Cloning of the PYR3 Gene of Ustilago maydis and Its Use in DNA Transformation

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The Ustilago maydis PYR3 gene encoding dihydroorotase activity was cloned by direct complementation of Escherichia coli pyrC mutations. PYR3 transformants of E. coli pyrC mutants expressed homologous transcripts of a variety of sizes and regained dihydroorotase activity. PYR3 also complemented Saccharomyces cerevisiae ura4 mutations, and again multiple transcripts were expressed in transformants, and enzyme activity was regained. A 1.25-kilobase poly(rA)* PYR3 transcript was detected in U. maydis itself. Linear DNA carrying the PYR3 gene transformed a U. maydis pyr-3-1 pyrimidine auxotroph to prototrophy. Hybridization analysis revealed that three different types of transformants could be generated, depending on the structure of the transforming DNA used. The first type involved exchange of chromosomal mutant gene sequences with the cloned wild-type plasmid sequences. A second type had integrated linear transforming DNA at the chromosomal PYR3 locus, probably via a single crossover event. The third type had integrated transforming DNA sequences at multiple sites in the U. maydis genome. In the last two types, tandemly reiterated copies of the transforming DNA were found to have been integrated. All three types had lost the sensitivity of the parental pyr-3-1 mutant to UV irradiation. They had also regained dihydroorotase activity, although its level did not correlate with the PYR3 gene copy number.

Ustilago maydis is a basidiomycete fungus, and its value in studies of DNA repair, recombination, and replication has been well documented (19, 24, 26, 46). These studies have been largely at the genetic, physiological, and biochemical levels. In addition to these approaches, however, an ability to clone specific gene sequences and then to transform them, either unmodified or modified in a defined way, back into the organism would be a powerful tool for further detailed molecular studies in these and other areas. It is perhaps the availability of this combination of approaches that is the major advantage of lower eucaryotes in studies of RNA and DNA metabolism (for a review, see reference 30). Furthermore, as U. maydis is a pathogen of Zea mays, the development of a range of recombinant DNA techniques would perhaps promote investigations into host-parasite interactions. Recently, an efficient transformation system based on hygromycin resistance was reported (42).

In this study, we describe the cloning of the U. maydis PYR3 gene, which encodes dihydroorotase (DHOase; EC 3.5.2.3.) activity, and its uses in DNA transformation studies. This enzyme catalyzes the conversion of N-carbamyl-L-aspartate to dihydroorotate in the de novo pyrimidine biosynthetic pathway, a pathway which is highly conserved (22). It was chosen because pyrimidine auxotrophs of U. maydis are sensitive to a range of DNA-damaging agents, and these auxotrophs have therefore been isolated in association with studies on DNA repair mechanisms (18, 31, 32). One such strain containing the pyr-3-1 auxotrophic mutation has been used in the work reported here as a recipient for transformation to prototrophy by the cloned PYR3 gene. The evidence presented identifies three types of transformants; the structure of the transforming DNA determines which type predominates. We also show that the PYR3 gene can also complement mutants of both Escherichia coli and Saccharomyces cerevisiae which lack their own DHOase activity.

MATERIALS AND METHODS

Strains. The E. coli strains used were as follows: RB2262 (pyrC46 hfr thi-1 relA1 lac-243 λ+), RB567 (pyrD34 F- thi-1 his-68 trp-45 galK35 malA1 xyl-7 mtl-2 recA1 tyrA2 λ · λ-), and RB2160 (pyrE60 F− leuB6 proA2 his-4 argE3 lacY1 galK2 ara-14 xyl-5 mtl-l supE44 chlG6 rpsL31 mK− mK− λ · λ-), kind gifts of R. S. Buxton; MB1000 (pyrF Trp− Lac− rK− nR−), a kind gift of R. Davis; MC4100 (araD139 lacYI−169 rpsL6 Thi−), S91263 (same as MC4100 but ΔpyrC), S91763 (same as MC4100 but pyrC::Mu1cts[Blal lac−]), kind gifts from K. F. Jensen; and DH1 (recA1 F− endA1 gyrA96 thi-1 supE44 rK− mK−), a kind gift from S. Sedgwick. U. maydis 521 (wild-type, AB) and 241 (pyrL-1 panL-1 nrtl-1 AB) were kind gifts from the stocks of R. Holliday. A haploid S. cerevisiae trpl ura4 strain was constructed by standard genetic methods (33) from a trpl and a ura4 strains provided by F. Lacroute.

