MOLECULAR AND CELLULAR BIOLOGY, Sept. 1989, p. 4052–4055
0270-7306/89/094052-04$02.00/0

Vol. 9, No. 9

Cloning and Disruption of *Ustilago maydis* Genes

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Received 27 March 1989/Accepted 11 May 1989

We have demonstrated that genes from *Ustilago maydis* can be cloned by direct complementation of mutants through the use of genomic libraries made in a high-frequency transformation vector. We isolated a gene involved in amino acid biosynthesis as an illustrative example and showed that integrative and one-step disruption methods can be used to create null mutations in the chromosomal copy of the gene by homologous recombination. The results of this investigation make it clear that one-step gene disruption will be of general utility in investigations of *U. maydis*, since simple, precise replacement of the sequence under study was readily achieved.

Key for success in molecular genetics is the ability to move DNA in and out of a cell, altering it at will. Exemplar of this dictum is the transformation system of the yeast *Saccharomyces cerevisiae*, whose power derives from a sophisticated array of multifunctional plasmid vectors and depends upon the homologous recombination apparatus in the cell (2). In yeast cells, the ability to return an altered DNA sequence to the exact location from which the parental sequence was derived has led to a general method for inducing a null mutation in any yeast gene that has been cloned. Gene disruption techniques have provided means for establishing identity of a cloned fragment with a specific gene and for determining whether a cloned gene is essential (12, 13).

We have had a long-standing interest in the mechanism of genetic recombination in *Ustilago maydis*, the first fungus in which mutants altered in recombination were isolated (9). Our work has centered on biochemical analysis of recombination in *U. maydis* (see, for example, reference 10). As part of our endeavor to understand the process in more depth, we have begun a molecular genetic analysis of genes controlling recombination.

U. maydis has a classic genetic system, but until recently there has been no characterization of its molecular biology. An efficient but low-frequency transformation system based on hygromycin resistance was developed by Wang et al. (15), who reported integration of the transforming vector by predominantly nonhomologous recombination. Banks and Taylor (1) took a different approach and developed a heterologous transformation system. They cloned the *U. maydis PYR3* gene, encoding dihydroorotase, indirectly by complementing *pyrC* mutants of *Escherichia coli*. Our approach has been to set up a system of general utility for cloning *U. maydis* genes and for introducing DNA altered in vitro back into the cell.

The strategy of cloning we chose as most suitable for our purposes was direct complementation of *U. maydis* mutants, using gene banks prepared in a high-frequency transformation vector (14). As a test system to explore the feasibility of our strategy, we chose to focus on a simple auxotrophic mutant that could be scored easily. Since reversion to prototrophy would obscure identification of complementing transformants, we elected to study the *leu1-1* mutant, which reverts at $<10^{-8}$, a frequency low enough to ensure that clones obtained would likely have arisen by complementation.

In this paper, we show that the *LEU1* gene can be cloned by complementation using a high-frequency transformation vector, altered by manipulation in vitro, and returned to the homologous location in the genome, demonstrating the general utility of these procedures for studies of *U. maydis*. In a more general sense, these findings make it clear that the power of molecular genetics can be brought to bear on the genetic system of *U. maydis*.

Isolation of the *LEU1* gene. We prepared a genomic library by partially digesting DNA from *U. maydis* wild-type strain 518 (a_2b_2) with endonuclease *Sau*3A, selecting fragments in the size range of 2 to 6 kilobase pairs (kbp), and ligating them into the unique *Bam*HI site of the high-frequency transformation vector pCM54. This vector contains the bacterial hygromycin phosphotransferase gene driven by a *U. maydis* heat shock promoter as the selectable marker (15) and the 383-bp *Ssp*I-*Ssp*I active subfragment of the autonomously replicating sequence UARS1 (14). The transformation frequency to hygromycin resistance with use of pCM54 is approximately 1×10^4 to 2×10^4/μg of DNA. The genomic library was used to transform the Leu$^-$ strain 87 (a_2b_2 *ad1-1 leu1-1*) to leucine prototrophy. Leucine prototrophy was detected on minimal medium plates as described by Holliday (8) except that sucrose replaced glucose and glycine (100 μg/ml) was added to enhance growth of the *leu1-1* mutant.

