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The topoisomerase I gene from *Ustilago maydis*: sequence, disruption and mutant phenotype

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ABSTRACT

The *Ustilago maydis* genomic *TOP1* gene encoding DNA topoisomerase I was cloned by amplifying a gene fragment using the polymerase chain reaction, and using this fragment to search a genomic DNA library by hybridization. The predicted peptide sequence exhibited 30–40% identity to other eukaryotic *TOP1* genes, yet differed in several features. First, an unusually long acidic region was identified near the amino terminus (28/29 residues are acidic), which resembles other nucleolar peptide motifs. Second, an atypical carboxy-terminal 'tail', absent in other *TOP1* genes, followed the active site tyrosine residue. A *top1* gene disruption mutant was constructed by replacing the genomic *TOP1* gene, with a *top1::HygR* null allele. This mutant lost the abundant topoisomerase I activity evident in wild-type *U.maydis*, and displayed a subtle coloration phenotype evident during cell senescence.

INTRODUCTION

DNA topoisomerases are versatile enzymes which facilitate DNA replication (23), transcription (13), chromatin condensation (19) and recombination (50). All eukaryotic topoisomerases can relieve superhelical stress in DNA, yet in yeast and in human cells this task is shared by topoisomerases encoded by three (or more?) genes (50). Thus, overlapping activities have made it challenging to define the role played by each enzyme in these cellular processes.

Clues to the duties of various topoisomerases are provided by the unique biochemical properties of each enzyme. Topoisomerases are classified as type I or type II, according to transient formation of single strand or double strand breaks in DNA. The known eukaryotic type II topoisomerases require ATP for catalysis, whereas eukaryotic type I enzymes are ATP-independent. Eukaryotic type I topoisomerases remove a positive or negative supercoil by covalently bonding to the 3′ phosphate group of DNA, passing the intact DNA strand through the resultant break, and resealing the broken strand, thereby changing the linking number in steps of one (8, see 49 for review). Null mutations in the topoisomerase I gene are not lethal in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (46), but are lethal to a developing *Drosophila melanogaster* embryo (25). Type II topoisomerases catalyze a double stranded break in DNA, covalently bond to both 5′ phosphate groups, pass a double strand DNA through the break and reseal both strands (reviewed in 26). The ability to pass one helix through another allows topoisomerase II to resolve intertwined and entangled DNAs. This activity of topoisomerase II is required for chromosome condensation and decondensation, at the start and finish, respectively, of mitosis (19,47). A null mutation in the *Schizosaccharomyces pombe* or *S.cerevisiae* topoisomerase II gene is lethal, apparently because interlocked chromosomes are not properly partitioned at mitosis (19,47).

Topoisomerases are attractive candidates for enzymes involved in genetic recombination. This hypothesis stems from their ability to cleave single- or double stranded DNA, and subsequently religate DNA termini. These events are common features of virtually all recombination models. *In vitro* evidence indicates that mammalian topoisomerase I can transfer a DNA strand to a nonhomologous DNA molecule and ligate the molecules together (16). Similarly, a eukaryotic topoisomerase I enzyme expressed in *E.coli* can mediate a form of illegitimate recombination measured by bacteriophage λ excision (39). *In vivo* data suggesting roles for type I and type II topoisomerases in recombination has been obtained in *Saccharomyces cerevisiae* (see below).

The yeast, *Saccharomyces cerevisiae*, expresses type I and II topoisomerases encoded by the *TOP1* and *TOP2* genes, as well as a gene designated *TOP3* which is homologous to prokaryotic type-I topoisomerases. These genes have been isolated and sequenced, and their gene products biochemically characterized (15,43,48, summarized in 50). Mitotic recombination rates between direct or inverted repeats are not affected by mutations in *TOP1* or *TOP2* (except for rDNA direct repeats; 10). In contrast, a *top3* mutant displayed increased mitotic recombination between inverted repeats, as well as direct repeats (1,48). Mitotic recombination between rDNA repeats increases approximately 50–200 fold in a *top1* or *top2* mutant background, indicating a suppression of rDNA recombination by topoisomerases I and II (10). The intensive transcriptional activity associated with rDNA suggests that suppression may not be a dedicated activity of topoisomerases I and II, but rather a result of the accumulation

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of superhelical tension generated by RNA polymerase I (50). Indeed, topoisomerase I seems to accumulate preferentially in nucleoli (33). Finally, some illegitimate recombination events observed in *S.cerevisiae* have been postulated to be topoisomerase I-mediated (38). Despite such data from the *S.cerevisiae* system, it is still unclear whether topoisomerases perform specialized functions in double strand break repair, meiotic recombination or illegitimate recombination *in vivo*.