Growth media. E. coli was grown in either M9 salts or Luria broth (LB) at 37°C (28). U. maydis was grown in nitrate minimal medium (NM) or complete medium (CM) at 32°C. Solid media contained 1.5% agar (31). The additions and their final concentrations, when required, were ampicillin at 50 μg/ml, tetracycline at 15 μg/ml, uridine at 10 μg/ml, leucine at 100 μg/ml, and other amino acids at 50 μg/ml. S. cerevisiae was grown in YEPE complete or YNB minimal media at 32°C (33). Tryptophan and uracil (both at 20 μg/ml) were added when required.

Transformation. E. coli was transformed by the calcium chloride method (27). U. maydis was transformed by modification of the method described previously (5). Novozyme 234 (Novo Industries, Bagsvaerd, Denmark) at 1 mg/ml was used to generate spheroplasts. After treatment with DNA and polyethylene glycol, spheroplasts were incubated for 60
min at 32°C in 2× CM containing 1 M sorbitol before the spheroplasts were spread onto selective agar containing 1 M sorbitol. Transformants could be picked after 4 to 6 days. S. cerevisiae was transformed by the method of Ito et al. (20) with lithium acetate.

**DNA manipulations.** DNA was cleaved by restriction endonucleases in buffers supplied by the manufacturers, Gibco-BRL (BRL Life Technologies Inc., Gaithersburg, Md.). When necessary, restricted DNA was dephosphorylated by calf intestinal phosphatase (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) treatment. DNAs were ligated by T4 DNA ligase in a solution containing 66 mM Tris hydrochloride (pH 7.6), 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM ATP, and 0.25% Nonidet P-40 at 14°C. Restriction fragments were extracted from low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) gels, essentially by the method of Wieslander (44). Plasmid DNA probes for hybridization were labeled with [α-³²P]dCTP by nick translation (39). Fragment DNA probes were labeled by the random oligonucleotide primer method (13).

**U. maydis recombinant DNA library.** High-molecular-weight DNA isolated from nuclei of strain 521 was purified by cesium chloride density centrifugation in the presence of ethidium bromide. The resulting DNA was partially digested by Sau3AI and fractionated on a 10 to 40% sucrose gradient (28). Fractions containing 5- to 15-kilobase (kb) fragments (identified by agarose gel electrophoresis) were pooled, and the DNA was ligated into YEp13 vector DNA (10), which had been digested with BamHI and dephosphorylated. This preparation was used to transform E. coli to ampicillin resistance. About 24,000 transformants, 80% of which carried insert DNA, were collected into six pools.

**Extraction of DNA.** Small-scale plasmid DNA preparations were done by the method of Birnboim and Doly (8). On a large scale, plasmid DNA was purified by the sodium dodecyl sulfate lysis procedure of Godson and Vapnek (14), as modified by Maniatis et al. (28). U. maydis DNA was extracted from transformants described previously (5) and modified in this laboratory by A. Spanos. Briefly, spheroplast lysates were extracted with phenol-chloroform, nucleic acids were ethanol precipitated, RNA was digested by RNase A at 100 µg/ml, the mixture was again phenol-chloroform extracted, and the DNA was ethanol precipitated. The resulting DNA was fully digested by all restriction endonucleases tested. Plasmid DNA was extracted from *S. cerevisiae* by the method of Hoffman and Winston (17).

**Extraction of RNA.** Total RNA was extracted from E. coli by the method of Albin and Silverman (1). Total RNA was extracted from *U. maydis* by a method based on that of Cathala et al. (12). Cells from a 200-ml CM culture at 5 × 10⁹/ml were suspended in 2 ml of a solution containing 50 mM Tris hydrochloride (pH 7.5), 5 mM guanidine monothioctetane, 10 mM EDTA, and 8% (vol/vol) 2-mercaptoethanol. The suspension was passed twice through a French pressure cell at 18,000 lb/in² at room temperature. Cell debris was removed by centrifugation, and RNA was precipitated by LiCl and held on ice overnight. The precipitate was collected by centrifugation on a SW28.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 90 min at 4°C and 9,500 rpm and resuspended in 3 M LiCl. After centrifugation, the RNA was dissolved by suspension of the pellet in 2.5 ml of a solution containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. This suspension was extracted with phenol-chloroform, and RNA was then precipitated from the aqueous phase by ethanol after the addition of 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. RNA solutions were stored in aliquots at −70°C. Poly(rA)*RNA was extracted from total RNA by one cycle of chromatography on oligo(dT)-cellulose (28). Total RNA was extracted from *S. cerevisiae* as described by Aves et al. (2).

