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Isolation and characterization of mutations that affect nuclear migration for dikaryosis in *Coprinus cinereus*

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Abstract We identified 14 *Coprinus cinereus* variants defective in nuclear migration for dikaryosis after restriction enzyme-mediated integration mutagenesis. All the variants were able to donate nuclei but failed to accept nuclei in compatible matings. Of the 14 variants, six were due to single-gene mutations. We characterized *num1*, a gene responsible for one of the mutations. The *num1* ORF interrupted by three introns is predicted to encode a protein of 217 amino acids. The Num1 protein has two leucine-zipper motifs, one at the N-terminal region and the other at the C-terminal region. Num1 is also predicted to form a coiled-coil structure in its C-terminal half. The *num1-1* mutant allele is predicted to produce a truncated peptide with only one leucine-zipper motif at the N-terminal region. A plasmid carrying a sequence for the truncated peptide could inhibit nuclear migration when introduced into a wild-type strain, showing that the *num1-1* mutant allele is dominant negative. The transcription of *num1* is down-regulated when the pathway regulated by *B* mating-type genes is activated.

Keywords Basidiomycete · Mating type · Nuclear migration · Dikaryosis

Introduction

Sexual development in the homobasidiomycete *Coprinus cinereus* is under the control of *A* and *B* mating-type loci (Kimura 1952). Basidiospores, meiospores produced in

the fruiting-body cap, germinate to give rise to haploid homokaryons, which are normally sterile on their own. When two homokaryons with compatible alleles at both *A* and *B* loci are mated, both *A*- and *B*-regulated pathways operate to give rise to a fertile dikaryon, which is a prolonged mycelial phase and produces the fruiting body under proper environmental conditions (see Raper 1966; Casselton and Olesnicky 1998; Kües 2000; Brown and Casselton 2001; Kamada 2002). The *A* mating-type genes regulate conjugate division of the two nuclei from the mating partners, associated with the formation of clamp connections, to maintain the dikaryotic state. The *B* mating-type genes regulate exchange and reciprocal migration of nuclei between the two mating partners for dikaryosis. Nuclear migration is much more rapid than hyphal growth and nuclei migrate through hyphae over a long distance across the mycelial colonies of the mating partners to produce the dikaryon from the colony margins. In addition, compatible *B* alleles promote the cellular fusions that complete clamp connections during growth.

The *B* mating-type genes have been shown to encode pheromones and their receptors in *C. cinereus* (O'Shea et al. 1998) and in another homobasidiomycete, *Schizophyllum commune* (Wendland et al. 1995). However, little is known about the downstream events of the *B* mating-type genes, except the three findings that: (1) microtubules are involved in nuclear migration for dikaryosis in *C. cinereus* (Kamada et al. 1989; for a review, see Raudaskoski 1998), (2) the pheromone receptor is very likely coupled with G protein in *C. cinereus* (Olesnicky et al. 1999) and *S. commune* (Fowler et al. 1999), and (3) transcription of the *brt1* gene of *S. commune* is regulated by *B* mating-type genes (Lengeler and Kothe 1999). One promising approach to this problem is the isolation and characterization of mutants affecting *B*-regulated development. Dubovoy (1976) identified *S. commune* mutants that only donate nuclei and do not accept them in matings. However, no genes responsible for the mutations have yet been characterized at the molecular level.

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The present study aimed to identify genes that participate in the pathway from pheromone signaling to the generation of the machinery driving nuclear migration for dikaryosis in *C. cinereus*. We first developed a reliable method to test for nuclear migration for dikaryosis. We then examined 2,703 strains after restriction enzyme-mediated integration (REMI) mutagenesis for strains defective in nuclear migration. We isolated and genetically analyzed 14 strains that donate nuclei but fail to accept nuclei in compatible matings. Furthermore, we cloned and characterized *num1*, a gene responsible for one of the six mutations identified.

Materials and methods

Strains, culture conditions, and genetic techniques

Strains of *C. cinereus* listed in Table 1 were used. Malt extract/yeast extract/glucose (MY) medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in 9-cm Petri dishes was used for routine mycelial cultures. Slants of the same medium in test tubes were used for cultures for fruiting, and the same medium without agar in 9-cm Petri dishes was used for mycelial cultures for extraction of DNA and RNA. MY medium was supplemented with 100 mg of tryptophan/l for culturing tryptophan-requiring strains. The minimal medium was that of Shahriari and Casselton (1974), modified by Bininger et al. (1987). Cultures were maintained at 28 °C throughout this study. Genetic analysis was performed as described by Inada et al. (2001).

Mutagenesis

Homokaryotic strain 5302 was mutagenized by REMI, using plasmid pPHT1 (carrying the hygromycin-B resistance gene as a positive selectable marker in *C. cinereus*; Cummings et al. 1999) and restriction enzyme *HindIII*, as described by Inada et al. (2001). Hygromycin B-resistant transformants were transferred onto minimal medium containing 50 µg of hygromycin B/ml, to purify the transformed mycelium, and were examined for nuclear migration for dikaryosis as described in the next section.