By direct measurement using diphenylamine to assay DNA, we estimated the haploid genome size of *U. maydis* to be approximately 10^4 kbp (T. Tsukuda and W. K. Holloman, unpublished data). For DNA of this complexity, a gene library containing DNA fragments with an average size of 2 to 6 kbp should contain about 12,000 fragments to represent a complete genome at a probability of 0.99 (3). Among 10^5 hygromycin-resistant (HYG^R) transformants, we found three leucine prototrophs. Analysis of plasmid DNA extracted from the Leu$^+$ transformants revealed common sequences in the DNA inserts from the three candidates. One selected for further study contained *leu1*-complementing activity on a 6.5-kbp fragment. By restriction mapping and subcloning into pUC vectors containing the *U. maydis* autonomously replicating sequence, we isolated the DNA sequence containing the *leu1*-complementing activity on a 3.0-kbp-long *Eco*RI-*Hin*dIII fragment. Plasmid pCM117

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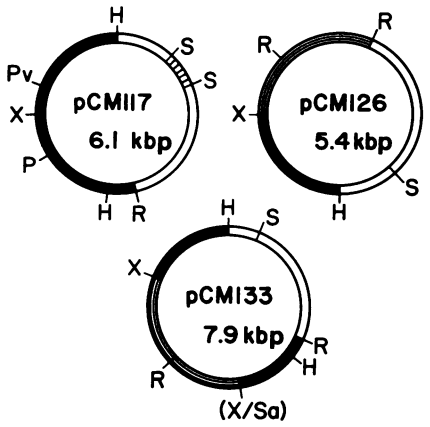

FIG. 1. Structure of transforming plasmids. Plasmid pCM117 has the 3.0-kbp fragment containing the *LEU1* gene (▬) and the 383-bp fragment (▥) containing the autonomously replicating sequence in pUC12. pCM126 contains the internal sequence of the *LEU1* gene (▬) and the *hsp70*-hygromycin phosphotransferase gene fusion HYG^R (▤) in pUC18. pCM133 contains the *LEU1* gene (▬) interrupted by the HYG^R cassette (▤) in pUC12. Restriction endonuclease sites: H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*I; S, *Ssp*I; R, *Eco*RI; Sa, *Sal*I; X, *Xho*I.

(Fig. 1) containing this fragment was found to transform the *leu1-1* strain to leucine prototrophy at a frequency of 1×10^4 to 4×10^4/μg of DNA.

Marker rescue of *leu1-1*. The lesion in the *leu1-1* allele was localized by marker rescue, a technique used widely for genetic mapping of viruses. Fragments of a gene that are incapable of complementation may rescue a marker if they span the lesion. Marker rescue depends on the homologous recombination system of the cell to direct correction of a genetic defect by using an added DNA fragment and therefore occurs at a much lower frequency than does complementation. Experimentally, this amounted to four to six transformants per dish in the positive cases and none in the negative cases. Overlapping fragments spanning the 3.0-kbp *Hin*dIII-*Eco*RI sequence containing the *LEU1* gene were cloned into pUC vectors lacking the autonomously replicating sequence. Although the 3.0-kbp *Hin*dIII-*Eco*RI fragment was the minimal sequence that we found to be active in complementing the *leu1-1* mutation, several subfragments were found to rescue the phenotype. The smallest was a 1.4-kbp *Pvu*I-*Hin*dIII fragment (clockwise from *Pvu*I to *Hin*dIII in Fig. 1).

