Pathways of Transformation in *Ustilago maydis* Determined by DNA Conformation

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Manuscript received August 18, 1989
Accepted for publication December 26, 1989

ABSTRACT

Ustilago maydis was transformed by plasmids bearing a cloned, selectable gene but lacking an autonomously replicating sequence. Transformation was primarily through integration at nonhomologous loci when the plasmid DNA was circular. When the DNA was made linear by cleavage within the cloned gene, the spectrum of integration events shifted from random to targeted recombination at the resident chromosomal allele. In a large fraction of the transformants obtained using linear DNA, the plasmid DNA was not integrated but was maintained in an extrachromosomal state composed of a concatameric array of plasmid units joined end-to-end. The results suggest the operation of several pathways for transformation in *U. maydis*, and that DNA conformation at the time of transformation governs choice of pathways.

ASIDE from the enormous utility provided for molecular genetics, integration of nonreplicating plasmids into the genome of *Saccharomyces cerevisiae* has been used as a model system for studying recombination (SZOSTAK *et al.* 1983). The double-strand break model was conceived in part from the finding in this system that double-strand DNA breaks are recombinogenic (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). A plasmid with a yeast DNA sequence can be made to transform yeast at a high frequency after cutting the plasmid within that sequence. Integration of the plasmid then takes place almost invariably by homologous recombination at the corresponding chromosomal locus.

Transformation by nonreplicating plasmids has also been observed in other fungi. Almost without exception in the familiar fungi used in the laboratory for genetic analysis, integration has been found to take place for the most part nonhomologously. In *Neurospora crassa* (PAIETTA and MARZLUF 1985; CASE 1986) and *Aspergillus nidulans* (MILLER, MILLER and TIMBERLAKE 1985), for example, some homologous integration takes place, but 70–95% of the plasmid integrants were found at nonhomologous loci. Typically, transformants were found to contain tandem and rearranged copies of the transforming plasmid. Furthermore, little difference in transformation frequency was observed with linear plasmid DNA compared with circular plasmid DNA.

In mammalian plasmid transformation systems the predominant mode of integration is nonhomologous (THOMAS, FOLGER and CAPECCHI 1986; KATO, ANDERSON and CAMERINI-OTERO 1986; ROBINS *et al.* 1981). Nevertheless, it is apparent that linear molecules are much more recombinogenic than uncut plasmid (LIN, SPERLE and STERNBERG 1984; SONG *et al.* 1985), a finding similar to that in yeast.

We have been interested in developing transformation in *Ustilago maydis* as a model system for analyzing the mechanism of recombination. *U. maydis* is well suited for such an analysis because mutants altered in recombination and repair have been isolated (HOLLIDAY 1965, 1967) and also because some aspects of the biochemistry of recombination have been studied (KMIEC and HOLLOMAN 1982). To explore the mechanism we have begun investigating how plasmid DNA recombines with genomic DNA sequences during transformation. In the experiments in this paper, we demonstrate the mode of transformation by plasmid DNA in circular and linear form. We show that integrative transformation takes place primarily through nonhomologous recombination when plasmid DNA is circular, but through homologous recombination when the same plasmid is made linear by cutting within the *U. maydis* sequence. We also describe a novel replicative mode of transformation by linear DNA lacking an autonomously replicating sequence.

MATERIALS AND METHODS

Cell culture and transformation: *Ustilago maydis* strain 87 (*ad1-1 leu1-1* a_2b_2) was obtained from R. HOLLIDAY, CSIRO, N. Ryde, Australia. *ad* and *leu* refer to auxotrophic requirements for adenine and leucine, respectively. Minimal medium used was described by HOLLIDAY (1974) with the

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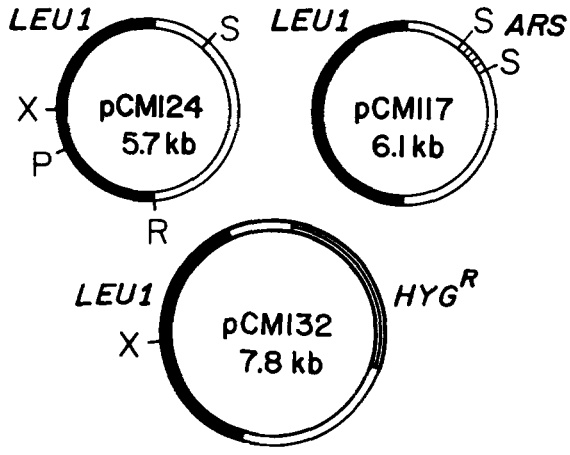

FIGURE 1.—Plasmids containing the *LEU1* gene. Plasmid pCM124 has a 3.0-kb fragment of the *LEU1* gene (solid) in pUC12 (open). pCM117 is identical to pCM124, but with the 383 bp autonomously replicating sequence (radial hatches). pCM132 contains the *LEU1* gene (solid) and a 2.1 kb fragment with the *hsp70*-hygromycin phosphotransferase gene fusion (striped) in pUC18 (open). Restriction endonuclease sites are X (*Xho*I), S (*Ssp*I), P (*Pst*I), and R (*Eco*RI).

exception that sucrose replaced glucose. Minimal medium was supplemented with glycine at 100 μg/ml for growth of leucine auxotrophs (HOLLIDAY, 1961). Rich YEPS medium contained 1% yeast extract, 2% peptone and 2% sucrose. Transformation was performed essentially as described before (TSUKUDA *et al.* 1988) with the following modifications: protoplasts were prepared from cells of strain 87 by treatment with Novozyme 234 (NovoBiolabs) at 50 μg/ml for 10 min until >95% of the cells had been converted to protoplasts. Use of top agar was eliminated. Protoplasts were spread directly onto regeneration agar plates containing 1 M sorbitol with no adverse effect on regeneration. The transformation efficiency of strain 87 was about 50% of the level observed with the wild-type strain 518.