Test for nuclear migration

To test for nuclear migration for dikaryosis, a small agar block (2×2 mm) with mycelium from a homokaryon was first inoculated on a plate and incubated for 3 days until the colony became about

Table 1 *Coprinus cinereus* strains used in this study. AT8A42 and AT8B42 were constructed by transforming AT8 (*A43B43 ade8-1 trp3-1*) with the *A42* and *B42* genes, respectively (L.A. Casselton, unpublished data)

Strain	Genotype	Source
5302	<i>A2B2</i>	This laboratory
5401	<i>A1B1</i>	This laboratory
5308	<i>A8B8</i>	This laboratory
5104A	<i>A7B7 his5-1</i>	This laboratory
KF ₂ #1	<i>A91B92</i>	This laboratory
292	<i>A3B1 trp1-1,1-6</i>	P.J. Pukkila
8T20	<i>A3B1 num1-1 trp1-1,1-6/progeny of NUM8×292</i>	This study
AT8A42	<i>A43/A42 B43 ade8-1 trp3-1/trp3⁺</i>	L.A. Casselton
AT8B42	<i>A43 B43/B42 ade8-1 trp3-1/trp3⁺</i>	L.A. Casselton

1 cm in diameter. Then, the inoculum in the center of the colony was replaced by an inoculum (2×2 mm) from a compatible homokaryon to be mated, using a surgical knife. After incubation for 4 days, nuclear migration, which leads to the emergence of dikaryotic mycelium with a characteristic colony morphology and characteristic clamp connections, was examined by first observing the mycelial colony macroscopically and then examining mycelia at the colony margin under a light microscope for the presence or absence of clamp connections. Tests for nuclear migration for dikaryosis were made reciprocally: each homokaryon was examined to determine whether it donated nuclei in compatible matings when it accepted nuclei.

The hygromycin B-resistant transformants isolated after REMI mutagenesis were first tested for nuclear migration, using the compatible strain 5104A (*A7B7 his5*). The transformants that exhibited defects in nuclear migration were further tested using three other compatible strains, 5401 (*A1B1*), 5308 (*A8B8*), and KF₂#1 (*A91B92*). Transformants that did not exhibit normal nuclear migration with any of the strains were stored as potential mutants defective in nuclear migration.

Plasmid rescue

Genomic DNA (5 µg) from NUM8 was digested with *BglIII* in 10 µl of reaction mixture at 37 °C for 4 h. The mixture was treated first with phenol/chloroform and then with SEVAG [chloroform:isoamylalcohol, 24:1 (v/v)]. The DNA sample was precipitated with ethanol, washed with 70% ethanol, and then resuspended in 450 µl of Tris-EDTA (TE). The DNA fragments thus obtained were self-ligated with 350 units of T4 ligase in 500 µl of reaction mixture at 16 °C overnight. DNA in the reaction mixture was precipitated with ethanol, washed with 70% ethanol, and resuspended in 2 µl of TE plus 2 µl of distilled water. This suspension (1 µl) was then introduced into *Escherichia coli* DH10B cells (Invitrogen) by electroporation and plasmids were isolated from the resulting ampicillin-resistant transformants.

Cosmid library screening

DNAs of 3,744 clones (96 clones × 39 plates) from a cosmid library of *C. cinereus* (May et al. 1991) were pooled. The DNA pool was introduced into DH10B and the resulting transformants were subjected to colony blotting and hybridization analysis, using the 2-kb *EcoRI-EcoRI* genomic fragment from plasmid pRES as the probe. Of about 1,600 colonies in the library, one colony screened positive and was named LIB1.

5'- and 3'RACE experiments

num1 cDNA was amplified from an existing cDNA library (Muraguchi and Kamada 1998), using a set of nested gene-specific primers, *num1a* (TTCACCCTCTACCGTCTTCTGCTTTC), *num1b* (AACTCTTCGGCTAAGCTCTCAACCTG), *num1E* (TGTTGAAAGCAGAGTTGGATCGAGT), and *num1e* (TGC-GCTACCAACTACCTGCGCCAACATC). PCR was performed with the Advantage cDNA PCR Kit (Clontech) and PCR products were cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instruction.

Plasmid construction

To construct plasmid pRM51, the 418-bp sequence extending from 270 bp upstream of the predicted transcriptional start site of *num1* to 4 bp downstream of the *HindIII* site, where pPHT1 was integrated in the *num1-1* mutant, was amplified using LIB1 as the template and primers H + *num1*#1 (TGCGAAGCTTTACCTCTTGACAC-

ATTTGAG) and H+num1#2 (TTCAAAGCTTTCAATTCG-CGCGCCAATTCT). Primer H+num1#1 was designed so that the amplified sequence has a 10-bp 5' extension containing a *Hind*III site. The PCR product was inserted in the multicloning site of the pPHT1 after digestion with *Hind*III.