**Cell protein extracts.** *E. coli* and *U. maydis* extracts for enzyme assays were prepared by suspending cells in cold 100 mM Tris hydrochloride (pH 8.0)–10% glycerol, followed by passage through a French pressure cell twice at 18,000 lbs/in² and 4°C. The resulting suspensions were centrifuged for 20 min at 15,000 rpm and 4°C. Supernatant fractions were stored at −70°C in small aliquots. *S. cerevisiae* extracts were prepared as described previously (6). Protein concentrations were estimated by the method of Bradford (9).

**Assay of DHOase activity.** Samples of the above extracts were incubated in 1 ml of 100 mM Tris hydrochloride (pH 8.5)–3.5 mM dihydroorotic acid at 30°C for 20 min. The reaction was stopped by the addition of 0.1 ml of 4 M perchloric acid, the precipitate was removed by microcentrifugation, and the carbamyl aspartate in the supernatant was measured essentially as described by Prescott and Jones (38). Extracts to be compared were prepared and assayed together to ensure maximum reproducibility. For each extract, enzyme activity was measured as a function of protein concentration and of time to ensure proportionality. Production of carbamyl aspartate was linear for at least 35 min.

**Cell survival after UV irradiation.** Cells from CM cultures at about 2 × 10⁹ cells/ml were suspended in sterile water to a density of 5 × 10⁹ cells/ml. They were irradiated by a Hanovia germicidal lamp (low-pressure Hg vapor at 254 nm) at a dose rate of 1.02 J/m² per s. The cells were diluted before spreading on CM plates for survival determination. All operations after the irradiation were carried out under subdued light.

**Hybridizations.** Hybridization of DNA digests was carried out by standard procedures (28). Gels containing genomic digests were routinely stained with ethidium bromide to ensure that digestion was complete. RNA was denatured (29), electrophoresed, and transferred to GeneScreen for hybridization.

**RESULTS**

**Complementation of *E. coli* pyrC auxotrophs.** Purified plasmid DNA from the *U. maydis* recombinant library was used to transform competent cells of *E. coli* mutants defective in the *pyrC, pyrD, pyrE, or pyrF* gene product. These genes encode DHOase, dihydroorotate dehydrogenase (EC 1.3.99.11), orotate phosphoribosyltransferase (EC 2.4.2.10), and orotidine-5'-phosphate decarboxylase (EC 4.1.1.23), respectively (3). Pyrimidine prototrophic or Amp* transformants were selected. No prototrophs resulting from transformation of *pyrD, pyrE,* or *pyrF* mutants were obtained from more than 10² Amp* transformants, but three Amp* prototrophs were obtained after transformation of the *pyrC* mutant. Plasmid DNA, pMH2001, extracted from one such transformant contained 12 to 14 kilobases (kb) of DNA inserted into the YEp13 vector, and it retransformed the *E. coli pyrC* mutant to prototrophy at a high frequency. Subcloning the *pyrC*-complementing insert sequences by partial *PstI* digestion of pMH2001 DNA and ligation into pBR322 gave plasmids pMH2002 and pMH2003 (Fig. 1A). These are identical except for the orientation of the 3.2-kb insert. Further manipulations generated pMH2004, in which an EcoRI fragment containing both insert and pBR322 sequences had been
deleted from pMH2003 (Fig. 1A). All three of these plasmids transformed the E. coli pyrC mutant to pyrimidine prototrophy and tetracycline resistance at high frequencies. Each of the two PstI insert fragments of pMH2002 was also separately ligated into pBR322 and used to transform the E. coli pyrC mutant. Restriction analysis of transformant plasmid DNA minipreparations showed that each fragment had been cloned in both possible orientations (results not shown). None of these four plasmid DNAs transformed the pyrC mutant to pyrimidine prototrophy, even though they did so efficiently to antibiotic resistance. This suggests that the internal PstI site is located within the pyrC-complementing sequences.

The nature of the pyrC mutation in E. coli RB2262, used to select the complementing sequences, is unknown. To rule out the possibility of complementation by nonspecific suppression, we transformed E. coli Sφ1263 and Sφ1793 with plasmids pMH2001, pMH2002, and pMH2003. Sφ1263 contains a deletion of the pyrC gene, while in Sφ1793 it is interrupted by a Mu bacteriophage (21). Both strains were transformed to pyrimidine prototrophy at high frequencies by all three plasmids.

