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Deletion of *nudC*, a Nuclear Migration Gene of *Aspergillus nidulans*, Causes Morphological and Cell Wall Abnormalities and Is Lethal

Ya-Hui Chiu, Xin Xiang, Angus L. Dawe, and N. Ronald Morris*

Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-5635

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Nuclear migration is required for normal development in both higher and lower eukaryotes. In fungi this process is mediated by cytoplasmic dynein. It is believed that this motor protein is anchored to the cell membrane and moves nuclei by capturing and pulling on spindle pole body microtubules. To date, four genes have been identified and shown to be required for this process in *Aspergillus nidulans*. The *nudA* and *nudG* genes, respectively, encode the heavy and light chains of cytoplasmic dynein, and the *nudF* and *nudC* gene products encode proteins of 49 and 22 kDa. The precise biochemical functions of the *nudF* and *nudC* genes have not yet been identified. In this report we further investigate NUDC protein function by deleting the *nudC* gene. Surprisingly, although deletion of *nudA* and *nudF* affect nuclear migration, deletion of *nudC* profoundly affected the morphology and composition of the cell wall. Spores of the strain deleted for *nudC* grew spherically and lysed. The thickness of the cell wall was increased in the deletion mutant and wall polymer composition was abnormal. This phenotype could be repressed by growth on osmotically buffered medium at low temperature. Similar, but less severe, effects were also noted in a strain depleted for NUDC by down-regulation. These results suggest a possible relationship between fungal cell wall biosynthesis and nuclear migration.

INTRODUCTION

Nuclear migration is required for growth and development in many different eukaryotic organisms (Beckwith *et al.*, 1995b; Morris *et al.*, 1995). Much of the recent progress in understanding this phenomenon has come from genetic and morphological studies in fungal systems. In the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, mutations that inhibit nuclear migration severely inhibit the rate of colony growth (Morris, 1976; Plamann *et al.*, 1994). In *Saccharomyces cerevisiae* similar mutations affect the ability of the postmitotic daughter nucleus to move into the bud. Characterization of these mutations has shown that the main motor for nuclear migration is cytoplasmic dynein (Eshel *et al.*, 1993; Li *et al.*, 1993; Plamann *et al.*, 1994; Xiang *et al.*, 1994). Also needed are dynactin, a multiprotein complex required for dynein to mediate vesicle movement (Clark and Meyer, 1994; Muhua *et al.*, 1994; Plamann *et al.*, 1994; Robb *et al.*, 1995; Bruno *et al.*, 1996), and a functional microtubule track (Oakley and Morris, 1980; Sullivan and Huffaker, 1992).

Four nuclear migration genes, *nudA*, *nudC*, *nudF*, and *nudG*, so named because they are required for normal nuclear distribution along the mycelium, have been described in *A. nidulans* (Beckwith *et al.*, 1995b; Morris *et al.*, 1995). Temperature-sensitive (ts) mutations in *nudA*, *nudC*, *nudF*, and *nudG* cause a failure of nuclear migration into young germlings, which become growth inhibited but are otherwise morphologically normal. *nudA* and *nudG*, respectively, encode the heavy and the 8-kDa light chains of cytoplasmic dynein (Xiang *et al.*, 1994; Beckwith *et al.*, 1995a). *NudC* encodes a 22-kDa protein, whose sequence provides no clue as to function (Osmani *et al.*, 1990). *NudF* encodes a 49-kDa β-transducin-like WD40 protein

* Corresponding author.

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similar to the LIS1-encoded platelet-activating factor acetyl hydrolase subunit involved in human brain development (Reiner *et al.*, 1993; Hattori *et al.*, 1994; Xiang *et al.*, 1995a). The NUDC and NUDF proteins do not resemble any of the known components of cytoplasmic dynein or dynactin (Holzbaur and Vallee, 1994; Schafer *et al.*, 1994; Schroer, 1994), nor do they cosediment with dynein in sucrose density gradients (Xiang *et al.*, 1995a; Beckwith, Roghi, and Morris, unpublished observations). We therefore have suggested they may play a regulatory role in the nuclear migration process (Xiang *et al.*, 1995a). The cytoplasmic dynein heavy chain has been located at the growing tips of the mycelium by immunocytochemistry (Xiang *et al.*, 1995b). We have been unable to localize the NUDC and NUDF proteins.