Integrative disruption. Proof that the cloned DNA fragment encodes the *LEU1* gene was provided by disrupting the gene and performing complementation analysis. Two approaches were taken. In the first, the integrative disruption strategy of Shortle et al. (13) was followed, using plasmid pCM126 (Fig. 1). This plasmid was a pUC18 derivative containing the noncomplementing 0.8-kb *Xho*I-*Hin*dIII internal sequence of the cloned *leu*1-complementing fragment and a 1.2-kbp fragment of the *hsp70* promoter from pHL1 (15) in fusion with the 1.0-kbp hygromycin phosphotransferase gene from pLG90 (6) but lacking the 383-bp autonomously replicating sequence. Leu$^+$ cells were transformed with pCM126, and HYG^R transformants were picked and screened for leucine auxotrophy. From 21 HYG^R transformants, 1 was found to be simultaneously leucine requiring, as expected for recombination of the plasmid by a single crossover at the endogenous *LEU1* gene. The low frequency of Leu$^-$ clones was not surprising in light of the low frequency of homologous integration events we have observed when circular DNA is used for transformation of wild-type cells (S. Fotheringham and W. K. Holloman, submitted for publication).

Southern hybridization analysis confirmed homologous integration of the transforming DNA into the genome (Fig. 2A). Genomic DNA from the HYG^R Leu$^-$ transformant was isolated as described by Hoffman and Winston (7), digested with *Bam*HI, which does not cut the plasmid, transferred to a Zeta Probe membrane (Bio-Rad Laboratories) by the alkaline blotting method of Reed and Mann (11), and hybridized, using as a probe the 1.0-kbp *Xho*I-*Eco*RI fragment of the cloned *leu1*-complementing sequence labeled to high specific activity by the method of Feinberg and Vogelstein (5). Homologous integration was recognized by disappearance of the endogenous fragment hybridizing with the probe and concomitant generation of a new fragment larger by the length of one plasmid DNA sequence. Digestion with a second enzyme, *Xho*I, which cuts once within the plasmid at one end of the probe sequence, confirmed homologous integration. In this case, a fragment containing part of the endogenous sequence plus a fragment containing one unit length of plasmid DNA were generated, as expected for homologous integration.

The mitotic stability of this transformant, UCM91, was examined by growing it for a number of generations in nonselective medium and then determining the fraction of cells no longer resistant to hygromycin. After 30 generations of growth, cells were streaked to single colonies and 100 individual clones were tested on media with and without hygromycin. The mitotic stability of UCM91, the disrupted strain, was 100% compared with that of a transformant containing the autonomously replicating vector pCM54, which was 4%. UCM91 was mated on plates containing hygromycin and 1% charcoal (4) with compatible tester strains to form diploids for complementation analysis. The Leu$^-$ phenotype was complemented by a *LEU1* tester but not by a *leu1-1* tester. The disrupted strain was also crossed with a compatible wild-type tester strain for meiotic analysis. In 13 tetrads examined by the individual-products-of-meiosis method of Holliday (8), there was tight linkage of *leu* and HYG^R.

One-step gene disruption. The second approach taken to disrupt the gene was a one-step procedure (12). A 2.0-kbp *Xho*I-*Sal*I fragment from pHL1 (15) spanning part of the *hsp70* promoter and all of the hygromycin phosphotransferase gene was inserted into the *Xho*I site of pCM124 (pCM117 without the autonomously replicating sequence), disrupting the *leu1*-complementing activity. The resulting plasmid, pCM133 (Fig. 2B), was digested with *Hin*dIII to liberate the disrupted *leu1*-complementing fragment and transformed into a Leu$^+$ strain. Among 12 HYG^R transformants obtained in one experiment, 3 were simultaneously Leu$^-$. These results confirmed that the *LEU1* gene was encompassed within the 3.0-kbp cloned DNA sequence.