In order to characterize the origin of the DNA sequences which complemented the pyrC mutations, we digested U. maydis chromosomal DNA with PstI, EcoRI, or SalI and each digest was electrophoresed and then transferred to nitrocellulose membrane for Southern hybridization. The oligonucleotide-labeled 1.95-kb EcoRI insert fragment of pMH2003 was used as the probe (Fig. 1B). The hybridization pattern for the EcoRI digest demonstrates that the two PstI fragments are adjacent in the chromosome, as well as in the cloned sequences (Fig. 2). The probe also hybridized to a single 6.1-kb SalI fragment, consistent with the lack of this site in the cloned sequences.

**DHOase enzyme levels in E. coli transformants.** DHOase levels were assayed in cell extracts of E. coli MC4100 and Sφ1263, which are isogenic except for the pyrC deletion in the latter (21). In addition, enzyme levels were determined in Sφ1263 transformed by plasmids pMH2002, pMH2003, and pMH2004 (Table 1). Only 2% of the enzyme level in MC4100 was detected in the mutant, whereas after transformation this level increased to 35, 43, and 29%, respectively. Attempts were made to confirm that DHOase activity in the transformant extracts had originated from the plasmid DNA sequences, but although several reaction parameters of both E. coli and U. maydis enzyme activities were investigated, no differentiating property was found (results not shown).

*E. coli* transformant RNA homologous to *U. maydis* DNA. RNA was extracted from *E. coli* MC4100 and from Sφ1263 transformed by plasmids pBR322, pMH2002, and pMH2003 (1). It was denatured by glyoxylolation (29) before electrophoresis and then transferred to GeneScreen membrane for Northern (RNA) hybridization by using the 1.95-kb EcoRI fragment as the probe. No hybridization was detectable to RNA from untransformed and the pBR322-transformed cells at the stringency used here (Fig. 3A, lanes 1 and 2), but multiple hybridization bands were detected in RNA from both the pMH2002 and pMH2003 transformants (Fig. 3A, lanes 3 and 4). Their sizes ranged from about 2.0 down to 0.5 kb.

**U. maydis** poly(rA)* RNA transcripts. Total RNA was extracted from log-phase *U. maydis* 521 cells (12), and a

<table>
<thead>
<tr>
<th>Organism and strain</th>
<th>DHOase activity</th>
<th>% Wild-type activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MC4100</td>
<td>3.15</td>
<td>100</td>
</tr>
<tr>
<td>Sφ1263</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td>Sφ1263(pMH2002)⁶</td>
<td>1.11</td>
<td>35</td>
</tr>
<tr>
<td>Sφ1263(pMH2003)⁶</td>
<td>1.36</td>
<td>43</td>
</tr>
<tr>
<td>Sφ1263(pMH2004)⁶</td>
<td>0.90</td>
<td>29</td>
</tr>
<tr>
<td>U. maydis 521</td>
<td>0.78, 0.81, 0.71, 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>241</td>
<td>0.13, 0.12, 0.13, 0.12</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td>898</td>
<td>0.72, 0.72</td>
<td>92, 90</td>
</tr>
<tr>
<td>904</td>
<td>0.46, 0.50</td>
<td>58, 61</td>
</tr>
<tr>
<td>920</td>
<td>0.80, 0.74</td>
<td>102, 91</td>
</tr>
</tbody>
</table>

<sup>a</sup> Micromoles of carbamyl aparatase produced per milligram of total protein in the standard assay.

<sup>b</sup> Strains with plasmid numbers in parentheses are transformants.

<sup>c</sup> All activities in a column were extracted and prepared together.

<sup>d</sup> Three independent transformants are shown.

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**FIG. 1.** (A) DNA restriction map of the cloned PYR3 region of *U. maydis*. The restriction sites shown are PstI (P), PvuII (P'), NdeI (N), NdeI (N<sup>+</sup>), EcoRI (E), SphI (S), HindIII (H), and Clal (C). In plasmid pMH2002, the fragment orientation is such that the right-hand-terminal PstI site shown in the figure is closest to the EcoRI site of the pBR322 vector. In pMH2003, the fragment is in the opposite orientation. pMH2004 is identical to pMH2003, except for the deletion of the EcoRI fragment containing both insert and vector sequences. (B) 1.95-kb EcoRI probe fragment.

