to the 11-bp *S. cerevisiae* ARS consensus sequence 5′-(A/T)TTTAT(A/G)TTT(A/T)-3′ (7, 22). However, there are several notable features in the sequence, including a 12-bp polypurine stretch (residues 102 to 113), an 11-bp A-T stretch (residues 196 to 206), two 30-bp direct repeats lying end to end, an 8-bp unit (5′-TGATTCPuPy-3′) repeated seven times, and five 11-bp units having 8 of 11 residues in common with the yeast consensus sequence. One of these 11-bp units in each orientation flanked the polypurine stretch. The other three lay within or overlapped the two 30-bp repeats.

Heterologous DNA segments have been found to be capable of autonomous replication in yeast cells (30). In a number of instances these segments have been found to contain stretches close in sequence to the yeast consensus (35). Nevertheless, regardless of identity in 8 of 11 nucleotides between the yeast consensus and the above-mentioned sequences in the UARS1 subfragment, the 383-bp stretch did not function as an ARS in yeast. When *S. cerevisiae* was

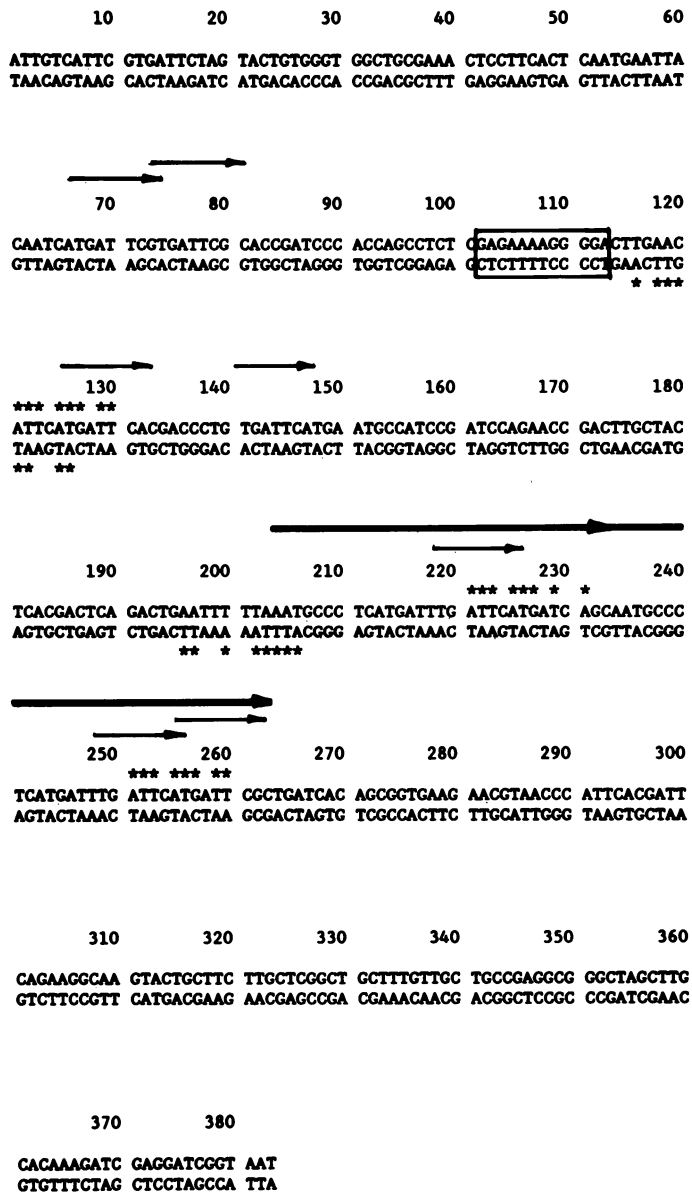

FIG. 5. DNA sequence of the 383-bp UARS1 subfragment. The 383-bp *Ssp*I-*Ssp*I fragment from UARS1 was cloned into the single *Hin*cII site of pUC18 in both orientations. Nucleotides 82 to 283 were read from both strands; the remaining sequence was read from two reactions on only one strand. The boxed residues 102 to 113 indicate the 12-bp polypurine stretch. The 11-bp A+T stretch is within the region 196 to 206. The long bold arrows lie over the 30-bp direct repeats. The short thin arrows lie over the 8-bp direct repeats. The stars indicate stretches in both orientations with partial identity to the 11-bp yeast consensus sequence.

transformed with a derivative of the integrative vector YIp5 containing the 383-bp fragment, no increase in transformation frequency over that of YIp5 alone was observed.

Chromosomal location of UARS1. Chromosome-sized DNA molecules from *U. maydis* were separated by CHEF gel electrophoresis. Bands representing molecules in the size range of approximately 300 to 1,600 kb were separated (Fig. 6), although clusters of molecules of approximately 600 and 1,600 kb were not well resolved. The UARS1 probe hybridized to chromosomes in these unresolved clusters. The signal from the faster-migrating chromosome cluster was approximately five times more intense than that from the more slowly migrating cluster.

DISCUSSION

UARS1 is a genetic element that promotes high-frequency transformation of plasmids in *U. maydis*. It is 1 of 12 such elements we isolated by screening plasmids containing random genomic fragments for high-frequency transformation. UARS1 compares favorably to the most powerful of the yeast ARSs, transforming at a frequency approaching 10^5 per microgram of DNA. Thus, the UARS1 element will prove useful in the construction of high-efficiency transforming vectors for molecular genetic studies of *U. maydis*.