Plasmid construction: Plasmid pCM117 was a pUC12 derivative that contained a 3.0-kb *Hin*dIII-*Eco*RI *U. maydis* DNA fragment encompassing the *LEU1* gene (FOTHERINGHAM and HOLLOMAN 1989) and the 383-bp *Ssp*I-*Ssp*I active subfragment from the autonomously replicating sequence UARS1 (TSUKUDA *et al.* 1988) inserted into the *Ssp*I site. The 383 bp *Ssp*I-*Ssp*I fragment was removed to yield pCM124. Plasmid pCM132 contained a *hsp70*-hygromycin phosphotransferase gene fusion on a 2.1-kb fragment inserted into the *Ssp*I site of pCM124. The 2.1-kb sequence was derived from a pUC18 construct containing the 1-kb bacterial hygromycin phosphotransferase gene from pLG90 (GRITZ and DAVIES 1983) in fusion with approximately 500 bp of the *hsp70* promoter sequence from pHL1 (WANG, HOLDEN and LEONG 1988). All plasmids were prepared and maintained in *Escherichia coli* DH1.

Blot hybridization: DNA from *U. maydis* to be analyzed by conventional blot hybridization was isolated according to the method described by HOFFMAN and WINSTON (1987). After digestion with the appropriate restriction endonuclease, DNA fragments were separated by electrophoresis in 1% agarose gels containing 40 mM Tris-acetate, pH 7.6, 5 mM sodium acetate, 1 mM ethylenediaminetetraacetate (EDTA). Fragments were transferred to Zeta Probe membrane (Bio-Rad Laboratories) by the alkaline blotting method of REED and MANN (1985). DNA to be analyzed by pulse field gel electrophoresis (CHEF) was prepared as previously described (TSUKUDA *et al.* 1988) and transferred to Zeta Probe membrane as described by VOLLRATH *et al.* (1988). Hybridizations were performed at 68° in a solution containing 0.27 M sodium chloride, 15 mM sodium phosphate (pH 7.5), 15 mM EDTA, 1% sodium dodecyl sulfate, 0.5% nonfat dry milk, 1 mg per ml denatured salmon sperm DNA, and 10^6 cpm per ml ^{32}P-labeled DNA probe. The probe used was the 1.0-kb *Xho*I-*Eco*RI fragment encompassing part of the *LEU1* gene unless specified otherwise. It was labeled to high specific activity by the method of FEINBERG and VOGELSTEIN (1983).

TABLE 1

DNA conformation influences frequency of transformation

Plasmid	Frequency (per μg DNA)	Number	Mode of transformation I	II	III	Episomal
Experiment 1						
pCM117, uncut	4×10^4					
pCM124, uncut	8	22[a]	0	14	7	0
pCM124, X-cut	8.9×10^2	23[b]	3	0	0	15
pCM124, S-cut	9.0×10^2	22[c]	0	0	0	19
Experiment 2						
pCM132, X-cut	2.4×10^2	12	5	1	4	2
pCM132, X-cut[d]	4.9×10^2	11	5	0	0	6

Cells of Leu$^-$ strain 87 were transformed to Leu$^+$ with the indicated plasmid DNA in either the circular form or the linear form cut with the indicated restriction endonuclease. DNAs from transformants were analyzed by blot hybridization. The results presented, representing a summary of all of the studies performed, are based on Southern analysis and in some cases mitotic stability. Restriction enzymes used were X (*Xho*I), and S (*Ssp*I).

[a] One ambiguous, probably type I.
[b] Five ambiguous.
[c] Three ambiguous.
[d] Selection was for Leu$^+$ and HYG^R.

RESULTS

Efficiency of transformation: We investigated the ability of plasmid molecules containing a cloned, selectable gene to transform *U. maydis*. We constructed plasmid pCM124 (Figure 1) containing the DNA sequence encoding the *LEU1* gene of *U. maydis* (FOTHERINGHAM and HOLLOMAN 1989). This plasmid lacks an autonomously replicating sequence (ARS). Consequently, transformation with this plasmid would be expected to take place by recombination with genomic sequences and thus should be infrequent. As predicted, transformation by the nonreplicating plasmid pCM124 was much less frequent than transformation by the same plasmid containing an ARS (Table 1). Cutting the nonreplicating plasmid DNA to a linear form by digestion with a restriction enzyme increased the frequency of transformation 100-fold. The same increase in frequency was observed regardless of whether the plasmid was cut within the *U. maydis* sequence with *Xho*I or cut outside this sequence with *Ssp*I.

Transformation by circular DNA: We examined the structure of DNA from transformants obtained

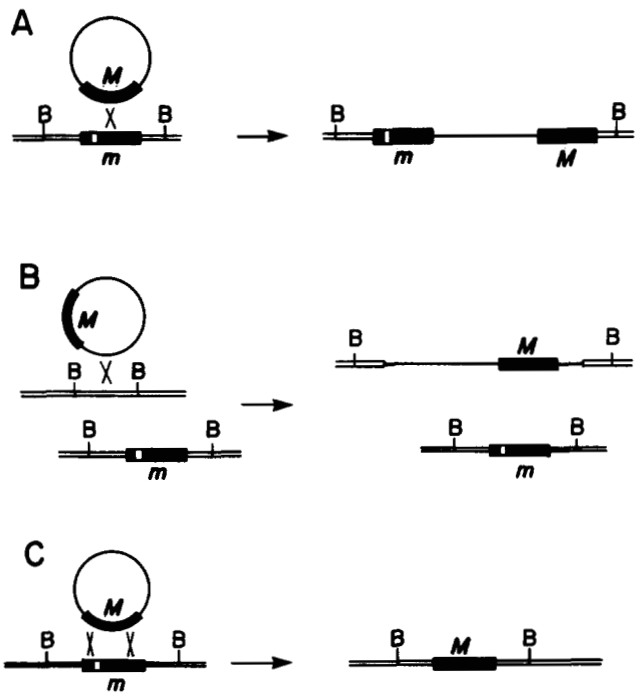

FIGURE 2.—Modes of transformation. Plasmid carrying a selectable marker *M* (solid bar) transforms cells of genotype *m* (interruption in bar) by recombination. *A*, Type I, single homologous crossover to yield a tandemly integrated copy; B, type II, nonhomologous integration of plasmid; C, type III gene conversion or replacement of mutant locus.

using circular and linear plasmid DNA and categorized recombination events as one of the three types defined by HINNEN, HICKS and FINK (1978). A type I event refers to homologous integration of the complete plasmid into the *leu1* locus in the genome (Figure 2) resulting in gene duplication. Type II transformation means the plasmid integrates at some site other than the resident *leu1* allele. Type III represents no change in DNA hybridization pattern from the untransformed host, indicating a replacement event.