**FIG. 2.** Chromosomal DNA restriction fragments which hybridize to cloned PYR3 sequences. DNA from *U. maydis* 521 was digested by EcoRI (lane 1), PstI (lane 2), and SphI (lane 3). In control experiments, plasmid pMH2002 was digested by EcoRI (lane 4), pMH2003 was digested by EcoRI (lane 5) or PstI (lane 6). Plasmid and chromosomal digests were run on the same gel; different exposures of the resulting filter are shown. The 1.95-kb EcoRI probe was used. A 1-kb DNA ladder (GIBCO-BRL) provided the size markers.
sample was subjected to one cycle of oligo(dT)-cellulose chromatography (28). This RNA was analyzed by Northern hybridization with the same probe as described above. A major transcript of 1.25 kb hybridized with the probe, as well as a minor one of 1.65 kb (Fig. 3B).

**Transformation of* U. maydis* pyrimidine auxotrophs.** *U. maydis* 241, containing the pyr3-1 mutation, is able to grow on NM supplemented with dihydroorotic acid but not with carbamyl aspartate, which is consistent with its possessing a mutation in the DHOase gene (31). This strain has now been shown by direct biochemical assay to contain wild-type levels of orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase activities (results not shown) but reduced levels of DHOase activity compared with that of the wild type (Table 1).

Spheroplasts of *U. maydis* 241 were treated with different linearized plasmid DNAs. These linearized plasmid DNAs were pMH2003 that had been digested by *NheI* to give the *pyrC*-complementing sequences on a 6.3-kb fragment (Fig. 4A), pMH2002 that had been partially digested by *NheI*, and the 7.5-kb full-length linear molecules isolated after gel electrophoresis (Fig. 5A). Transformation has also been effected by a 23-kb fragment derived from pMH2001 by *NcoI* digestion, but as the results were similar to transformation by the digest of pMH2003, the results will not be presented here. Potential transformants appeared as large colonies against a background of minute ones after 4 to 6 days of incubation at 32°C on selective medium. Transformation frequencies were 40 to 90/μg of transforming DNA for the first of the DNAs just described and about 200/μg per 5 × 10^6 viable spheroplasts for the second. Single colonies were picked after regrowing the transformant colonies on NM agar. All transformants tested were stable in that there was no loss of pyrimidine prototrophy after growth for 8 to 10 generations in nonselective CM (results not shown).

Total DNAs from 20 of the 6.3-kb *NheI* fragment transformants were digested with *EcoRI*, electrophoresed, and transferred to a nitrocellulose membrane for Southern hybridization analysis. Nick-translated pMH2002 DNA was used as a probe which should hybridize to a 1.95-kb fragment and to two junction fragments (with respect to the cloned sequences) in the *EcoRI*-digested chromosomal DNA. These junction fragments extend from each of the two cloned *EcoRI* sites to adjacent sites in the chromosome which were not cloned. Junction fragments of 6.8 and 9.5 kb were found for both the wild-type strain 521 and the *pyr3*-1 strain 241 (Fig. 4B). One set of 12 transformants displayed a hybridization pattern identical to that of the parent strain (Fig. 4C). When a probe containing only vector sequences (nick-translated pBR322 DNA) was used, there was no detectable hybridization to these restricted transformant DNAs and none, as expected, to 521 and 241 DNAs (results not shown).

A second set of seven transformants had gained extra *EcoRI* fragments homologous to pMH2002 sequences. Many of these, and also the 1.95-kb *EcoRI* fragment itself, were present at copy numbers much greater than those of the single-copy parental junction fragments (Fig. 4D). In addition, most of these extra fragments (13 of a total of 17 in the seven transformants) contained sequences which hybridized to pBR322 vector sequences (results not shown). A single transformant contained just two extra *EcoRI* fragments of 1.1 and 0.65 kb that were homologous to pMH2002 sequences. The hybridization intensity of both fragments was, however, lower than would be expected if they were present at the same copy number as the parental fragments (results
not shown). It is possible that these fragments were not present in all cells of the culture; alternatively, perhaps they were a second pair of junction fragments, and only a short sequence in each was homologous to the pMH2002 probe sequences.

Total DNAs from 19 transformants generated by the two full-length 7.5-kb NheI fragments were also analyzed in an identical manner. DNA of 10 transformants had two EcoRI fragments which hybridized to the probe in addition to the three parental fragments (Fig. 5C). Their sizes of 1.4 and 4.1 kb are those expected if the transforming DNA integrated into the chromosome by homology at the NheI site via a single crossover event. In agreement with this suggestion, both of the novel fragments hybridized to pBR322 vector sequences (results not shown). The hybridization intensities of these fragments (and that of the 1.95-kb fragment) relative to the parental junction fragments, in many cases, indicate substantial tandem insertion of multiple copies. One transformant had gained multiple EcoRI fragments, again suggesting multiple nonhomologous insertions of DNA to generate novel junction fragments (Fig. 5D). An additional eight transformant DNAs had hybridization patterns which appeared to be a combination of these two (Fig. 5E).