Non-REMI transformation of *C. cinereus*

Transformation of *C. cinereus* cells by DNA without the addition of restriction enzyme was carried out as described by Binninger et al. (1987), except that the digestion of oidia for protoplasts was performed with Sigma L-1412 lysing enzymes.

Hybridization

DNA and RNA were transferred to Hybond-N and Hybond-N+ (Amersham, Arlington Height, Ill.), respectively. Bacterial colonies were lysed and bound to Hybond-N+ according to Sambrook et al. (1989). The ECL direct system (Amersham) and the Gene Images system (Amersham) were used for probe-labeling and detection for Southern and Northern analysis, respectively.

DNA and RNA manipulations

Cosmid and plasmid DNAs were isolated with the FlexiPrep kit (Amersham Pharmacia Biotech). Genomic DNA from *C. cinereus* was prepared as described by Zolan and Pukkila (1986). Total RNAs from *C. cinereus* mycelia were prepared as described by Inada et al. (2001).

Results

Isolation and genetic analysis of mutants defective in nuclear migration for dikaryosis

To identify mutations affecting nuclear migration for dikaryosis, we needed a reliable method to test for nuclear migration. In conventional mating, two inocula from compatible homokaryons are placed side-by-side on an agar plate (Fig. 1A). However, we found that, in

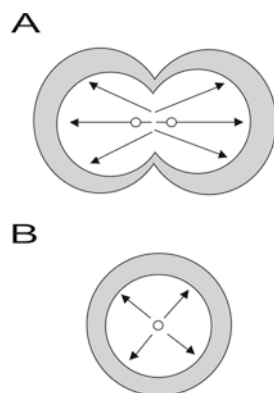


Fig. 1 Schematic representation of the conventional method for mating (**A**) and a new mating method used in this study (**B**). *Small and large white circles* represent inocula and mycelial colonies formed before dikaryotic mycelia emerge, respectively. *Arrows* indicate the direction of nuclear migration. *Shaded portions* indicate dikaryotic mycelia produced after nuclear migration

the side-by-side mating, nuclei sometimes failed to migrate across the mycelial colony of the mating partner even in matings between wild-type homokaryons. We considered this failure might be due to the fact that, in side-by-side mating, the direction of nuclear migration is opposite to the direction of hyphal growth during the early phases of migration. In fact, it has been observed that, in side-by-side mating, nuclei migrate slowly until they reach the center and thereafter they migrate rapidly toward the opposite side of the colony (Kamada et al. 1989). To get rid of this problem, we devised a new mating method (described in the Materials and methods) in which the direction of nuclear migration is the same as the direction of hyphal growth all the way (Fig. 1B). In a preliminary experiment, 53 of 630 REMI transformants failed to accept when mated with the tester strain, 5104, using the conventional method, but 51 of the 53 transformants accepted nuclei from the same tester when tested again using the new method. From these results, we chose to use the new mating method for the screening of mutants defective in nuclear migration. Figure 2 shows the results of matings between the parental wild-type strain (5302; *A2B2*) and a compatible wild-type strain (5401; *A1B1*) and between NUM8, one of the mutants isolated (see below), and strain 5401.

In total, 2,703 hygromycin-resistant transformants, which include the 630 transformants described in the previous section, were isolated and tested for nuclear migration for dikaryosis. All transformants donated nuclei when mated with a compatible homokaryotic strain, 5104A, producing the dikaryon from the margin of the colony of 5104A. However, 14 of the 2,703 transformants, named NUM1~NUM14, failed to accept nuclei in all of the matings using four tester strains, including 5104A. Six of the 14 strains exhibited defects in vegetative growth in addition to the defects in nuclear migration: NUM5, NUM10, and NUM11 showed much slower vegetative growth than the parental strain; and NUM1, NUM7, and NUM14 showed reduced aerial hyphal development. The remaining eight strains did not exhibit any remarkable defect in mycelial growth.

We then mated the 14 transformants defective in nuclear migration to the wild-type homokaryon, KF₂#1

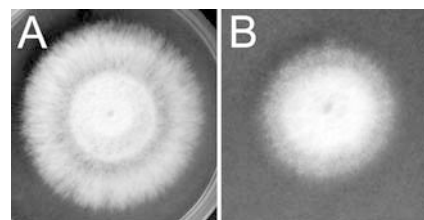


Fig. 2A, B Colonies formed after matings. **A** The wild-type homokaryon (5302; *A2B2*) × compatible, wild-type homokaryon (5401; *A1B1*). **B** NUM8 (*A2B2 num1-1*) × 5401 (*A1B1+*). Strain 5401 was first grown for 3 days and then mated with 5302 or NUM8, as described in the Materials and methods. Note that, in mating **A**, the vigorous dikaryotic mycelium is produced in the peripheral region of the colony, whereas in mating **B**, it is not

and analyzed the F₁ progeny (Table 2). In six of the 14 strains (NUM1, NUM6, NUM7, NUM8, NUM9, NUM11) progeny defective in nuclear migration and progeny exhibiting normal nuclear migration occurred in a 1:1 ratio, indicating that the defects in nuclear migration in the six strains are due to single mutations in chromosomal genes.