The ts mutations in *nudA*, *nudC*, or *nudF* mutation decrease the rate of mycelial growth to 10–20% of the wild-type growth rate, and nuclear migration is similarly inhibited by all of these mutations. Deletion of either *nudA* or *nudF* produces the same phenotype. Doubly mutant strains that combine a ts *nudF* mutation with a Δ*nudA* deletion are phenotypically indistinguishable from the parental singly mutant strains, suggesting that NUDF is on the same pathway as cytoplasmic dynein. Also consistent with this idea is the fact that mutations in the heavy chain of cytoplasmic dynein act as bypass suppressors of *nudF* ts and deletion mutations (Willins *et al.*, 1997). Before the current work, only one mutation in *nudC* had been described, *nudC3*, a ts missense mutation with a phenotype similar to that of the *nudA* and *nudF* mutations (Chiu and Morris, 1997). The ts *nudC3* mutation causes a decrease in the intracellular level of NUDF protein at restrictive temperature. The ts *nudF* mutations also cause a decrease in the intracellular level of NUDF protein at restrictive temperature. This suggests that the NUDC protein may regulate dynein function indirectly by decreasing the amount of NUDF protein in the cell (Xiang *et al.*, 1995a). In summary the data suggest a putative dynein/dynactin regulatory pathway in which the NUDC protein regulates NUDF protein, which in turn modulates the dynein/dynactin motor system that mediates nuclear migration (Xiang *et al.*, 1995a). In this report we further investigate NUDC protein function by manipulating and deleting the *nudC* gene. Surprisingly, although deletion of *nudA* and *nudF* affect nuclear migration, deletion of *nudC* affects the composition and morphology of the cell wall and is lethal.

MATERIALS AND METHODS

Aspergillus Strains, Culture Conditions, and Strain Construction

A. nidulans strains used in this study are listed in Table 1. Media used for culturing *A. nidulans* were as previously described (Chiu and Morris, 1995). Standard *A. nidulans* genetics techniques were used to construct strains (Käfer, 1977). Transformation of *A. nidulans* was performed as previously described (Chiu and Morris, 1995). Growth conditions for Δ*nudC* strains are summarized in Table 2. Minimal medium supplemented with glycerol as a carbon source was used in the transformation of strains YH61 and YH62 by the C-del and pKK-nc plasmids, respectively. Transformants were selected for pyrimidine or arginine prototrophy, as appropriate.

Table 1. *A. nidulans* strains

Strain	Genotype
R153	*wA3, pyroA4*
GR5	*pyrG89, wA2, pyroA4*
AO1	*nudC3, wA2, nicA2, pabaA1, pyrG89, chaA1*
XX20	*nudF6, pyrG89*
XX41	*alcA(p)::nudF::pyr4, pabaA1, chaA1*
XX60	Δ*nudA::pyrG; pyrG89*
XX62	*alcA(p)::nudC::pyr4, pyroA4, wA2, pyrG89*
ΔF54	Δ*nudF::pyr4, pyrG89, wA2, pyroA4*
20.3.10	*fwA1, pabaA1, argB2, pyrG89*
YH60	Δ*nudC::pyrG, pyrG89, wA3, pyroA4*
YH61	Δ*nudC::pyrG, pyrG89, fwA1, pabaA1, argB2*
YH62	Δ*nudC::pyrG, alcA(p)::nudC::argB, pyrG89, fwA1, pabaA1, argB2*
YH70	Δ*nudA::pyrG; pyrG89, nudC3*
YH80	Δ*nudF::pyr4, pyrG89, nudC3*