An abundant type I topoisomerase expressed by the basidiomycete fungus, *Ustilago maydis*, has been biochemically characterized (5,37,42). Following isolation of the first eukaryotic DNA recombination mutants in this fungus (17), *Ustilago* has become an important eukaryotic model for studying the genetics and biochemistry of recombination. *Ustilago maydis* differs from *Saccharomyces cerevisiae* in demonstrating an abundant end-ligation activity (12) resembling that of human cells (8). In order to better understand the relationship between topoisomerases and various forms of recombination, we are characterizing the topoisomerase genes as well as enzymes from *Ustilago*. Here we present the cloning and sequence of the *TOP1* gene, along with preliminary phenotypic results from a *top1*-disruption mutant.

MATERIALS AND METHODS

Strains, plasmids and media

Ustilago maydis UCM5 (ab, leu1-1, ade1-1) was obtained from Dr W.Holloman, Cornell University Medical College. DNA cloning was carried out in *E.coli* XL1 Blue (*recA1, endA1, gyrA96, thi1, hsdR17, supE44, relA1, lacI*^{q}, [F′ *proAB, lacI*^{q}D*M15*, Tn*10* (*tetR*)].; Stratagene, La Jolla, CA). pBluescript and pBC were obtained from Stratagene, Inc. pCM449 (a generous gift from Dr W.Holloman) is a p34H (44) derivative, containing the *hygromycin resistance* gene positioned between promoter and terminator fragments from a *U.maydis hsp70* gene. *U.maydis* was grown in Yeast Extract Peptone Sucrose medium (YEPS) or Holliday Minimal Medium (18).

Polymerase chain reaction

The following DNA oligonucleotide primers ('I' represents deoxy-inosine residues) were made using an ABI Model 394 at the Jefferson Cancer Institute:

1+ 5′ GAC/TACIGTIGGITGC/TTG
2+ 5′ AAA/GGTIC/TC/TIA/CGIACITAC/TAAC/TGC
2− 5′ GCG/ATTG/ATAIGTICT/GIG/AG/AIACT/CTT
3− 5′ TGG/ATGG/ATTG/ACAIAG/AIATIGC

Polymerase chain reaction (PCR) was carried out on either a Perkin Elmer Model 9600, or an Ericomp 'Powerblock' thermal cycler. Fifty microliter reactions contained 100 μM each primer, 200 μM each dNTP, 1× PCR Buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 2.5 units Taq polymerase and approximately 10 ng *Ustilago* genomic DNA. Amplification temperature cycles were as follows: 2 min at 95°C; 35 cycles of 30 sec at 94°C, 40 sec at 48°C, 2 min at 72°C; and a final 8 min at 72°C.

Library construction

Ustilago genomic DNA was isolated by the method of Hoffman and Winston (16), partially digested with *Sau3A*, and size-fractionated on sucrose gradients to isolate fragments >30kb. (31). These fragments were ligated to *Bam*HI-digested pHC79 cosmid DNA and packaged into bacteriophage λ heads *in vitro* (Stratagene '*Gigapack Gold*'), prior to transfection into *E.coli* XL1 Blue MRF′ according to manufacturers instructions. The cDNA library representing a mating population of *U.maydis* was a generous gift of S.Gold and J.Konstad, University of Vancouver.

Southern hybridizations

DNA digests were resolved on 0.9% agarose gels, transferred to Zeta Probe membranes in 0.4 M NaOH, and hybridized with a ^{32}P -labelled random-primed DNA probe according to the manufacturer (Bio-Rad). Hybridization and washing were performed at moderate stringency (55°C) to address the possible existence of additional *TOP1* alleles. Genomic and cDNA libraries were searched by hybridization according to manufacturers instructions (New England Nuclear, Colony/Plaque Screen).