In NUM8, one of the six strains, the nuclear-migration phenotype and the hygromycin sensitivity co-segregated in the progeny (Table 2), suggesting that the defect in nuclear migration in this strain is a direct result of the insertion of plasmid pPHT1.

Cloning of the *num1* gene

On the basis of the above result suggesting that the gene responsible for the mutation in NUM8, named *num1*, is tagged by pPHT1, we performed a plasmid-rescue of a sequence adjacent to pPHT1 in the genome of NUM8 in order to clone *num1*. Twenty self-ligated products of the *Bgl*III digest of the genomic DNA were rescued as plasmids, using ampicillin resistance as the marker. One of the 20 plasmids isolated, named pRES1, displayed a single band of 13 kb when digested with *Bgl*III, while the remaining 19 plasmids all exhibited a single band of 5.8 kb, which is the same in size as pPHT1, when digested with the same enzyme (Fig. 3). When the 20 plasmids were digested with *Eco*RI, plasmid pRES1 showed 6.5-, 4.5-, and 2-kb bands, while the other 19 plasmids showed 4.0-, 1.2-, and 0.6-kb bands. These results indicate that pRES1 carries a 7.2-kb (13 kb minus 5.8 kb) fragment from the genomic DNA, while the other 19 plasmids had the intact pPHT1 (5.8 kb; Cummings et al. 1999), carrying no genomic DNA. In addition, these results, together with the 1:1 segregation of hygromycin-sensitive and hygromycin-resistant progeny of the cross between NUM1 and the wild type (see Table 2), suggest tandem integration of at least two pPHT1 plasmids in one site in the NUM8 genome.

Single, tandem integration of this plasmid in the genome of *C. cinereus* has been reported to be a common event in REMI mutagenesis (Cummings et al. 1999).

To examine whether the genomic fragment rescued in plasmid pRES1 represents the *num1* locus, we performed a RFLP analysis, using the 2-kb *Eco*RI-*Eco*RI genomic fragment from plasmid pRES1 as the probe (Fig 4). Sixteen progeny from the cross between the NUM8 and KF₂#1 were scored, which showed that the 2-kb genomic region is closely linked to the *num1* locus (recombinants: 0/16).

To clone the whole sequence of the *num1* gene, we screened a cosmid genome library by colony blotting and hybridization using the 2-kb *Eco*RI-*Eco*RI fragment as the probe and identified a positive clone, named LIB1.

num1 ORF

Sequencing of the *num1* region in cosmid clone LIB1, together with 5'- and 3'RACE experiments, identified an ORF interrupted by three introns, which is predicted to encode a protein of 217 amino acids (Fig. 5). The 5' splice sites agree with the consensus sequence GTRNGT found for filamentous fungi, and the 3' splice sites with the consensus sequence YAG (Gurr et al. 1987), except that the first 3' splice site is AAG. The *num1* mRNA is predicted to have a 27-nt 5'- and a 93-nt 3'-untranslated region. The promoter region contains a CAAT-box-like sequence 67–64 nt upstream of the predicted transcriptional start site.

The predicted Num1 protein contains two leucine-zipper motifs, one at the N-terminal region and the other at the C-terminal region (Fig. 6). Num1 is also predicted to form the coiled-coil structure in its C-terminal half (Fig. 6).

Database searches using the BLAST procedure (Altschul et al. 1990) revealed that the Num1 protein has similarities to the following proteins with probabilities of between 1e–23 and 1e–12 and identities of 29–31%:

Table 2 F₁ analysis of the crosses between the 14 strains defective in nuclear migration for dikaryosis and the wild-type homokaryon, KF2#1. χ^2 values are for 1:1 segregation of normal and deficient strains in nuclear migration. + Migration occurred, – migration absent, *hyg*^S hygromycin B-sensitive, *hyg*^R hygromycin B-resistant