We examined 22 Leu$^+$ strains generated by transformation of the *leu1-1* strain 87 with uncut plasmid pCM124. To establish the mode of transformation we digested DNA from transformants with *Bam*HI restriction endonuclease, which does not cut within the plasmid DNA sequence, and used an internal sequence from the *LEU1* gene or else pUC18 DNA as hybridization probe (Figure 3). About one-third of the transformants lacked integrated vector sequences. It is unlikely that this class represented revertants because the reversion frequency of the *leu1-1* allele is quite low, less than 10^{-8} (FOTHERINGHAM and HOLLOMAN 1989). Such substitution transformants most probably resulted from gene conversion, or else double-crossover. In two-thirds of the transformants, two fragments were observed when the *LEU1* gene sequence was used as hybridization probe of Southern blots. One was the fragment containing the endogenous *leu1* allele. The other contained vector DNA sequences and appeared to be a different size in each case. These were type II transformants and probably resulted from integration by nonhomologous recombination. In only one case did more than one nonhomologous integration event occur (Figure 3, lane e). In no case was there clear evidence for homologous integration although one case (Figure 3, lane v) might be explained by homologous integration accompanied by partial deletion. Confirmation of the nonhomologous mode was obtained by digesting DNA from transformants with *Pst*I, which cuts the plasmid pCM124 once in the *LEU1* DNA sequence. Homologous integration would be revealed by appearance of one band corresponding to linear plasmid DNA in addition to the two fragments arising from the endogenous *leu1* allele. However, what we observed in every case in question was the appearance of at least two new bands, none corresponding in size to linear plasmid DNA, but different in each individual transformant, a result indicative of nonhomologous integration (data not shown).

Transformation by linear DNA: We examined 23 Leu$^+$ transformants chosen at random that were generated using linear pCM124 DNA cut in the *LEU1* gene with *Xho*I. While in no case in this set of transformants did we obtain evidence for type III or substitution transformation, 3 out of 23 cases were recognized immediately from the *Bam*HI pattern obtained after Southern blot analysis to result from homologous integration. The endogenous fragment hybridizing with the *LEU1* probe had disappeared and was replaced by a larger fragment (*e.g.*, transformant X-5, Figure 4, lane c). The remaining samples all appeared identical to *Bam*HI hybridization patterns in that after digestion a band corresponding to the endogenous fragment remained and a new band of slower mobility was detected (*e.g.*, transformants X-7 and X-4, Figure 4, lanes d and e). This pattern was reminiscent of that obtained above when circular transforming DNA was used, with the exception that the signal from the upper band was 25 times more intense on the average. Tandem integration of the plasmid at a nonhomologous location could account for this pattern. Such a mechanism for integration was proposed before by WANG, HOLDEN and LEONG (1988).

Additional restriction enzyme analysis was done to examine further the structure of the DNA from the transformants. Southern blot analysis of DNA from the untransformed cells after digestion with *Pst*I revealed two junction fragments resulting from splitting the resident *leu1* allele by cleavage within its sequence at the single *Pst*I site (Figure 4, lane f). If transformation were to take place through integration at a nonhomologous locus, two additional fragments would be generated. Concatamer formation accom-

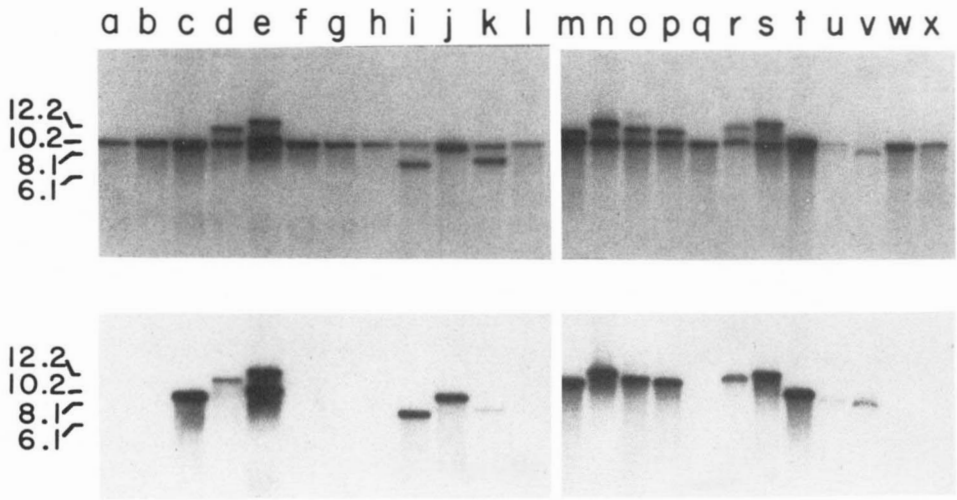

FIGURE 3.—Structure of DNA from transformants obtained using circular plasmid. Leu⁻ strain 87 was transformed to Leu⁺ with uncut pCM124. DNAs from 22 independent transformants (b–w) were cut with *Bam*HI (which does not cut the plasmid) and analyzed by Southern blot hybridization using (upper) the *LEU1* *Xho*I-*Eco*RI fragment from pCM124 as probe. The blotted membrane was then stripped by rinsing three times with boiling 0.1 × SSC, 0.5% SDS, and rehybridized with (lower) pUC18 as probe. The endogenous *leu1* allele is contained on a 10.2-kb fragment (lanes a and x). Type II events are recognized by hybridization to two fragments by the *LEU1* probe in the upper panel, and to one fragment by the pUC18 probe in the lower panel. Type III events are recognized in the upper panel by a single band with no change in mobility and in the lower by the absence of hybridization.