Ustilago transformation

Spheroplast preparation and transformation of *U.maydis* followed Fotheringham and Holloman (12).

DNA sequencing and analysis

The *Ustilago TOP1* gene was subdivided for sequencing by a combination of subcloning from pUT1-R9 and pUT-H12, and synthesis of oligonucleotide primers for regions lacking restriction sites. Sequencing reactions were performed using the dye-linked dideoxynucleotide termination method (Applied Biosystems Inc.). Sequence compilation and analysis employed the University of Wisconsin 'GCG' software programs.

Topoisomerase I assays

U.maydis cell extracts were prepared as previously described (42). Polyethylene glycol-clarified extracts were assayed for ability to relax negatively supercoiled plasmid DNA (42). Twenty-five microliter reactions contained 300 ng pBluescript SK+ DNA (Stratagene, Inc.), 25 mM Tris−HCl buffer pH 7.5, 2 mM EDTA and 25−200 ng total protein. After 30 min at 31°C, reactions were treated with SDS and proteinase K, and analyzed by agarose electrophoresis and ethidium bromide staining, as described (42).

RESULTS AND DISCUSSION

Cloning TOP1

Our strategy for cloning the *U.maydis TOP1* gene was to identify peptide domains which are conserved among eukaryotic topoisomerase I genes, to design oligonucleotide primers based on these domains, and to use the polymerase chain reaction (PCR) to amplify the intervening *TOP1* DNA fragment from genomic template DNA. This gene fragment can then be used to select a full length gene from genomic or cDNA libraries (see Fig. 1). Comparison of peptides encoded by the human, *Drosophila* and *Saccharomyces* topoisomerase I genes (21,25,43) revealed several regions of sequence identity which are conserved among the eukaryotic topoisomerase I peptides. Three conserved regions were selected to serve as 'templates' to design oligonucleotide primers for use in the polymerase chain reaction (PCR). These conserved peptide motifs: 1) DTVGCC, 2) KVFRTYNAS, and 3) AILCNHQ, were selected based on amino acid sequence conservation, proximity to one another, and paucity of highly degenerate codons. Peptides '2' and '3' were also selected by Hsieh *et al.* (20) to design PCR-primers used to amplify part of the *TOP1* gene from *Drosophila melanogaster*. Degenerate

positions within codons were represented by both deoxynucleotides at positions of two base degeneracies, and by a deoxyinosine residue (I) at three or four base degeneracies (24). Primers '1+', '2+', '2−' and '3−' were synthesized based on the coding strands for peptides 1 and 2, and the noncoding strands for peptides 2 and 3 (see Materials and Methods) and used for PCR.

The four primers were used in PCR in all three coding strand/noncoding strand pairs to amplify *U.maydis* genomic DNA fragments. Primers '2+' and '3−' directed amplification of a 160 bp DNA fragment from a template of *U.maydis* genomic DNA. This fragment was rendered blunt-ended with the Klenow fragment of *E.coli* DNA polymerase I (31), and ligated into the *Sma*I site of pBluescript SK+, yielding pUT1-2/3. The DNA sequence of this fragment predicted an amino acid sequence showing limited homology to other topoisomerase I peptides. This fragment was radiolabelled and used to probe a cosmid DNA library containing 30−40 kb fragments of *U.maydis* genomic DNA.

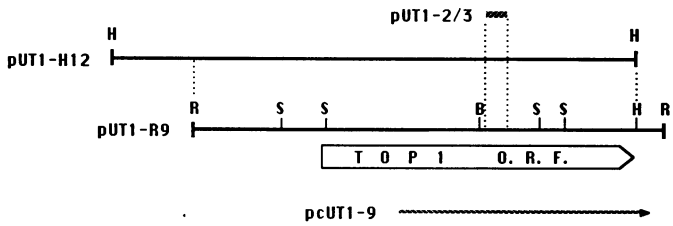

Figure 1. Physical map of *TOP1* gene region from *U.maydis*. The open reading frame is labelled TOP1 O.R.F. Genomic *TOP1* clones are labelled pUT1-2/3 (PCR product), pUT1-R9 and pUT1-H12. The largest cDNA clone obtained is labelled pcUT1-9. Restriction sites are indicated as H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; and B, *Bam*HI.