**DHOase levels in U. maydis transformants.** DHOase enzyme levels in extracts from the wild type and the pyr3-1 mutant were compared with the levels in a representative of each of the three main types of transformants described above. Strain 898, a transformant with a parental EcoRI restriction pattern only (Fig. 4C, lane 3) and strain 920, a transformant probably generated by homologous insertion of DNA (Fig. 5C, lane 3), had both regained wild-type enzyme levels. Strain 904, a transformant arising from multiple nonhomologous insertion (Fig. 4D, lane 3), had regained substantial wild-type levels (Table 1).

**UV sensitivity of U. maydis transformants.** The UV resistance of each of the transformants used for the determination of DHOase enzyme levels was compared with that of the parent, mutant, and wild-type strains (Fig. 6). All three transformants regained virtually full wild-type resistance, with mutant strain 241 showing the marked sensitivity reported previously (31).

**Complementation of S. cerevisiae ura4 mutations.** In order to determine if the cloned PYR3 gene could be expressed in S. cerevisiae and thus complement a ura4 mutation (DHOase; 15, 25), the 3.2-kb double PstI fragment (Fig. 1A) was ligated in YRp7 at the PstI site of the pBR322 sequences. The multicopy YRp7 includes a TRP1-ARS1 fragment in the EcoRI site of pBR322 and thus replicates autonomously in S. cerevisiae (40). An S. cerevisiae trp1 ura4 strain was transformed by the resulting plasmid, pMH2011. Transformation to tryptophan prototrophy was as efficient as a control YRp7 transformation, ca. 1.5 × 10^7 transformants per μg of DNA for both. The growth requirements of 94 such pMH2011 transformants were tested, and all grew on YNB agar without both tryptophan and uracil. None of 47 control YRp7 transformants grew unless supplemented with uracil. Five pMH2011 transformants and three YRp7 transformants were grown in YNB liquid medium, and the doubling times for the former transformants were found to be from 5.6 to 6.0 h, whereas for the latter they were >15 h. In YNB supplemented with uracil, the doubling times for the pMH2011 transformants varied from 3.4 to 4.5 h compared with 3.0 to 3.8 h for the YRp7 transformants.

Southern hybridization analysis confirmed that all five of the pMH2011 transformants contained unarranged
pMH2011 plasmid DNA (determined by PstI and EcoRI digestion) and all control transformants contained YRp7 (results not shown). Northern hybridization analysis of total RNA extracted from pMH2011 transformants revealed multiple transcripts which hybridized to the 1.95-kb EcoRI PYR3 probe used (Fig. 7, lanes 1 through 5). None of the YRp7 transformants contained PYR3-homologous transcripts (Fig. 7, lanes 6 through 8) even though a chromosomal HIS3 transcript was detected at levels similar to those in the pMH2011 transformants (results not shown).

**DISCUSSION**

We have described the cloning of the PYR3 gene of *U. maydis* by direct complementation of pyrC mutations of *E. coli*. Because the clone bank used was a genomic one, it can be concluded that the PYR3 gene, encoding the enzyme DHOase, is not interrupted by introns; the prevalence of interrupted genes in *U. maydis* is not yet known. The inability of the clone bank to complement any of the other *E. coli* pyrimidine auxotrophs tested may, for example, because the intact genes are not in the bank, because they contain introns, or because their regulatory sequences are incompatible with expression in bacteria. These are situations well documented for other eucaryotes (for a review, see reference 30). The observation that the gene is expressed in both orientations within pBR322 might suggest that *U. maydis* regulatory sequences, and in particular those of its promoter, are recognized in *E. coli*. This would be consistent with the orientation-independent pattern of the PYR3 homologous transcript distribution. However, the diversity of transcript sizes in these transformants indicates that initiation and termination of transcription occur at multiple sites. Promoter activity directing transcription toward an insert has been detected on both sides of the *PstI* site of pBR322, which could be responsible for orientation-independent expression of PYR3 (41). We have recently found that the 1.95-kb EcoRI fragment of pMH2002 (Fig. 1A), when inserted into pBR322, will complement pyrC mutations, but only in an orientation-dependent way.