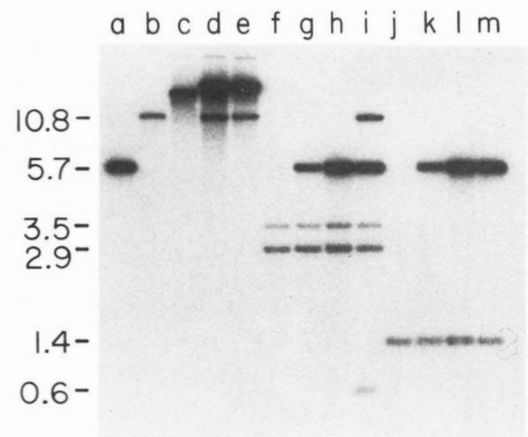

FIGURE 4.—Structure of DNA from representative transformants obtained using linear plasmid. Strain 87 was transformed to Leu⁺ with pCM124 cut to a linear form with *Xho*I. DNAs from representative transformants were digested with *Bam*HI (lanes b–e), *Pst*I (lanes f–i), and *Xho*I (lanes j–m) and analyzed by Southern blot hybridization using the *LEU1* probe. pCM124 cut with *Xho*I (lane a). Each panel of four includes untransformed strain 87 (lanes b, f and j); transformant X-5 (lanes c, g and k); transformant X-7 (lanes d, h and l); and transformant X-4 (lanes e, i and m).

panying transformation would be manifested by release of a fragment of 5.7 kb, representing full length linear plasmid.

Examination of the DNA structure from the 23 Leu⁺ transformants after digestion with *Pst*I revealed the two endogenous *leu1* junction fragments and also indicated concatamer formation in every single case as evidenced by the 5.7-kb fragment. The three transformants judged by the *Bam*HI hybridization pattern to result from homologous integration yielded, as expected, only the two junction fragments from splitting the endogenous *leu1* allele plus the 5.7-kb fragment (*e.g.*, transformant X-5, Figure 4, lane g) diagnostic of concatamers. In nineteen transformants additional fragments were also detected. However, in most cases the sizes of these additional fragments were not random as might be expected from a nonhomologous integrative mode. The transformants had one or both fragments that were 10.8 kb or 0.6 kb in length (*e.g.*, transformant X-4, Figure 4, lane i). Fragments this size would be predicted to result from *Pst*I digestion of a concatameric structure containing head-to-head and tail-to-tail tandem duplications of the linear transforming DNA (Figure 5). *Pst*I digestion would release unique terminal fragments of 5.4 and 0.3 kb if the concatameric structure was a linear array of repeated units. However, no such fragments were observed. In the one remaining transformant, X-7, concatamer formation was evident by appearance of the 5.7-kb fragment, but only the two endogenous junction fragments were detected (Figure 4, lane h). This concatamer contained tandem repeated units arranged only in the head-to-tail orientation.

Additional evidence supporting the notion of a concatameric structure composed of direct and inverted tandem repeats (Figure 5) in many of the transformants was garnered by analysis with a different restriction enzyme. Only a single 1.4-kb fragment was observed after DNA from untransformed cells was digested with *Xho*I (Figure 4, lane j), which cuts once within the *leu1* sequence, because the probe is comprised of only part of the *LEU1* sequence terminating at the *Xho*I site. When DNA from transformant X-4 (see above, Figure 4, lanes e and i) was digested with

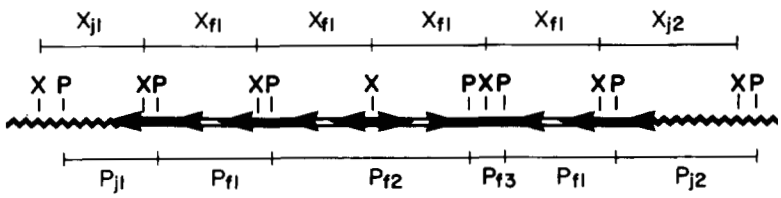

FIGURE 5.—Iterant array of direct and inverted repeats. Schematic representation is shown of an integrated plasmid structure formed by concatamerization of linear plasmid units into an array of direct and inverted repeats. A monomer unit is represented by a solid arrow with an open bar. End-to-end joining of monomer units cut at X followed by homologous integration into the genome at a site represented by a solid arrow would yield the indicated structure. When digested with enzyme P, monomer length fragments of size Pf1 would be released. Fragments Pf2 and Pf3 formed by head-to-head and tail-to-tail joining would also be released. If integration took place through homologous recombination, the junction fragments, Pj1 and Pj2, would be formed. When digested with enzyme X, only monomer units Xf1 and junction fragments Xj1 and Xj2 would be released.

*Xho*I, only the single 1.4-kb junction fragment and the 5.7-kb fragment representing full length linear plasmid DNA could be detected (Figure 4, lane m). This result provides strong support for the origins of the 10.8-kb fragment and the 0.6-kb fragment as the junctions between inverted tandem duplications, not as unique junctions resulting from nonhomologous integration. The same hybridization pattern was observed when DNAs from the representative homologous transformant X-5 (Figure 4, lane k) and transformant X-7 (Figure 4, lane l) were examined. The concatamers formed upon transformation with plasmid made linear with *Xho*I were completely reduced to 5.7 kb, the length of monomer linear plasmid, after digestion with *Xho*I (Figure 4, lanes l and m). This indicated that no degradation of the ends of the input DNA occurred, at least in the fraction that participated in the formation of the concatamers. Five transformants exhibited one or two additional faint bands, but we were unable to interpret for certain whether they arose from rearrangements, nonhomologous integration, or more than one type of event. Thus, analysis of DNA structure from Leu$^+$ transformants obtained using linear transforming DNA provided evidence for a concatameric structure composed of tandem repetitions of plasmid DNA units in direct and inverted orientations and also evidence in three out of 23 cases for homologous integration. More importantly, no unambiguous evidence for nonhomologous integration was obtained.