One of the transformants, UCM95, chosen for further study was completely stable after growth for 30 generations in nonselective medium. When UCM95 was mated with tester strains to form diploids, the Leu$^-$ phenotype was complemented by the *LEU1* tester but not by the *leu1-1* tester. Analysis of the DNA structure of transformant UCM95 revealed that a single copy of the *Hin*dIII fragment from the plasmid had replaced the resident fragment. After digestion with *Bam*HI, which does not cut within the plasmid sequences, the endogenous fragment recognized by the *LEU1* probe was replaced by a second band larger by 2.0

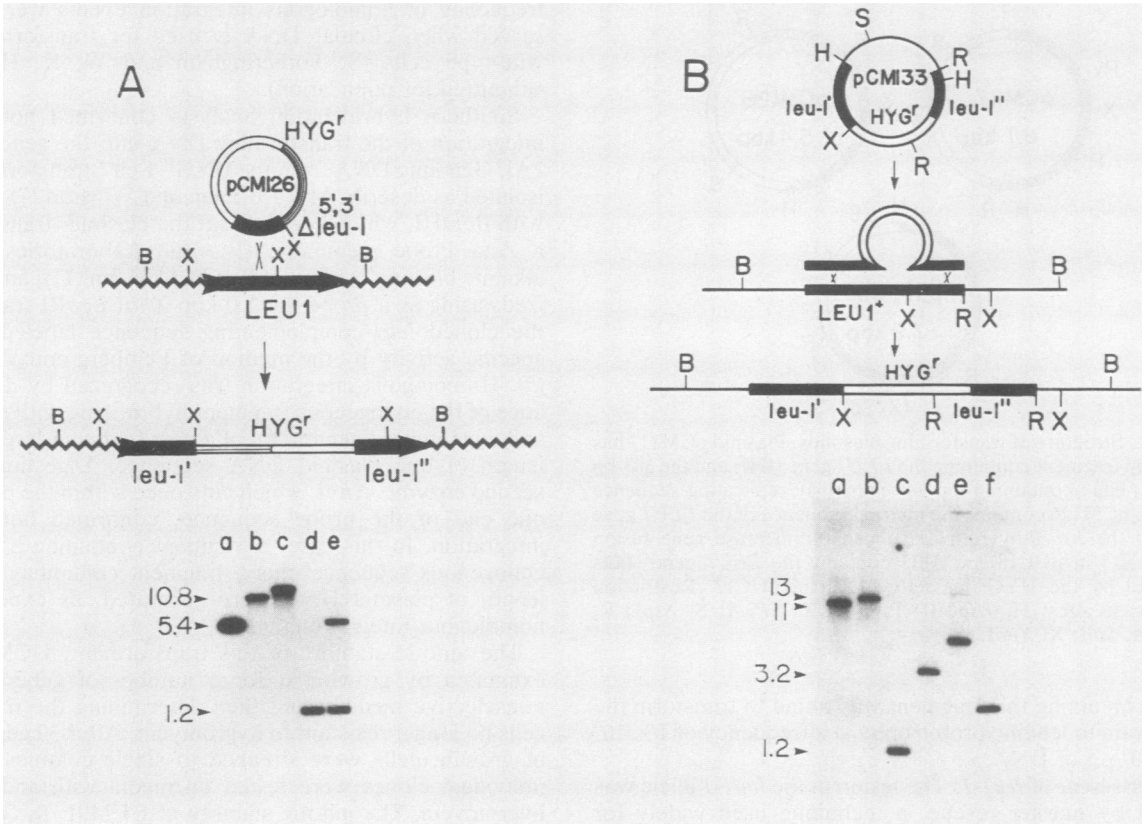

FIG. 2. Gene disruption. (A) Structure of the *LEU1* gene interrupted by integration of pCM126. *LEU1*$^+$ strain 521 (a_1b_1) was transformed to hygromycin resistance and leucine auxotrophy with pCM126. DNA extracted from the transformant UCM91 was digested with *Bam*HI (B), which does not cut within the plasmid sequences, or with *Xho*I (X), which cuts at the indicated sites, and then analyzed by Southern blot hybridization, using the 1.0-kbp *Xho*I-*Eco*RI fragment encompassing part of the *LEU1* gene as a probe. Lanes: a, pCM126 cut with *Xho*I; b, control DNA from untransformed strain 521 cut with *Bam*HI; c, transformant DNA cut with *Bam*HI; d, control DNA cut with *Xho*I; e, transformant DNA cut with *Xho*I. (B) One-step gene disruption. *LEU1*$^+$ strain 521 was transformed to hygromycin resistance and leucine auxotrophy with pCM133 cut with *Hin*dIII. DNA extracted from transformant UCM95 was analyzed by blot hybridization after digestion with *Bam*HI (B), *Xho*I (X), or *Eco*RI (R), using the 1.0-kbp *Eco*RI-*Xho*I fragment as a probe. Lanes: a, control DNA from untransformed strain 521 cut with *Bam*HI; b, transformant DNA cut with *Bam*HI; c, control DNA cut with *Xho*I; d, transformant DNA cut with *Xho*I; e, control DNA cut with *Eco*RI; f, transformant DNA cut with *Eco*RI.