Manipulation of DNA

Plasmids were constructed by standard methods (Sambrook *et al.*, 1989) and maintained in *Escherichia coli* DH5α. Vent polymerase (New England Biolab, Beverly, MA) was used for polymerase chain reactions. *Aspergillus* genomic DNA was isolated as previously described (Oakley *et al.*, 1987) with the following modifications. Pulverized freeze-dried hyphal material was extracted with 50 mM EDTA (pH 8.0), 0.2% SDS, and 0.1% (vol/vol) diethylpyrocarbonate at 65°C for 20 min. After being centrifuged at 3000 × *g* for 10 min, the supernatant was mixed with 0.5 volume of 5 M potassium acetate and incubated on ice for 15 min followed by centrifugation at 10,000 × *g* for 5 min. DNA in the supernatant was precipitated with isopropanol for further purification as follows. The pellet was

Table 2. Lethality of Δ*nudC* can be rescued by high concentrations of osmotic stabilizers and low temperature

	Temperature			
Medium	18°C	26°C	32°C	37°C
YAG	–*	–	–	–
YAG/sorbitol	++	+/–	–	–
YAG/KC1	++	+/–	+/–	–
YAG/sorbitol/KC1	++	++	+	–

Growth response of YH60 on solid medium was recorded as a range from no growth (–) to strong growth (++). Growth responses on liquid medium were the same except under the condition indicated (*). In this case YH60 was unable to form a colony on solid medium but could germinate in the liquid equivalent. Both the wild-type (GR5) and the *nudC3* mutation (AO1) were able to grow strongly under all of these conditions. Concentration of the medium supplements was as follows: sorbitol, 1.0 M; KC1, 0.6 M.

dissolved in 3.3 M LiCl and kept on ice for 15 min, followed by centrifugation to remove residual RNA and protein. DNA in the supernatant was then precipitated with isopropanol.

Construction of the ΔnudC and alcA(p)::nudC strains

The Δ*nudC* strain of *A. nidulans* was constructed by replacing the entire *nudC* gene with the orotidine-5'-phosphate decarboxylase gene (*pyrG*; Oakley, *et al.*, 1987) as described below (Figure 1A for details). The *nudC*-containing *Bam*HI–*Xba*I genomic fragment was first cloned into pBluescript (SK) to make plasmid pBXa-5. pC-del, the Δ*nudC::pyrG* plasmid used to generate the *nudC* deletion strain, was constructed as follows. The 1.9-kb 5' flanking region of *nudC* was amplified by polymerase chain reaction using primers YH1 (5'-TTTGGATCCAGCG-GCACAGCCCAACAC-3'), whose sequence was obtained by sequencing the insertion in BXa-5 plasmid near the *Bam*HI site, and YH2 (5'-CCCGAATTCGGAGAAAGTTGGAATTAGC-3'), which contained the complementary sequences of the 5' untranslated region of the genomic sequence of *nudC* (Osmani *et al.*, 1990). The restriction endonuclease sites are underlined. Amplified products were then digested with *Bam*HI and *Eco*RI to create the *Bam*HI–*Eco*RI fragment (fragment A). A *Eco*RI–*Nde*I fragment that contained the *pyrG* selectable marker gene was purified from a pUC19-*pyrG* plasmid. The BXa-5 plasmid was digested with *Ase*I and *Xba*I to create the *Ase*I–*Xba*I fragment (fragment B) that contained the 2.3-kb downstream flanking region of *nudC*. Fragments A and B and the *Eco*RI–*Nde*I *pyrG* fragment were then mixed together and ligated to the *Bam*HI–*Xba*I sites of the pBluescript (SK) to construct the Δ*nudC* plasmid pC-del (Figure 1A). The plasmid pC-del was linearized with *Not*I and *Xba*I and transformed into wild-type strains GR5 or 20.3.10 of *A. nidulans*. Site-specific integration of linear pC-del at the *nudC* locus was confirmed by Southern blotting. The Δ*nudC* strains were designated YH60 and YH61 (Table 1).