We carried out a similar analysis of transformation with pCM132, a plasmid containing the *LEU1* gene and an *hsp*70-hygromycin phosphotransferase gene fusion conferring resistance to hygromycin B (Figure 1). After transformation with this plasmid made linear with *Xho*I, selection was for leucine prototrophy and resistance to hygromycin, or simply leucine prototrophy. The DNA isolated from these transformants was analyzed in a manner similar to that above (data not shown) and is summarized in Table 1. As expected, addition of hygromycin B to the medium eliminated replacement events. Of 23 transformants examined, analysis of the DNA structure indicated plasmid integration by homologous recombination had occurred in 14 cases, by nonhomologous recombination in one case, and plasmid concatamer formation had occurred in eight. While integration events were found to have taken place predominantly through homologous recombination, as was seen with pCM124 cut with *Xho*I, there was a swing in the spectrum of events compared with those observed with pCM124. Whereas 80–85% of the pCM124 transformants were concatamers, only 35% concatamers were observed for linearized pCM132. Furthermore, for the latter plasmid type I events predominated. We do not understand the basis for the difference in frequencies of events seen with pCM124 and pCM132, but the presence of the *hsp*70 promoter and the size difference in plasmids might be factors contributing to the shift in events.

Transformation by a novel replicative mechanism: Several lines of evidence led us to the unanticipated conclusion that many of the Leu$^+$ transformants we obtained arose through autonomous replication of the linear transforming DNA even though no canonical ARS was present.

One line of evidence was drawn from a series of analyses of transformant DNA structure carried out in parallel with that above except that pCM124 cut to a linear form with *Ssp*I, which cuts within the vector sequence, was used. As noted before (Table 1) the frequency of transformation to Leu$^+$ was the same regardless of whether the plasmid DNA was cut within the *LEU1* sequence or outside the sequence. This observation contrasts with findings in yeast where there may be an order of magnitude difference in transformation frequency depending on where the plasmid is cut (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). When DNAs from 22 Leu$^+$ transformants chosen at random were digested with *Bam*HI and analyzed by blot hybridization, in no case was there the diagnostic shift of the endogenous fragment indicative of homologous integration. The pattern ob-

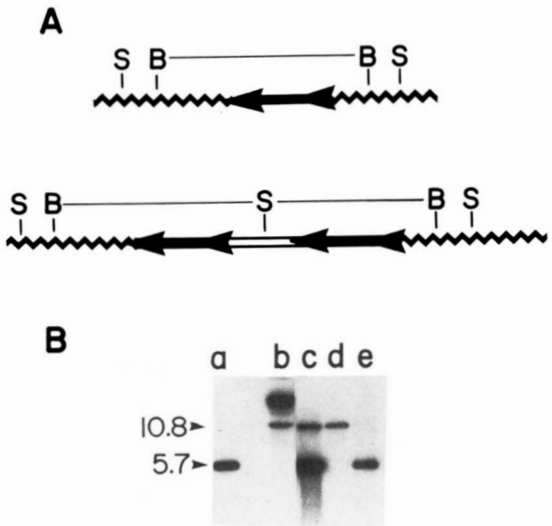

FIGURE 6.—DNA structure from an extrachromosomal transformant. A, Schematic representation of the structure resulting from a single homologous crossover of a plasmid into a target locus. The solid arrow represents the target gene bounded by jagged cellular sequences. Integration results in tandem duplication of the gene interrupted by open vector sequences. Digestion of transformant DNA with *Ssp*I (S) and *Bam*HI(B) would result in generation of two new junction fragments. B, Strain 87 was transformed to Leu$^+$ using pCM124 cut to a linear form with *Ssp*I. DNA from representative transformant S-1 was digested with *Bam*HI with and without *Ssp*I and analyzed by blot hybridization using the *LEU1* probe. Since *Ssp*I cuts *U. maydis* DNA rarely, analysis of DNA was performed by double-digestion with *Bam*HI to reduce the size of the products. (lane a), pCM124 cut with *Pst*I; (lane b), S-1 DNA *Bam*HI cut; (lane c), S-1 DNA *Bam*HI, *Ssp*I cut; (lane d), strain 87 DNA, *Bam*HI cut, *Ssp*I cut; (lane e), pCM124 cut with *Ssp*I.

tained in every case (Figure 6B, lane b) was the same as that noted above (*e.g.*, Figure 4, lanes d and e), an intense band of high molecular weight above a less intense band representing the fragment containing the endogenous allele. Analysis of the DNA after digestion with *Pst*I and *Xho*I indicated formation of concatamers with monomer plasmid units arranged in direct and inverted orientation, but did not reveal convincingly the existence of junction fragments that would arise from nonhomologous integration. Furthermore, analysis of DNA by digestion with *Ssp*I, which cuts once in the vector sequence, indicated no junction sequences of any kind, homologous or nonhomologous. The concatameric DNA was reduced to unit length monomers, indicating also that the ends of the linear transforming DNA had not been damaged by exonucleolytic activity. In this experiment the DNA was additionally digested with *Bam*HI, which does not cut the plasmid (*e.g.*, Figure 6, lane c). Because *Ssp*I sites surrounding the *LEU1* gene give rise to a fragment too large to resolve by conventional gel electrophoresis, the additional enzyme digestion was necessary to reduce the fragment size. The results obtained could be explained if the transforming DNA had never integrated. Concatamerization of the transforming DNA by end-to-end joining, maintenance of the complex in an episomal state, autonomous replication and transmission to daughter cells during cell division would constitute a model consistent with all these results.