Three cosmid clones were identified which hybridized to the putative *TOP1* probe; these were called pUH-1c, pUH-3c and pUH-4c. Restriction mapping indicated that these three cosmids were identical (data not shown). Subsequent Southern hybridization identified a 4.6 kb *Eco*RI fragment and a 5.2 kb *Hin*dIII fragment containing the pUT1-2/3 probe fragment. These fragments were subcloned into pBluescript SK+, forming pUT1-R9 and pUT1-H12, respectively. These plasmids were further subcloned to generate DNA sequencing templates and probes for genomic Southern blots. Sequencing of these subclones revealed an open reading frame with highly significant homology to other *TOP1* genes.

Predicted TOP1 peptide

The genomic *TOP1* gene was sequenced completely along both strands using the dye-linked dideoxy-nucleotide termination method (Applied Biosystems Inc.) to allow comparisons to previously sequenced topoisomerase I genes. Restriction digests of cosmid pUH-1c and subclones pUT1-R9 and pUT1-H12 were compared to genomic Southern blots to ensure that each clone accurately represented the corresponding *U.maydis* genomic region. Sequence analysis identified an open reading frame (ORF) of 3057 bases encoding a putative peptide of 1019 amino acid residues (GenBank accession # L32017). A search for potential introns was conducted using consensus splice sites: 5'GTA/GAGT and C/TAG3' compiled from 18 introns identified in 8 *Ustilago* genes described in the GenBank database (4,6,15,28,40,45). These canonical sequences are very similar to those derived for a variety of filamentous fungi (2), however

A

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Hs  150 KKIKTEDTK.KEKKRKLEEEEDGKLKKPKNKDKDKKVPEPDNKKKKPKKEEEQKWKWWEEERYPEGI 215
Sc   88 KKIKKEDGDVKVKTTKKEEQENEKKKREEEEEEDKKA.....KEEEEEYKWWEKENEDDTI 143
Um  102 KKSNGSDDDEDDDDDDDEGDDDDEEDDDDDDDDDDKPLSKSSKSNRKPPKTMSITGSGEK..... 161
            ****************************

Hs  216 KWKFLEHKGPVF 227
Sc  144 KWVTLKHNGVIF 155
Um  162 KWDVLIHKGPRF 173
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B

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Hs  716 LGTSKLNYLDPRITVAWCKKWG..... 737
Sc  720 LGTSKINYIDPRLSVVFCKKYD..... 741
Um  815 LSTSKLNYIDPRITLAWLKEWDDRLSDLGQGKAAPKKKVKKEEEENDIKPKKKDAKGAASKKRAAKTG 882

Hs  738 .....VPIEKIYNKTQREKFAWAIDMADEDYEF*..... 765
Sc  742 .....VPIEKIFTKTLREKFKWAIESVDENWRF*..... 769
Um  883 LANSTGDSEKMELGLQVMNISQFFANALQKKFKWA.ASGDDGRDISAKWVFVKDAQSKMRKLDSAERKG 950

Hs      ..........
Sc      ..........
Um  951 QKGGSMAAMTDAADSKEAQPKVNCVLKKQTSADRKMSKPIKAVDKTEESDDDLSSDSSDDGDKPLASVV* 1019
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Figure 2. Contrast between amino termini (**A**), and carboxy termini (**B**), of topoisomerase I peptides from *Homo sapiens*, *S.cerevisiae* and *U.maydis* (labelled *Hs*, *Sc* and *Um*, respectively. Gaps, displayed as '...', were introduced into each peptide using the University of Wisconsin GCG 'GAP' program to maximize alignment. Amino acid residues conserved by all three organisms are shown in bold type, and the long acidic motif in the *U.maydis* sequence is underscored with asterisks. Amino acid residues are numbered to indicate relative positions of conserved domains. The translational stop codon for each peptide is represented as a single asterisk.

a consensus 2′ 'branch site' could not be derived with confidence. Two potential intron 5′ splice sites, each with a single mismatch from the consensus, were identified at bp 2966 and bp 3324.