UARS1 appears to confer autonomous replication on the plasmid which harbors it. Free plasmid with the mobility

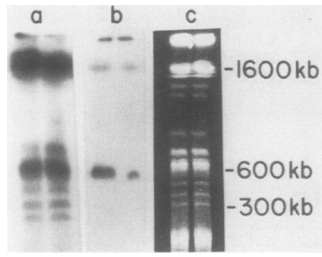

FIG. 6. Localization of UARS1 sequence to chromosome-sized DNA molecules. Chromosome-sized DNA molecules from *U. maydis* were separated by CHEF gel electrophoresis. Before DNA transfer to a Zeta probe membrane, the gel was soaked in 0.25 M HCl for 45 min and then transferred in 0.4 M NaOH. The hybridization probe was the 383-bp *Ssp*I-*Ssp*I fragment. To visualize the separated chromosomes on the Southern blot and correlate them with the bands stained with ethidium bromide, *U. maydis* genomic DNA cut with *Hin*dIII and labeled by random priming was used as the probe in separate lanes. Approximate sizes indicated are based on *S. cerevisiae* chromosome standards (11). Lanes: a, total *U. maydis* DNA used as probe; b, *Ssp*I fragment used as probe; c, ethidium bromide-stained gel.

expected of circular DNA and without any gross structural alterations can be detected in transformants. Analysis of plasmid DNA by a combination of methylation-sensitive and methylation-resistant restriction endonucleases strongly suggests transmission through replication. While it remains a possibility that a cellular demethylase is responsible for the loss of adenine methylation from the transforming DNA, it seems likely that unmethylated sites arise simply from new synthesis of plasmid DNA.

Plasmids containing the UARS1 element are mitotically unstable in transformants, as demonstrated after growth under nonselective conditions, and are lost over the course of several generations. After 15 generations, less than 20% of transformed cells retained the plasmid. In contrast, yeast plasmids containing ARS1 (31) or ARS2 (19) are lost more rapidly, only 1 to 5% of transformed cells maintaining the plasmids after 15 generations. An exception to instability is the TRP1 R1 circle of yeast cells (36). After 15 generations in nonselective medium, 75 to 80% of transformants retained the plasmid. The mitotic stability in this case has been attributed to the high copy number of TRP1 R1 circles, 200 per cell as opposed to 20 to 50 copies per cell for other ARS plasmids. However, it is thought that there may be another factor important to stability involving segregation bias. Pedigree analysis (23) has revealed strong segregation bias in the case of ARS plasmids but no bias in the TRP1 R1 circle. Thus, plasmid molecules are distributed between mother and daughter cells with little disparity. We estimate the copy number of the UARS1 plasmid to be 25 per cell, which is similar to estimates for ARS plasmids in yeast. However, the *U. maydis* plasmid is more stable in mitosis, perhaps reflecting greater parity in distribution during cell division.

The concept of separate, functional domains emerged from deletion analysis of yeast ARSs (8, 21, 22, 28, 32). Within ARS1 of yeast, there is a 14-bp core sequence containing the ARS consensus element essential for replicator activity. Besides this domain, there are two others flanking the core sequence that are important for efficient, stable replication. In ARS 17 of the fission yeast *Schizosaccharomyces pombe*, two sequences separated by 100 bp but lying within a 220-bp stretch appear to be necessary for ARS activity. Thus, full ARS activity may result from contributions by several distinct DNA sequences. The UARS1 is localized on a 1.7-kb fragment and contains a cluster of ARS elements. At least three domains lying within this 1.7-kb fragment can independently confer autonomous replication activity, an observation distinct from the finding that yeast ARSs can be separated into domains of different function. However, our results are reminiscent of findings in which yeast ARS clusters were noted in telomeric regions of chromosomes (10), the reiterated array of rDNA replicons of chromosome XII (27), and the mating type cassettes (7). Assuming that these ARS sequences contained within UARS1 represent origins, such clustering might help ensure that the replication machinery initiates with high probability at this site.

The nucleotide sequence of the smallest of the three active domains of UARS1 does not contain the 11-bp consensus found in *S. cerevisiae* ARS elements (22) or the 11-bp consensus found in *S. pombe* ARS elements (21). However, it does contain some interesting features. There is a polypurine stretch of 12 residues, seven virtually identical 8-bp direct repeats, and two 30-bp direct repeats lying end to end. Near the ends of each 30-bp repeat and next to the polypurine stretch lie sequences in both orientations in which there is identity in 8 of 11 bp with the yeast consensus. Whether any of these sequences plays a functional role in conferring autonomous replication activity remains to be determined. It is interesting that the 8-bp repeat unit is nearly identical to the consensus binding sequence of the yeast GCN4 regulatory protein (17).

The 383-bp subregion of UARS1 is specific for *U. maydis*. It has no demonstrable activity in *S. cerevisiae*. However, it may not be an entirely unique sequence in *U. maydis*. At the level of hybridization used in Southern blot analysis, more than one chromosome-sized DNA molecule was seen to show sequence similarity with it.

We have a long-standing interest in the genes controlling genetic recombination in *U. maydis*. As an approach to isolating and studying these genes, we wanted to establish a transformation system of general utility in *U. maydis*. As demonstrated in this paper, high-frequency transformation and recovery of freely replicating plasmid have been achieved. These desirable features will make gene isolation from *U. maydis* by complementation of mutations a practical strategy. We have begun such studies and have isolated DNA fragments that can complement recombination-deficient mutants.

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