TABLE 2

Mitotic stability of transformants

Transforming plasmid	Mitotic stability (%)
pCM117	1.5
pCM124, uncut[a]	
T-2	100
T-3	96
T-21	100
pCM124, *Xho*I cut	
X-4	0
X-5	100
X-7	0
pCM124, *Ssp*I cut	
S-1	0
S-12	36
S-17	0

Individual Leu$^+$ transformants obtained using the indicated plasmids were streaked to single colonies on minimal medium (selective). Isolated colonies were transferred to 5 ml of YEPS (nonselective) and grown to stationary phase (*ca.* 10^8 cells/ml). Approximately 500 cells from this culture were used to inoculate a second 5-ml culture of YEPS and allowed to grow to stationary phase. By this regime cells will have proceeded through at least 30 generations. About 200 cells from the second culture were plated on minimal medium containing leucine. At least 100 colonies arising were transferred individually to minimal plates lacking or containing leucine. Mitotic stability is measured as the percentage of viable colonies on plates without leucine relative to the number on plates containing leucine.

[a] Southern analysis of these transformants in Figure 3. T-2, lane n; T-3, lane o; T-21, lane j.

Characterization of the extrachromosomal transformants: One characteristic of autonomously replicating genetic elements is instability in the absence of selective pressure. We examined the mitotic stability of transformants obtained using uncut plasmid DNA and plasmid DNA cut to a linear form with *Xho*I and *Ssp*I after growing three transformants from each class for 30 generations with no selection (Table 2). All three transformants obtained using circular plasmid DNA were completely stable, while transformants obtained using linear plasmid DNA were not stable. The one exception in the group tested was transformant X-5, but hybridization analysis revealed this one to have arisen through homologous recombination of the plasmid into the resident *leu1* locus. Thus, transformants whose DNA structure clearly indicated plasmid integration were mitotically stable, while others with DNA structures ambiguous for plasmid integration were mitotically unstable.

Evidence for replication of the transforming plasmid DNA, rather than passive transmission, was obtained by monitoring the sensitivity of the DNA to methyl-directed restriction endonucleases (PEDEN *et al.* 1980). Plasmid DNA was prepared from the *dam*$^+$ *E. coli* strain DH1 which methylates adenine in the

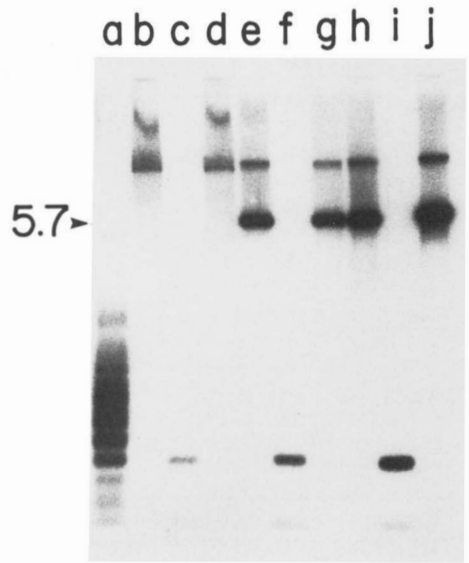

FIGURE 7.—Differential digestion of DNA with methyl-sensitive restriction enzymes. Strain 87 was transformed to Leu$^+$ with pCM124 either uncut or cut to a linear form with *Ssp*I. DNAs from a stable integrant obtained using circular pCM124 (T-21) and from two unstable transformants obtained using linear pCM124 (S-1 and S-17) were digested with *Ssp*I and either *Dpn*I or *Mbo*I as indicated then blotted and hybridized with the *LEU1* probe to analyze the methylation state of the DNA. The positive control was the transforming DNA pCM124 prepared in *dam*$^+$ *E. coli* coli strain DH1 and digested with *Ssp*I and *Dpn*I (lane a). The large number of bands are fragments arising from digestion by *Dpn*I. DNA isolated from T-21 (lanes b–d), S-1 (lanes e–g), and S-17 (lanes h–j) was digested with *Ssp*I (lanes b, e and h); *Ssp*I and *Mbo*I (lanes c, f and i); *Ssp*I and *Dpn*I (lanes d, g and j). The band at 5.7 kb is linear pCM124.

sequence GATC. This DNA was cut by *Dpn*I, but not by the isoschizomer *Mbo*I. However, after transformation and 30 generations of growth under selection, the plasmid DNA sequences had become resistant to digestion by *Dpn*I, but sensitive to *Mbo*I, indicating a loss of methylation (Figure 7). Seven different transformants were analyzed by this procedure and three representatives are shown, one nonhomologous integrant obtained using circular plasmid DNA, one homologous integrant obtained using linear plasmid DNA (X-5, see above), and one mitotically unstable transformant (X-7) obtained from using linear plasmid DNA. The loss in the pattern of methylation observed was the same with all the transformants, in keeping with a mode of transmission in which the plasmid sequences were replicated.

The results from Southern hybridization analyses suggested that the structure of the concatameric DNA was most likely circular. However, we were unable to establish the topology as circular by dye-buoyant density centrifugation. When DNA extracted from Leu$^+$ transformants obtained using linear plasmids were banded in cesium chloride gradients containing ethidium bromide, *LEU1* DNA sequences detected by dot-blot hybridization banded in a single mode at the density of relaxed DNA. While our analysis indicated the structure of the concatameric DNA as isolated was not covalently closed circular, it remains a possibility that the DNA molecules from these transformants were nicked during preparation.