In order to confirm or reject the existence of introns in the *U.maydis TOP1* gene, and to establish whether the gene is transcribed, a cDNA library was searched by hybridization to a *TOP1* DNA probe. Seven *TOP1* partial cDNA clones were identified among approximately 8×10^5 plaques, suggesting a steady-state message abundance of approximately 9×10^{-6}, or .0009%. The longest partial cDNA clone, designated pCUT1-9c, measured 2.3 kb in length (Fig. 1). This insert was sequenced and compared to the genomic *TOP1* gene. The *TOP1* partial cDNA sequences were found to be identical to the corresponding genomic regions, effectively ruling out the existence of the two potential introns postulated from genomic DNA sequence.

The predicted *U.maydis* topoisomerase I protein is rich in charged amino acids, in agreement with the amino acid compositions of other topoisomerase I proteins. Barring post-translational processing, this ORF predicts a peptide with a molecular mass of 115.3 kD, which is slightly larger than the 110 kD and 105 kD masses previously estimated from SDS−PAGE (5). Comparison of the *TOP1* ORF from *U.maydis* with *TOP1* peptides from human (21), plant (22), amphibian (34), insect (25) or other fungi (43,47) reveals significant sequence identities (30−40%). Codon usage was strongly biased in favor of C in the third position, with G favored over A in two base 'wobble' codons.

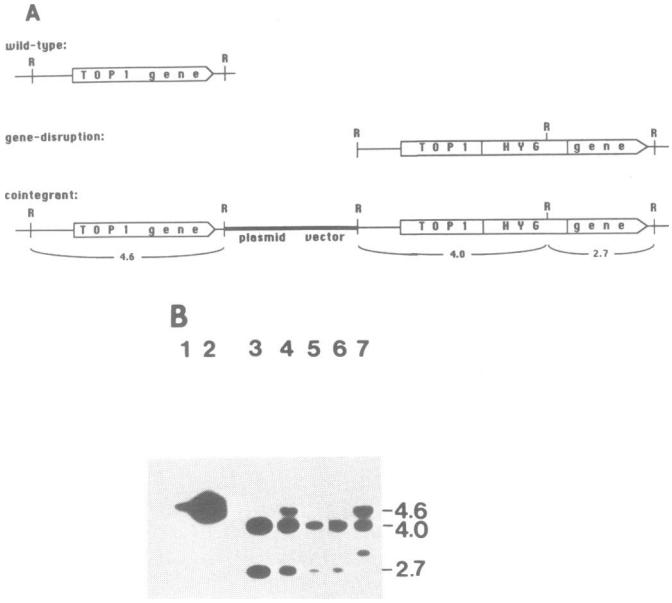

Figure 3. Disruption of the *U.maydis TOP1* gene. **A.** *Eco*RI (R) restriction maps of the genomic *U.maydis TOP1* gene showing wild-type, cointegrant and replacement (gene disruption) alleles, respectively. Sizes of *Eco*RI (R) restriction fragments are indicated in kilobases. **B.** Southern blot analysis of *TOP1::Hyg* transformants. *Eco*RI digests of control plasmids or genomic DNA from hygromycin resistant transformants was resolved by agarose electrophoresis, transferred to nylon membranes, and probed with the 2.1 kb *Sal*I fragment (Fig. 1) from *TOP1*. Lanes represent wild-type strain UCM5 (lane 1), cosmid pUH-1c (lane 2), plasmid pUT1-R9::*Hyg* (lane 3), cointegrant strain U5-2H (lane 4), gene disruption strains U5-2I and U5-3I (lanes 5 and 6, respectively), and illegitimate integrant strain U5-3Q which presumably arose by nonhomologous integration of the *TOP1::Hyg* construct (lane 7). DNA fragment sizes in kilobases are indicated at right.