Strain	Nuclear migration				χ^2 value	P value
	+		–			
	<i>hyg</i> ^S	<i>hyg</i> ^R	<i>hyg</i> ^S	<i>hyg</i> ^R		
NUM1	47	4	35	14	0.04	0.9–0.8
NUM2	46	12	1	17	21	<0.001
NUM3	41	0	14	0	13	<0.001
NUM4	76	0	5	0	62	<0.001
NUM5	34	20	1	5	38	<0.001
NUM6	9	14	14	12	0.18	0.7–0.5
NUM7	21	5	15	3	1.4	0.3–0.2
NUM8	29	0	0	24	0.47	0.5–0.3
NUM9	26	9	8	15	2.4	0.2–0.1
NUM10	51	1	20	0	14	<0.001
NUM11	33	0	24	0	1.4	0.3–0.2
NUM12	48	2	0	0	51	<0.001
NUM13	54	2	0	0	56	<0.001
NUM14	64	13	0	0	77	<0.001

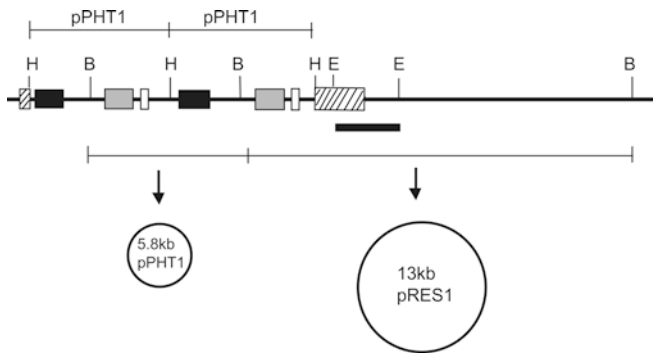


Fig. 3 Schematic representation of the integration of pPHT1 in the genome of NUM1 and of the plasmids rescued. The striped boxes represent the *num1* gene disrupted by pPHT1. The black, shaded, and white boxes indicate the hygromycin-B resistance gene, the ampicillin resistance gene, and COIE1 ori, respectively. H HindIII, B BglII, E EcoRI. The region indicated by the thick bar was used as the probe for the RFLP analysis shown in Fig. 4

the human DAM1 protein, *Arabidopsis thaliana* unknown protein AAM14351.1, *Drosophila melanogaster* LD24242p protein, *Caenorhabditis elegans* hypothetical protein T12A2.7 protein, and *Neurospora crassa* conserved hypothetical protein CAD21364.1. Thus, the protein seems to be conserved among the three eukaryotic kingdoms. However, the function of these proteins remains to be elucidated, although it is known that the DAM1 gene is up-regulated by amplification in human breast cancer cell lines (Nagasaki et al. 1999) and that the DAM1 protein is associated with the spliceosome (Neubauer et al. 1998).

num1-1 mutant allele

We amplified the *num1-1* mutant allele using primers designed based on the wild-type *num1* sequence and sequenced the amplified products directly, which revealed that, in the *num1-1* allele, pPHT1 linearized with HindIII was integrated in the HindIII site at 116–121 nt downstream of the translational start site of *num1*

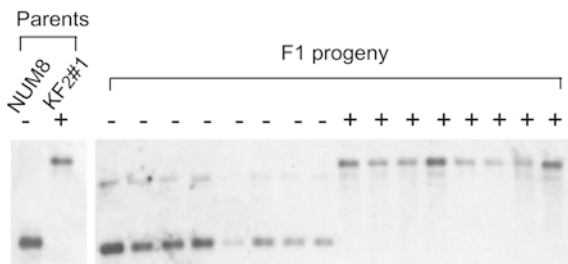


Fig. 4 RFLP analysis of the progeny from the cross NUM8 (*A2B2 num1-1*) × KF₂#1 (*A91B92*⁺). The progeny were scored for the wild-type accepting nuclei in compatible matings (+) and the mutant not accepting nuclei (-). A selection of eight wild-type and eight mutant progeny were then scored for RFLP, using enzyme EcoRI and the 2-kb EcoRI-EcoRI fragment from pRES1 (see Fig. 3)