Confirmation of the extrachromosomal location of the concatameric plasmid DNA was obtained from studies using pulsed field gel electrophoresis. DNA from stable and unstable transformants was analyzed in CHEF (contour-clamped homogeneous electric field) gels (CHU, VOLLRATH and DAVIS 1986). The chromosomal *LEU1* locus was physically mapped to chromosome *III*, the third smallest chromosome, with an estimated size of 450 kb. In stable transformants, known from conventional Southern hybridization analysis to have resulted from homologous integration of plasmid, *LEU1* DNA sequences appeared associated with chromosome *III* (Figure 8, lanes e and f) and in one case reiterated multiple times (Figure 8, lane f). In stable transformants obtained using circular plasmid DNA, *LEU1* sequences appeared associated with other chromosomes (Figure 8, lanes c and d). However, *LEU1* DNA sequences from unstable transformants were present in regions of the gel that did not correspond in position to any of the major bands representing chromosomes (Figure 8, lanes g–i). Present in transformants X-4 and X-7 were *LEU1* DNA sequences that migrated faster than the 6.1-kb pCM117 marker. Present in transformant S-17 was a series of slowly migrating bands containing *LEU1* DNA sequences. In light of the reports that circular DNA migrates anomalously in pulsed field gels (HIGHTOWER, METGE and SANTI 1987; BEVERLEY 1988) and in view of our own uncertainty regarding the transformant DNA structure, we are hesitant to assign a size to the DNA molecules from the unstable transformants. What is clear is that the *LEU1* associated DNA from these latter transformants is extrachromosomal.

We have attempted to propagate the extrachromosomal DNA in *recA* and *recBCsbcBrecF* strains of *E. coli* (BOISSY and ASTELL 1985), but have not been able to recover plasmid in any form other than monomer length form I circles. Such results may not be surprising in view of the instability of large palindromes in *E. coli* (COLLINS 1981; LILLEY 1981).

DISCUSSION

At work in *U. maydis* are several pathways for transformation. Besides the high frequency mechanism of transformation by circular plasmid molecules bearing an ARS (TSUKUDA *et al.* 1988), operating also are mechanisms for plasmid integration and replacement or gene conversion as well as a novel replicative mechanism. As we have demonstrated by the experiments in this paper, DNA conformation plays an important

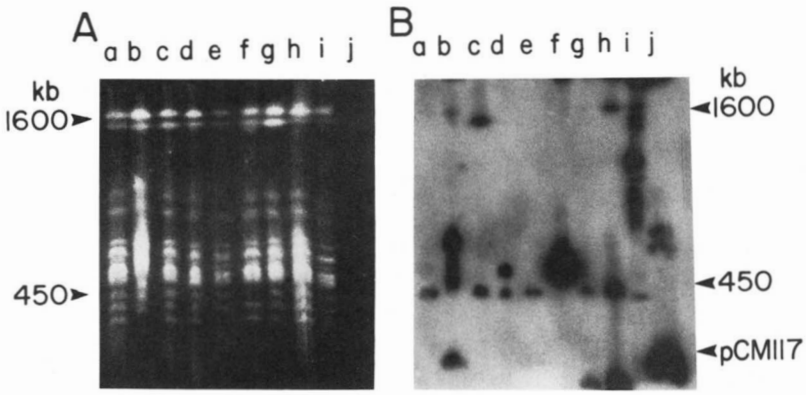

FIGURE 8.—CHEF gel analysis of DNA from Leu$^+$ transformants. Agarose plugs containing DNA from Leu$^+$ transformants were loaded in the wells of a 100 cm^2 gel of 1% agarose. The gel was run in a CHEF unit at 190 V for 23 hr at 9° with a switching interval of 90 sec. After staining in ethidium bromide (A), the gel was blot hybridized with the *LEU1* probe (B). (lane a) untransformed strain 87; (lane b) transformant with pCM117; (lane c) T-2, nonhomologous integrant obtained with circular pCM124; (lane d) T-21, nonhomologous integrant obtained with circular pCM124; (lane e) X-5, homologous integrant obtained with *Xho*I cut pCM124; (lane f) X-17 homologous integrant obtained with *Xho*I cut pCM124; (lane g) X-4, unstable transformant obtained with *Xho*I cut pCM124; (lane h) X-7, unstable transformant obtained with *Xho*I cut pCM124; (lane i) S-17, unstable transformant obtained with *Ssp*I cut pCM124; (lane j) 5 ng of pCM117 DNA. The indicated sizes were estimates based on the mobilities of *S. cerevisiae* chromosomes (TSUKUDA *et al.* 1988).

role in governing the pathway of transformation.

The predominant mode of transformation by circular plasmid DNA was found to occur through integration at nonhomologous sequences. In contrast transformants that arose from integration of linear plasmid DNA arose for the most part from homologous recombination. This dichotomy must reflect the operation of two pathways for integration in *U. maydis*.

In the yeast *S. cerevisiae* integration of transforming DNA takes place only by homologous recombination between cloned yeast DNA sequences in the transforming plasmid and the corresponding locus in the genome (BOTSTEIN and DAVIS 1982). The frequency of integration is influenced greatly by introduction of a double-strand break into the plasmid, but the mode of recombination remains unchanged, namely homologous (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981).

As we have demonstrated in *U. maydis*, the frequency as well as the mode of integration of plasmid DNA are governed by introduction of double-strand breaks (see Table 1 for summary). Cleavage of the transforming plasmid within the cloned *LEU1* gene changed the direction of integration from one of apparently random insertion into the genome to targeted recombination at the resident *leu1* allele. The magnitude of the change is difficult to assess because evaluation of the mode of integration was obscured by the prevalence of nonintegrated plasmid complexes in transformants. However, the change must be large. Approximately 15–40% of total transformants arising at a frequency of 890 per microgram represented homologous integration events when linear plasmid DNA was used compared to less than 5% of the cases examined when circular DNA was used (see also FOTHERINGHAM and HOLLOMAN 1989). In view of the 100-fold lower transformation frequency by circular DNA, we conclude that DNA ends are recombinogenic in *U. maydis*.