The predicted *U.maydis* protein maintains most of the peptide motifs conserved by *TOP1* genes from other eukaryotes, including the motif: 'LGTSKI/LNYIDPR' which forms a covalent tyrosine-3′ phosphate, enzyme−DNA intermediate during catalysis (5,11,29; Fig. 2). Eukaryotic topoisomerase I peptides typically contain a 'VPIEK' motif 14 amino acid residues carboxy-terminal to the active site tyrosine, and the translation stop approximately 23 amino acid residues further distant. The *U.maydis* ORF lacks this conserved sequence (although a weakly similar sequence occurs carboxy-terminal to the expected region; Fig. 2, residues 900−927), and is somewhat larger than other eukaryotic *TOP1* genes due to an additional carboxy-terminal 'tail' of 155 amino acids (Fig. 2). The significance of this 'tail' is unknown, since we are unable to find significant homology to sequences from the GenBank or EMBL databases. A search of this ORF for nuclear localization signals (36) is biased by the high content of lysine residues. Nevertheless, the minimal nuclear localization signal consensus 'KXXK/R' occurs 30 times in this ORF.

One somewhat unusual feature of the predicted *Ustilago* topoisomerase I protein is the unusually long series of acidic amino acids near the amino terminus (28 of 29 residues are aspartic acid or glutamic acid; Fig. 2). Although the predicted topoisomerase I proteins from various eukaryotes have highly charged, highly divergent amino termini, only the *U.maydis* gene has an acidic motif exceeding eight residues in length. This acidic motif, flanked by lysine-rich regions, resembles motifs found in nucleolar proteins such as nucleolin, NO38 and nucleoplasmin (32,35), an observation which is consistent with the reported accumulation of topoisomerase I in the cell nucleolus (33). The functional significance of this acidic region is unknown, but it may mediate interactions with positively charged nuclear proteins as suggested for recombinase REC2 (Rubin *et al.*, submitted). This explanation is attractive because it would help explain the functional interaction of *Ustilago* topoisomerase I with *Ustilago* histone H1 and high mobility group (HMG) proteins (37,42).

TOP1 promoter region

Comparison of the *U.maydis TOP1* promoter region to other fungal promoters reveals several interesting features. Promoters from previously reported *Ustilago* genes appear to lack canonical eukaryotic 'TATA-box/CAAT-box' features in the appropriate spatial context. Banks *et al.*, (3) suggest that CTTT and CATC repeats clustered at *Ustilago* transcription start sites may serve a promoter-function. These sequence motifs are markedly overrepresented in the upstream regions of *U.maydis* genes, and the transcription start sites mapped for five *U.maydis* genes all occur at CTTT or CATC sequences (30,40,41,45). The *U.maydis TOP1* gene displays three CTTT and nine CATC motifs in the 616 bp immediately upstream of the translation start site. A 12 bp direct repeat sequence (CTCGCATTTTGCATCTCGCATTTCGC) occurs 487 bp upstream from the translational start; as well as an imprecise inverted repeat sequence, positioned 336 bp upstream from the translational start, potentiating a stem-loop configuration in the mRNA. Two *Mlu*I restriction sites (5′ ACGCGT) occur in this sequence, 125 bp and 321 bp upstream of the putative translation start codon. In *Saccharomyces cerevisiae*, *Mlu*I sites can be recognized by a transcriptional regulatory factor which regulates gene expression in response to the cell growth cycle (27). Because this cell-cycle regulatory system has not been identified in Basidiomycetes such as *U.maydis*, the significance of these *Mlu*I sites is uncertain.

TOP1 gene disruption

In order to investigate the functions of topoisomerase I in *U.maydis*, the *TOP1* gene was inactivated by inserting the hygromycin resistance gene-cassette (*HygR*;11) within the *TOP1* coding region in the *U.maydis* genome (Fig. 3). First the *Bam*HI site was eliminated from pBC SK− (Stratagene) by 'filling in' the *Bam*HI-derived ssDNA ends using Klenow and religating the resultant blunt ends (31). Next the 4.6 kb *Eco*RI *TOP1* fragment was inserted into the *Eco*RI site of pBC SK−. The hygromycin resistance cassette, utilizing the *U.maydis* heat shock 70 gene promoter and terminator, was then excised from pCM449 using *Bam*HI, and ligated into the *Bam*HI site within the *TOP1* coding region. The resulting p*TOP1*::*HygR* construct (Fig. 3A) was transformed into *U.maydis* UCM5 in the circular, or *Hin*dIII-linearized form.