Northern hybridization identified a major *U. maydis* PYR3 poly(rA)* transcript of 1.25 kb. Although we have no quantitative data, this transcript appears to be of relatively low abundance, at about 50% of that of a heat shock transcript (F. Malik and G. R. Banks, unpublished observations). The transcript is sufficient to encode a polypeptide of some 40 kilodaltons, but the DHOase enzyme of *U. maydis* has not been characterized so as to establish its molecular identity. A comparison of reaction parameters was not able to distinguish between the *U. maydis* and *E. coli* enzymes. Subtle differences may be difficult to detect unless considerably purer preparations are used rather than the crude extracts in this work. DHOases may be a catalytically conserved group of enzymes and share the property of being zinc metalloenzymes (22, 23, 37, 43). At the amino acid sequence level, the *S. cerevisiae* enzyme shares 40% homology with both the *E. coli* and the *Salmonella typhimurium* DHOases and 23% homology with that from *Drosophila melanogaster*. Perhaps more strikingly, all four polypeptides share three highly conserved amino acid domains (15, 45). When DNA sequencing of the PYR3 gene is complete (A. Spanos and G. R. Banks, work in progress), further comparisons will be possible. The 40 kilodaltons predicted for the polypeptide encoded by PYR3 is a similar size to that predicted for the *S. cerevisiae* DHOase polypeptide (15) but much smaller than the multienzyme pyr1-3 polypeptide of animal cells (22). There is also genetic evidence that the genes encoding aspartate transcarbamylase and DHOase activities are unlinked (P. D. Moore, Ph.D. thesis, Council for National Academic Awards, London, United Kingdom, 1974), further evidence that *U. maydis* does not encode a multienzyme polypeptide incorporating DHOase activity.

The PYR3 gene was also able to complement an *S. cerevisiae ura4* mutation. The *URA4* gene encodes the *S. cerevisiae* DHOase activity (15, 25). Although multiple and abundant PYR3 homologous transcripts were detected in transformants, the majority may not be translationally competent in view of the extended doubling time of transformed cells growing in the absence of uracil and in view of the relatively low DHOase activity detected. All major transcripts in the transformants were, in fact, larger than the 1.25-kb *U. maydis* transcript.

A characteristic property of *U. maydis* pyrimidine auxotrophs is their marked sensitivity to DNA-damaging agents such as UV and γ irradiation and nitrosoguanidine (18, 31). As a result of these observations, mutants have been isolated which are defective in the pyrimidine biosynthetic pathway, from aspartate transcarbamylase to orotidine-5'-phosphate decarboxylase, and they have been characterized genetically and physiologically. One mutant, pyr3-1, was designated defective in DHOase activity on the basis of genetic complementation tests and its utilization of metabolic intermediates (31). This has now been confirmed by direct enzyme assays (Table 1). We have, therefore, used strain 241 containing the pyr3-1 mutation as the recipient for DNA transformation studies with the cloned PYR3 gene. Although inefficient transformation of *U. maydis* to neomycin resistance has been reported previously (5), transformation to hygromycin resistance occurs most efficiently when linear DNA encoding a bacterial resistance gene is driven by a *U. maydis* heat shock gene promoter (42).

In the present studies, plasmid pMH2003 was digested by *Nhe*I to give a linear transforming DNA of 6.3 kb carrying the pyrC-complementing sequences. It has one terminus of *U. maydis* and the other terminus is that of pBR322 DNA (Fig. 4A). In the absence of a unique restriction enzyme site within the insert of pMH2002, it was partially digested by *Nhe*I and the full-length 7.5-kb linear molecules were isolated to give a second transforming DNA. Half of these
molecules have *U. maydis* sequences at both termini and half have pBR322 sequences (Fig. 5A). In agreement with the findings of Wang et al. (42), we have found that linear DNA was some 2 orders of magnitude more efficient than covalently closed circular DNA in generating transformants. Although the regeneration frequency of spheroplasts from strain 241 was four- to fivefold lower than that from wild-type strain 521, a fact which might be responsible for the higher transformation frequencies reported by Wang et al. (42), pyrimidine prototrophic transformants were readily isolated. Transformation frequencies have varied from 40 to 200/µg; these to fourfold more efficiently than a

observed that a pyr3-1 strain that also bears the

transformation frequency, the distribution of different trans-

crhomosomal DNA. The stability of the *PYR3* transformants during mei-

osis has not yet been determined, but Wang et al. (42) have shown that the hygromycin resistance of *U. maydis* trans-

formants is stably inherited through meiosis.