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1  GGATTGGCGACGTAGGTTCTGTGGAATGGAGCTGCGAAGTAGCTTTGAGCAAAAAGTAA
61  GAAATATGGGTCAATTTCCACAGGTTGCGGGCTTACCTCTTGACACATTTGAGGAGTG
121  CAAGGACCGTTGGAGGGTATTCGAGAAGCCCAACGATGGCCGACGCTCTGGCGACGGT
181  TTGTAGACCGCTGCTGCGCGGAGGGCGATACGGGAGCCATTTGGCAGCCACTGAC
241  GATGGAAAGCAAAACGGGACGAGGTCGGTTGGCAGCGAACCCTGACTTCGGCAATTCGGG
301  CCTCTTCCCAAAACCCCGACCGCTGACCGCGAAGGGCGGAGTTTACCGCGACCACTC
361  AATTTCCAGACAACCACCTACAGTCTATGCGAAGGACCTAATTTCTGAATCTTCGACAGT
      M S Q G P N S E I F D S
421  CTACCGTACTATGACGATGACCTACAAAAGTACCCAAATCTGAATCTAAAGTCGACCAA
L P Y Y D D D L Q K Y P N L K S K V D Q
481  GAAATGGCGCGCAATTTGAAGCTTTGAATCCAACCGCGCGCTCCATCCACGCGTTCCC
E L A R E L K A L N P T A A L H P R V P
541  CCGCCTGTAGAATATTTGCTgttctgtgagcagctttatgcacagaaggaagtgtatgt
P P V E L F A
601  gctgacaaaatcgcgtttcaagGATGATACCGCTGTTGAAAGCAGAGTTGGATCGAGTCAA
      D R P L L K A E L D R V K
661  GGCCAGCCAGCCATTTCCATCGCTCGACACATTTGCGCTACCAACTACTGCGCCCAACATC
A S Q P F P S L D T L R Y Q L P A P T S
721  CACACGGCAACGGATGAGGAGTGAAGGACGCTCTAATAATGCCCGTGCCTCAATTTGCA
T P A T D E E W K A A L N N A R A Q L Q
781  ACACCGCGGATACGgtgcttcaatcacttgaacccctgtaattcttaactgatatt
H Q R I R
841  ggtactagCGCAGCAAAATGGGACGCTCCTTCAGACGTACGGAGCAAGCCCTGGGAATT
      Q T N G T L L Q T Y G A N A W R I
901  CAGAATTACCTCCTCGGTCACCGTGAAGCAGGTTGAGAGCTTTAGCCGAAGAGTTGAG
Q N Y L L G S T V K Q V E S L A E E L K
961  CAGAAGACGGTAGAGTGACAGGGAAGAAAGAACCGACCGAGTgaattcttctgtctg
Q K T V E V N R E R K N D Q
1021  gaactgtcatactgcaatgccccgtgacttggaaagagatattctgtatcaaccttcgact
1081  ttcctgggatctggtgaagggcgacgcttgcctccatgaaatgcaactggtctttgatgg
1141  cttgcgtttgtcctggagcctcgcatggttccccactgcttccggactatcgggagatgac
1201  cttgcaecceagataggtggtcctgttacgagtggtatatttccaagtgtgaaaggca
1261  ggtcagagctctagagactgcaacatgctgttgtaaggtcatctctggatggttgaccgg
1321  aatctaactctatccacacggagcatcaatattggggcggcagcagatgccatattccacgttg
1381  cagaagcagagtagctactgacattatcattagGAACCTCTCGGCAAGCAGTTAACTCATTTGG
      E R L G K Q L T S L E
1441  AAACAGTGTGGACGGAGCTGATTTCAAACATCCTCGAAATTTGAATGGCCAAACATCGCGC
T R W T E L I S N I L Q I E M A N I A L
1501  TGGACCGCAGAGATTGACCGATTGAATCAAAAGGAAGCCGAGCTGGCCGACGAAATATAGC
D A E I D R L N Q K E A E L A Q Q I *
1561  TGCTTTGCGTTGCTTCTCTTCTGCTTTGCGTTGCTTCTCTTCTGTTTCCAGTCCAC
1621  TCTGATTTTCAATATGACATTTTGTACTTGGAAACAGTCGCGGAGTTTCAGTCTTTGAA

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Fig. 5 Nucleotide and deduced amino acid sequences of the *num1* gene. The number of the first nucleotide of each line is indicated at the left margin and that of the last amino acid of each line is indicated at the right margin of the figure. The intron sequences are shown in lowercase letters. The HindIII site in which pPHT1 is integrated in the *num1-1* mutant allele is double-underlined. A CAAT-box like sequence in the promoter region is underlined. The genomic DNA sequence of *num1* and the deduced amino acid sequence were submitted to DDBJ (accession number AB113360)

(Fig. 5). This insertion creates a stop codon at 21–23 bp downstream of the HindIII site, truncating the protein. We confirmed that, in pRES1, a part of pPHT1 linearized with HindIII is connected with a part of *num1* at the same HindIII site.

num1-1 mutant allele is a dominant negative

To examine whether the wild-type *num1* gene rescues the *num1-1* mutation, cosmid clone LIB1, which carries the whole sequence of *num1* and the selectable marker *trp1*, was introduced into strain 8T20 (*num1-1 trp1-1,1-6*). Eighty-one *trp*⁺ transformants were isolated and mated with strain 5104A to test for nuclear migration. Unexpectedly, we found all the transformants were defective in nuclear migration. This result suggested the possibility that the *num1-1* mutant allele was dominant negative. To test this possibility, we co-transformed homokaryon 292 (*trp1-1, 1-6*) with a mixture of pRM51 carrying the sequence extending from 270 nt upstream of the