Resulting hygromycin resistant colonies were analyzed by genomic Southern blot analysis to select precise gene replacement or 'gene knockout' events. Genomic DNA from 16 stable hygromycin resistant transformants was digested with *Eco*RI, resolved by electrophoresis (0.9% agarose gels), and transferred to nylon membranes for hybridization. The *TOP1* hybridization probe was made from the 2.0 kb *SalI*−*SalI* fragment derived from pUT1-R9 (Fig. 1). Fig. 3 demonstrates the hybridization patterns expected and observed for homologous integration events, which generate a wild-type as well as a disrupted *TOP1* allele; and for double homologous recombination events, which generate only the gene replacement event. Three gene replacement events were detected (i.e. Fig. 3B; lanes 5, 6) as well as ten integration events (i.e. lane 4), and three aberrant recombination events (i.e. lane 7). Two *TOP1* gene replacement or 'knockout' mutants (UCM5-2I, and UCM5-3I) were selected for phenotypic testing to rule out possible effects of secondary mutations.

Haploid *Ustilago* carries a single copy of the *TOP1* gene

In order to determine whether additional *TOP1* alleles occur in the haploid *U.maydis* genome, restriction digests of genomic UCM5 DNA were hybridized with a *TOP1* DNA probe under low stringency conditions (Fig. 3B, and data not shown). Only the expected fragment sizes were detected by Southern hybridization, even using extended exposure times or digests with other enzymes. Thus, we find no evidence for additional *TOP1* alleles. This experiment does not address the possible presence of a *TOP3* gene in *U.maydis*, since the *S.cerevisiae TOP3* and *TOP1* genes are highly divergent from one another and would not be expected to cross-hybridize (48).

Topoisomerase I activity is absent in the *top1*-mutant

A type I topoisomerase activity has been purified from *U.maydis* and biochemically characterized (5,37,42). Thus, it is possible to compare the *top1*-mutant and *TOP1* parent strains to determine whether the knockout mutation eliminates the known topoisomerase I activity. Crude extracts were prepared from wild-type and *top1*-mutant strains (42), and assayed for the ability to remove negative supercoils from plasmid DNA. ATP was omitted, and EDTA was included, in these assays to inactivate other topoisomerases or nucleases in the extracts. Fig. 4, lanes 2−9 depict the major type I topoisomerase activity in the wild type extract. Comparable extracts from the *top1*-mutants demonstrated no detectable DNA topoisomerase activity under these reaction conditions (Fig. 4, lanes 11−18). In repeated attempts, we failed to detect this activity in extracts from *top1*-disruption strains U5-2I and U5-3I.

Growth phenotype of *top1*-mutant

During preliminary characterization, the *U.maydis top1*::*Hyg*^{R} mutants demonstrated a small colony phenotype on YEPS plates (Fig. 5A). Growth rates of the *wild-type* and *top1*-isogenic strains were subsequently evaluated by measuring doubling times for the two strains during logarithmic growth in YEPS broth at 31°C. Three separate cultures of the *wild-type* strain UCM5, and *top1*-mutant strains U5-2I or U5-3I, yielded average doubling rates of 202 min for wild-type, and 198 min for *top1*-mutant strains. The unimpaired doubling time of the *top1*-mutant strains

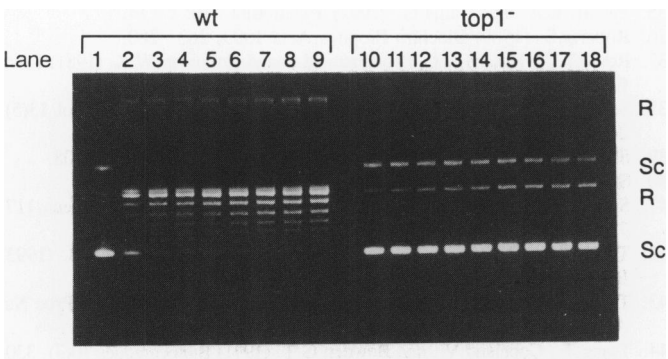

Figure 4. Loss of topoisomerase I activity in *U.maydis TOP1*-disruption mutant. Supercoiled pBR322 DNA (untreated, lanes 1 and 10) was treated with crude protein fractions (see text) from wild-type strain UCM5 (lanes 2−9) or *top1*-mutant strain U5-3I (lanes 11−18), prior to resolution by agarose gel electrophoresis. Total protein assayed was 25 ng (lanes 2, 11), 50 ng (lanes 3, 12), 75 ng (lanes 4, 13), 100 ng (lanes 5, 14), 125 ng (lanes 6, 15), 150 ng (lanes 7, 16), 175 ng (lanes 8, 17) and 200 ng (lanes 9, 18). Topological plasmid isoforms are labelled Sc for supercoiled, and R for relaxed (nicked). The series of bands below R represent topologically relaxed DNA, whereas the upper bands marked Sc and R represent plasmid dimers.