Transformants arising from the 6.3-kb *NheI* fragment of pMH2003 were examined by hybridization analysis of their DNAs after *EcoRI* restriction. Of these transformants, 60% exhibited only the hybridization pattern of the parental 241 and wild-type 521 strains. The possibility that these prototrophs were generated by spontaneous reversion of the *pyr3-1* allele is unlikely because the spontaneous reversion frequency of strain 241 is \( <5 \times 10^{-7} \) (S. Y. Taylor, unpublished observations), and we have not isolated prototrophs from control transformation experiments with pBR322. These transformants appear to correspond to the *S. cerevisiae* class III of Hinnen et al. (16) and could arise by a double crossover event between the homologous plasmid and chromosomal sequences or by gene conversion. This is consist-

ent with our observation that these transformants contain no detectable pBR322 vector sequences. Only one end of the transforming DNA was homologous to genomic sequences at the *PYR3* locus. We have also generated identical transformants using pMH2003 linearized by *BamHI*, an enzyme which cuts once only in the vector DNA (results not shown).

Therefore, terminal homology is not required to generate this type of transformant. Another 35% of the transformants had a much more complex *EcoRI* hybridization pattern than that of the parent. In addition to the three parental fragments, many novel fragments of different sizes had been generated. Most of these fragments also hybridized to pBR322 vector sequences. It is likely that these transformants arose by multiple, nonhomologous insertion of transforming DNA into *U. maydis* chromosomes, thereby gener-

ating novel junction fragments, a situation found after transformation of other fungi (4, 7, 11, 34, 36). We cannot, however, determine if these transformants have, in addition, undergone an event leading to the first type of transformant, an event which is responsible for prototrophy, while the

nonhomologous insertions only involve incomplete fragments of the *PYR3* gene. That this need not be the case is indicated by the hybridization intensity of the 1.95-kb fragment, suggesting insertion of multiple intact copies of *PYR3*. Three of these transformants also have a high-copy-number 4.3-kb fragment, a size expected if tandem direct repeats of the intact DNA had been inserted (Fig. 4D, lanes 2, 3, and 6). Integration of tandemly repeated sequences can facilitate subsequent recovery of transforming DNA (4, 47).

Transformants from the 7.5-kb partially *NheI*-restricted DNA were also analyzed as above. Of these transformants, 53% had, in addition to the three parental *EcoRI* fragments, one fragment of 1.4 kb and one fragment of 4.1 kb. These are sizes expected for homologous insertion of the half of the transforming DNA possessing *U. maydis* sequences at both termini (Fig. 5A). They could be generated by a single crossover event at the *NheI* site, analogous to the targeting of *S. cerevisiae* genes (35). This is consistent with our observation that these two fragments hybridized to pBR322 vector sequences. In almost all cases, the hybridization intensities of these fragments to the *PYR3* probe compared with the parental junction ones suggested that many copies of the DNA had been tandemly inserted homologously into the chromosome. The stability of these repeats is not yet known. Of the 4% transformants, 5% were similar to those apparently generated by multiple nonhomologous insertion of DNA as described above. The hybridization pattern of the remaining 42% was a combination of the two types in this set. Because the transforming DNA used in these experiments was a mixture of two permuted linear molecules (Fig. 5A), it is not possible to definitively ascribe a particular structure to a transformant type. However, it is likely that the homologous insertions arose via a single crossover between the *U. maydis* termini of one linear plasmid and the homologous *U. maydis* chromosomal sequences. We have recently found a unique restriction site within the *U. maydis* sequences of pMH2002, making it possible to analyze this more directly.

All three major classes of transformants described above had regained DHOase activity and resistance to UV irradiation. The latter finding implicates the pyrimidine auxotrophy directly in the generation of UV sensitivity. Both transformants that probably arose by homologous events had regained wild-type DHOase levels (Table 1). Because one of the transformants carried multiple tandem copies of the *PYR3* gene (Fig. 5C, lane 3), DHOase levels were not proportional to *PYR3* gene copy number, and we suggest that expression is regulated in some way. Conversely, although the transformant arising from multiple nonhomologous insertions also carried multiple copies of the gene, its DHOase levels were significantly lower than wild type. The location in the genome at which the *PYR3* gene becomes inserted may well dictate its level of expression. Transcript mapping for all three transformants might clarify some of these differences.

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**LITERATURE CITED**