kbp, the length of the sequence containing the *hsp70*-hygromycin phosphotransferase gene fusion. Digestion with additional enzymes confirmed that the endogenous *LEU1* allele had been replaced by a disrupted version. After digestion with *Xho*I, which cuts at the junction between the disrupted *LEU1* gene and the 2.0-kbp *hsp70*-hygromycin phosphotransferase gene fusion, we observed the *leu1* fragment in the transformant to be 2.0 kbp larger than the fragment in the wild-type cell. Digestion of DNA from the transformant with *Eco*RI resulted in production of a fragment smaller than the endogenous one present in the wild type because an additional *Eco*RI site was located within the DNA sequence disrupting the *LEU1* gene. Blot hybridization analysis of DNA from the two other Leu$^-$ HYG^R transformants revealed more complicated patterns, possibly because more than one copy of the plasmid DNA sequence was integrated into the genome.

Although the disruption vector used in the experiment described above was cleaved with *Hin*dIII to expose free ends homologous with the sequence of interest in the genome, we also observed targeted replacement when the DNA ends were not homologous with the target sequence. The frequency of Leu$^-$ HYG^R clones that arose was no different when cells were transformed with pCM133 cleaved with *Ssp*I or with *Hin*dIII (Table 1). In each case in this experiment, about half of the HYG^R transformants were simultaneously Leu$^-$. In two different experiments, only 1 of 19 HYG^R transformants was Leu$^-$ when uncleaved plasmid DNA was used. Thus, DNA ends are recombinogenic.

The low frequency of *LEU1* gene disruptions among the HYG^R transformants obtained in the integrative procedure may indicate, in a general sense, a limit to the usefulness of this system. If one has no selection for the gene of interest, there will be no certainty of isolating transformants with the

TABLE 1. Influence of DNA ends on one-step gene disruption[a]

Cleavage site	Frequency of transformation/μg of DNA	No. of transformants		Disruption (%)
		Leu$^-$	HYG^R	
Uncut	<1	1	19	5
*Hin*dIII	190	30	58	52
*Ssp*I	170	28	56	50

[a] Leu$^+$ cells transformed with pCM133 DNA cut with the indicated restriction endonuclease were selected for resistance to hygromycin in medium containing leucine. Resistant colonies were then picked to minimal medium lacking leucine to test for leucine auxotrophy.

target gene disrupted. On the other hand, the one-step disruption procedure as we performed it is equal to the yeast system in effectiveness. Among HYG^R transformants selected in two experiments, one quarter to one half were simultaneously Leu^-. One of three chosen for DNA structure analysis was revealed to result from a simple replacement of the endogenous sequence by the corresponding disrupted sequence, a result on par with findings reported in yeast cells (12). Versatility of the one-step procedure for studies of *U. maydis* should be broadened by the finding that the ends of the transforming DNA need not be homologous with the target sequence.

Continued efforts toward isolating more genes by these techniques are sure to bring investigations of the molecular genetic system of *U. maydis* to a high level of sophistication.

We thank Robin Holliday, CSIRO, Sidney, Australia, for providing strains of *U. maydis*. We are especially grateful to Robert Bauchwitz and Toyoko Tsukuda of this laboratory for helpful discussions.

This work was supported by Public Health Service grant GM 36327 from the National Institutes of Health.

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