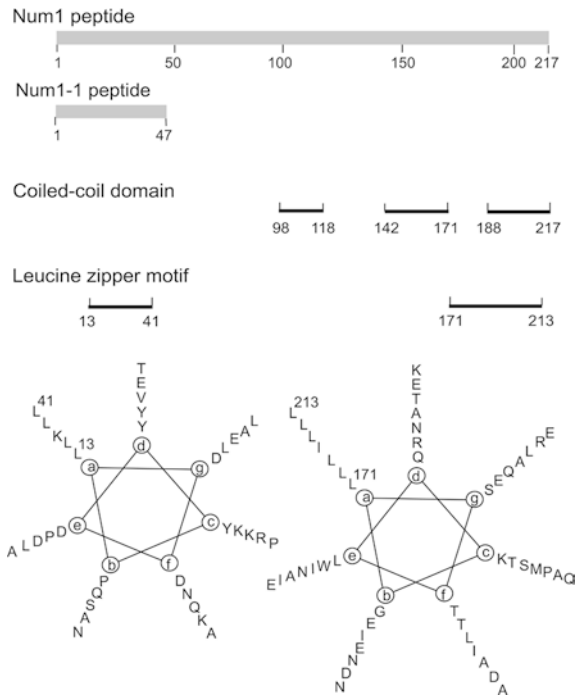


Fig. 6 Leucine-zipper motifs and predicted coiled-coil domains in the predicted wild-type Num1 peptide and the predicted mutant Num1-1 peptide. Note that, in the leucine-zippers, leucine residues are in the same position along the peptide chains forming the α helix, although one residue in one leucine-zipper is lysine instead of leucine and one residue in the other is isoleucine. Numbers indicate the amino-acid numbers. The probabilities of coiled-coil formation in the regions are 0.552–0.957, as calculated using the program COILS ver. 2.2 (MTIDK matrix, unweighted *a* and *d* positions, window 21) at http://www.ch.embnet.org/software/COILS_form.html (Lupas 1996)

transcriptional start site of *num1* to the *Hind*III site where pPHT1 is integrated, which is ligated in the *Hind*III site of pPHT1, and pCc1003 carrying the *trp1* gene (molar ratio 2:1). We found that 74.5% (35/47) of the *trp*⁺ transformants were defective in nuclear migration. As a control, strain 292 was co-transformed with the mixture of pPHT1 and pCc1003 (molar ratio 2:1): the 50 *trp*⁺ transformants isolated were all normal in nuclear migration. Furthermore, we found that the mutant produced a truncated transcript and that the *trp*⁺ transformants defective in nuclear migration transcribed both the wild-type *num1* gene and the *num1-1* mutant allele (Fig. 7, lanes 5–7). On the basis of these results, we concluded that disruption of the *num1* gene by the insertion of pPHT1 caused a dominant mutation affecting nuclear migration.

Developmental regulation of *num1* transcription

We examined the transcription of *num1* in various strains (Fig. 7, lanes 1–4). A transcript at about 800 nt, which is expected from the *num1* cDNA analysis, was identified in the parental homokaryon, 5302, the dikaryon, 5302×5401, the *A*-on homokaryon, AT8A42, and

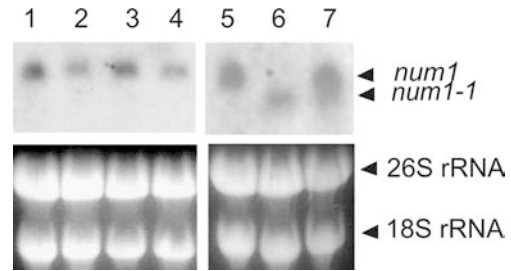


Fig. 7 Expression of the wild-type *num1* gene and the *num1-1* mutant allele. For each lane, 20 μ g of total RNAs from the following were electrophoresed in a 1.0% agarose formaldehyde gel: the wild-type homokaryon 5302 (lanes 1, 5), the dikaryon 5302×5401 (lane 2), the *A*-on strain AT8A42 (lane 3), the *B*-on strain AT8B42 (lane 4), NUM8 (lane 6), and the *num1-1* mutant strain 8T20 transformed with the wild-type *num1* gene (lane 7). After the ribosomal RNAs were visualized under UV, the gel was blotted and hybridized with the probe from the 674-bp cDNA covering the whole *num1* ORF

the *B*-on homokaryon, AT8B42. The level of the *num1* transcription was lower in the dikaryon and the *B*-on homokaryon, in which the *B*-regulated pathway is activated, than in the parental homokaryon and the *A*-on homokaryon, in which the *B*-regulated pathway is not activated.