It is likely that *U. maydis* has elements in its recombination machinery in common with *S. cerevisiae*, where integrative recombination is solely homologous, as well as with mammalian systems, where integration of transforming DNA takes place with little regard for homology. Further analysis of transformation in recombination deficient mutants might help us understand more about the elements controlling these pathways and give us insight into how the switch between nonhomologous and homologous modes of integration is thrown.

Gene conversion or replacement events were noted when both circular and linear DNA preparations were used in transformation although they were less frequent when the transforming DNA was linear. In the case of circular transforming DNA this result is perhaps surprising in view of the dependence of gene conversion on sequence homology, in contrast with the independence of the observed integration on sequence homology. In *S. cerevisiae* gene conversion is strongly correlated with crossing over (ORR-WEAVER and SZOSTAK 1983; BORTS and HABER 1987). In *U. maydis* the genetic data are less extensive due to the paucity of well characterized linked markers. Nevertheless, where examined, gene conversion accompanies mitotic crossing over (HOLLIDAY *et al.* 1976). We examined 22 transformants but did not observe a single homologous integrant, even though we counted seven gene convertants. Such dissociation of gene conversion from homologous crossing over has been noted before in *S. cerevisiae* (JACKSON and FINK 1981; KLEIN 1984; RAY *et al.* 1988; ROMAN and JACOB 1958; ROMAN and FABRE 1983) and in *U. maydis* (HOLLIDAY 1966) in cells stressed by ultraviolet radiation.

A remarkable feature of transformation by linear DNA molecules that came to light in our analysis that was not reported by two other groups studying trans-

formation in *U. maydis* (BANKS and TAYLOR 1988; WANG, HOLDEN and LEONG 1988) was the propagation of the transforming DNA extrachromosomally. When the transforming DNA was in the form of monomer length circles, no such propagation was observed. Extrachromosomal maintenance and replication of the plasmid in this form requires the presence of an ARS. Our finding of episomal maintenance of plasmid DNA in the absence of an ARS raises the question of whether concatamerization or structural features associated with a large molecule composed of tandemly iterated units can transcend the requirement for an ARS in the control of replication.

We estimate that the concatameric structure on the average is composed of 25 iterant units of plasmid DNA arranged in a tandem array of direct and inverted repeats. Several aspects of this structure are reminiscent of features observed in other transformation systems. End-to-end joining is not unique. It readily occurs during transformation of mammalian cells (CHANG and WILSON 1987). Neither is propagation of DNA containing large inverted repeats unique to *U. maydis*. In maize, an inverted duplication can be formed between the daughters of a broken chromosome (MCCLINTOCK 1939). In *S. cerevisiae* inverted dimers can be formed when linear plasmid DNA is introduced into the cell if the ends bear no homology to yeast DNA sequences (KUNES, BOTSTEIN and FOX 1985). However, the ends never form perfect inverted dimers.

Concatamerization is also a feature not uniquely associated with *U. maydis* transformation. Circular transforming plasmid DNA introduced into *Schizosaccharomyces pombe* may be transmitted in a polymeric form as large as a decamer (HEYER, SIPICZKI and KOHLI 1986; SAKAGUCHI and YAMAMOTO, 1982). DNA introduced into mammalian cells has also been found to persist in large concatameric structures (PERUCHO, HANAHAN and WIGLER 1980). Likewise, DNA microinjected into germline cells of *Caenorhabditis elegans* forms high molecular weight arrays that were found to be extrachromosomal and heritable (STINCHCOMB *et al.* 1985).

How does such a structure replicate in *U. maydis* independent of a canonical autonomously replicating sequence? In *S. cerevisiae* the requirements for ARS activity are stringent. An eleven base pair core sequence is absolutely essential for ARS function (CELNICKER *et al.* 1984; KEARSEY 1984; BOUTON and SMITH 1986), although a single core sequence by itself is not sufficient for activity. Multiple sequences that are near matches to the core are also required for activity (PALZKILL and NEWLON, 1988).

In other eukaryotic systems investigated, the sequence requirement in control of replication may be more relaxed. In Xenopus, there is no specific sequence essential for replication of plasmids (MECHALI and KEARSEY 1984). A variety of prokaryotic and eukaryotic plasmids can be replicated after injection into Xenopus eggs with a proficiency dependent merely on the size of the molecule. Similarly, in *S. pombe*, plasmids containing no *S. pombe* DNA can be propagated by replication. In this case extensive rearrangements of plasmid sequences have been noted, sometimes coupled with acquisition of chromosomal DNA sequences (HEYER, SIPICZKI and KOHLI 1986; MAUNDRELL, HUTCHISON and SHALL 1988). One explanation accounting for these findings is that any easily unwound DNA sequence constitutes a critical determinant satisfying part of the requirement for initiation of replication (UMEK and KOWALSKI 1988).

Reiteration of sequences may be a key prerequisite for replication in the absence of a canonical ARS. Possibly the plasmid used by us in this study contains some sequence such as a transcriptional element (DEPAMPHILIS 1988) that in the context of a monomeric unit has weak, or no activity in providing ARS function, but as part of a structure multipled in a colinear array through end-to-end joining of monomer plasmid units can overcome some threshold barrier to autonomous replication. Perhaps the tandem coupling is a device to enhance the probability that at least one surrogate sequence is recruited as an ARS by the replication machinery per round of cell division. How the cell might be served by autonomous replication of reiterated sequences is not at all clear. What is clear is that DNA end joining in *U. maydis* is quite efficient. In this regard it is not hard to imagine that the concatamer replication might be merely a secondary consequence of a promiscuous end-joining activity operating as a facet of a powerful DNA repair system (ROTH and WILSON 1988) in this very radioresistant organism (LEAPER, RESNICK and HOLLIDAY 1980).

We are most grateful to TOYOKO TSUKUDA and ROBERT BAUCHWITZ for their help during the course of this work. This study was supported in part by National Institutes of Health grant GM42548.

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Communicating Editor: R. L. METZENBERG