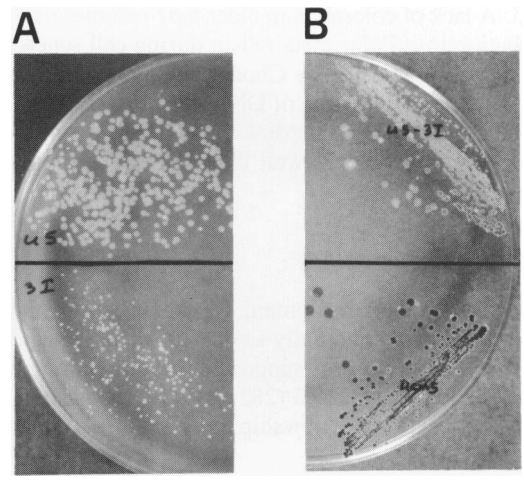

Figure 5. Small colony phenotype, and lack of coloration upon senescence phenotype, of *U.maydis top1*-mutants. **A.** *Wild type* UCM5 (top) and *top1*-mutant strain U5-3I (bottom) were spread on YEPS medium and incubated at 32°C for 4 days. **B.** *Wild type* UCM5 (bottom) and *top1*-mutant strain U5-3I (top) were streaked onto Holliday Minimal Medium + adenine and leucine and incubated at 31°C for 12 days.

in liquid medium was unexpected, but appears to reflect the different factors limiting cell growth in liquid versus solid media. *U.maydis top1-* and *wild-type* colonies grown on Holliday Minimal Medium (18) were of equal size, however after seven days a striking difference in coloration was evident (Fig. 5B). Whereas *wild-type* colonies developed a dark brown coloration beginning roughly seven days after plating, *top1*-strains remained a light tan color indefinitely. The physiological basis for this coloration phenotype is unknown, however it appears to coincidence with senescence, since coloration appears, and colony growth stops after approximately 7–8 days under these conditions. This browning phenotype may reflect the reported dependence of transcriptional repression on topoisomerase I during the cessation of growth in *Saccharomyces cerevisiae* (9).

CONCLUSIONS

We used conserved portions of topoisomerase I peptides from other organisms to design PCR primers for amplification of a *TOP1* gene fragment from *U.maydis*. PCR amplification was effective using primers 2+ and 3− which accurately represent the genomic sequence, but ineffective using primer 1+ which inaccurately represents the genomic sequence, due to protein sequence divergence. This approach to cloning genes from *U.maydis* has been successful for isolating several additional genes for which peptide sequence comparisons or direct protein sequencing data are available (Gerhold and Kmiec, unpublished).

DNA and predicted peptide sequences show the *Ustilago maydis TOP1* gene is a member of the family of conserved eukaryotic topoisomerase I genes, yet it possesses unique features as well. The long acidic motif near the amino terminus and the extended carboxy terminal 'tail' are unique to the *U.maydis TOP1* protein. Determination of the functions of these peptide domains awaits site-directed mutagenesis and phenotypic evaluation of the recombinant proteins.

A *TOP1* gene disruption mutant of *U.maydis* was constructed in order to investigate the role of topoisomerase I in cellular processes. Preliminary phenotypic evaluation of this mutant indicates that cell growth in most conditions is not seriously impaired. A lack of coloration in older *top1*-colonies may indicate a perturbation in cellular metabolism during cell senescence, as described for *S.cerevisiae* by Choder (9). This work enables us to further investigate the role of DNA topoisomerase I in various forms of recombination either directly in *U.maydis*, or indirectly, by expressing this gene in well characterized *top1*-mutants of *S.cerevisiae*.

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