Discussion

Proper nuclear movement is required for various phases of growth and development in eukaryotes. Examples of fungal nuclear movement include the movement of one of the daughter nuclei to the bud neck at the end of the G2 phase of mitosis in the budding yeast *Saccharomyces cerevisiae*, nuclear movement before karyogamy during the mating in the yeast, and the movement of nuclei into germ tubes during spore germination in filamentous fungi. Intensive genetic and cell biological studies on these nuclear movements have revealed that microtubule-based machinery drives nuclear movement in these processes (for reviews, see Fischer 1999; Morris 2000). Another remarkable example of fungal nuclear movement is the intercellular, long-distance migration of nuclei for dikaryosis in matings of heterothallic homobasidiomycetous species. Although it has been established that the *B* mating-type genes encoding pheromones and receptors control this nuclear migration, the downstream events of the *B* mating-type genes remain to be studied, as described above. It is not elucidated what the components of the machinery that drives the nuclear migration are, although genetic evidence shows that microtubules are involved in the migration (Kamada et al. 1989), nor is it clear how the pheromone signaling activates the machinery. Accordingly, it is not known how much the mechanism for this intercellular, long-distance nuclear migration overlaps with that for the rather short-distance nuclear movements. In the present study, we induced REMI mutations affecting nuclear migration for dikaryosis and

analyzed one of the mutations at the molecular level in an attempt to understand the downstream events of the *B* mating-type genes which lead to nuclear migration.

We identified 14 strains defective in nuclear migration among 2,703 strains after REMI mutagenesis. All 14 strains were defective in accepting nuclei, but not in donating nuclei, in compatible matings. It has been reported in *Schizophyllum commune* that pheromone and pheromone receptor play different roles in *B*-regulated nuclear migration (Wendland et al. 1995; Kothe 1996) and that acceptance of migrant nuclei is regulated by pheromone receptor, while donation of migrant nuclei is regulated by pheromone: strains having loss-of-function mutations in the pheromone receptor are unable to accept migrating nuclei but are able to donate nuclei (Vaillancourt et al. 1997; Fowler et al. 1998, 2001). Because the pheromone receptor is the gateway to the *B*-regulated pathway leading to nuclear migration, the majority of genes involved in nuclear migration would function downstream of the pheromone receptor and hence loss-of-function mutations in such genes would cause defects in accepting the migrant nuclei. Therefore, it seems reasonable that the mutations identified in this study are all defective in accepting nuclei.

Cloning and sequencing of the *num1* gene, together with RACE experiments, revealed an ORF encoding a protein of 217 amino acid residues. Although proteins showing extensive similarity to Num1 exist in human, *A. thaliana*, *D. melanogaster*, *C. elegans*, and *N. crassa*, the function of these proteins is unknown. However, analyses of the deduced Num1 peptide predicted that the Num1 peptide has two leucine-zipper motifs, one in the N-terminal region and the other at the C-terminal region, and that its N-terminal half forms the coiled-coil structure. The leucine-zipper in the N-terminal region has lysine in place of a leucine residue in the middle (see Fig. 6), but its significance is not known at present. The existence of two leucine-zipper motifs and the coiled-coil domain suggests that the Num1 protein works in interaction with other protein(s) and/or itself. It may be interesting to note that the *Aspergillus nidulans* NUDE protein, a homologue of the nuclear distribution protein RO11 of *N. crassa* (Minke et al. 1999), has a coiled-coil domain in the C-terminal region and interacts with another nuclear distribution protein, NUDF (Xiang et al. 1995; Efimov and Morris 2000).

In the *num1-1* mutant, plasmid pPHT1 was integrated such that the *num1-1* mutant allele is predicted to produce a truncated peptide with only one leucine-zipper motif at the N-terminal region. The wild-type *num1* gene introduced in a *num1-1* mutant strain did not rescue the defect in nuclear migration. Plasmid pRM51 carrying a sequence for the truncated peptide inhibited nuclear migration when introduced into the wild-type strain. It was also shown that the *num1-1* mutant transformed by the wild-type *num1* gene produced both the normal *num1* transcript and the *num1-1* truncated transcript. These results raise the interesting possibility that the truncated peptide binds a presumptive target protein

competitively with the normal Num1 protein, causing the dominant negative effect on nuclear migration. Since Num1 has two leucine-zipper motifs, it is possible that it is working as a scaffold for more than one protein. Because the analogous human protein, Dam1, has been described as spliceosome-associated (Neubauer et al. 1998), it is also possible that Num1 regulates nuclear migration by controlling splicing of a transcript(s) for a component(s) of the presumptive machinery of nuclear migration. In addition, it should be mentioned that when a gene involved in nuclear migration is expressed in both resident and migrant nuclei it is difficult to identify its recessive mutation.

Northern analysis revealed that the transcription of *num1* is down-regulated by activation of the *B*-regulated pathway. At present, this result offers no clue about how Num1 works in the mechanism of nuclear migration, although it indicates that the *B* genes do not control nuclear migration by promoting *num1* transcription. It may be interesting to note, however, that a gene down-regulated during *B*-regulated development, *brt1*, has also been identified in *S. commune* (Lengeler and Kothe 1999). To determine how Num1 works and what role it plays in the *B*-regulated pathway leading to nuclear migration, it would be necessary to identify presumptive protein(s) that interacts with Num1.

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