The pKK-nc plasmid used to complement the Δ*nudC* null mutation was constructed as follows (Figure 1B, right). The pHO2 plasmid (a gift from S. Osmani, Weis Center for Research, Geisinger Clinic) was constructed by inserting the blunt-end *nudC* cDNA into the *Sma*I site of pAL5. One *Kpn*I–*Bam*HI 0.8-kb fragment containing the intact *nudC* gene was excised from the pHO2 plasmid and ligated into the pKK12 plasmid (Kirk and Morris, 1993), which contains the *argB* selectable marker. The circular plasmid pKK-nc in which the wild-type copy of *nudC* was under the control of the *alcA* promoter was then transformed into a Δ*nudC* strain, YH61 (Δ*nudC::pyrG, pyrG89, fwA1, pabaA1, argB2*). The activity of *alcA* promoter was induced in ethanol, repressed in glucose, and intermediately expressed in glycerol. Transformants were screened for arginine prototrophy and recovery of viability on minimal medium by using glycerol as the sole carbon source.

To provide an inducible/repressible strain in which *nudC* is under the control of the *alcA* promoter, the *palcA(p)::nudC* plasmid was constructed as follows (Figure 1B, left). The *Kpn*I–*Xho*I fragment of pHO2 consisting of the 5' open reading frame of *nudC* was cloned in pBluescript KS(+). This clone was then digested by *Kpn*I and *Bam*HI to obtain a 0.5-kb 5' *nudC* fragment, which was inserted into the *Kpn*I–*Bam*HI sites of the pAL3 vector (Waring *et al.*, 1989). The resulting clone palcA(p)::nudC, in which the 3' truncated *nudC* gene was under control of the *alcA* promoter, was used to transform a wild-type strain (GR5). Site-specific integration of this plasmid by homologous recombination at the *nudC* locus resulted in a strain that has the *nudC* gene situated immediately 3' to the *alcA* promoter (Figure 2A). Transformed protoplasts were first selected on minimal medium with glycerol as the sole carbon source supplemented with pyridoxine (0.5 mg/ml) and 0.6 M KCl. Transformants were then restreaked and tested for growth on *alcA*-repression plates (glucose). Several transformants that grew much more slowly than wild-type strains were selected and analyzed by Southern blotting (Figure 2B). A strain with the predicted integration for a conditional pseudo "null" *nudC* mutant was selected and designated XX62 (Table 1). Its genotype was further confirmed by Western blotting, which showed that the NUDC protein level was decreased when XX62 was grown in repression medium (Figure 2C).

4,6-Diamidino-2-phenylindole (DAPI) Staining, Immunofluorescence, and Electron Microscopy

Liquid medium was inoculated with conidia at a density of 10^4–10^5 spores/ml in Petri dishes containing coverslips. At a certain time point, the coverslips with adherent germlings were removed for staining. Slides were photographed by using a Zeiss Axioplan microscope with a 100× Neofluar objective and Tmax 400 (Kodak) or Super HGV 400 (Fuji) films. DAPI and actin staining were as previously described (Liu and Palevitz, 1992), using 0.5 μg/ml DAPI. Chitin was stained with the same procedure as for DAPI staining except that 1 mg/ml Calcofluor White M2R (Sigma, St. Louis, MO) was used in the mounting buffer instead of DAPI. For actin staining the cell walls were digested at 28°C for 75 min with 2.5% driselase (Sigma), 1% lysing enzyme (crude preparation of novozyme from *Trichoderma harzianum*; Sigma), and 50% (vol/vol) egg white. Cells were then incubated at 28°C for 1 h with mouse anti-actin monoclonal C4 antibody (ICN Biomedical, Costa Mesa, CA) followed by incubation (in the dark) at 28°C for 1 h with goat anti-mouse IgG conjugated to CY3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Coverslips were then mounted in DAPI mounting buffer.

For electron microscopy, wild-type and mutant cells were grown in YG liquid medium at either 18°C for 67 h or 37°C for 8 h and fixed in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) at 4°C overnight. Cells were rinsed in the same phosphate buffer three times and postfixed in 1% osmium tetroxide for 1 h on ice in the dark, followed by a rinse in ice-cold H_2O for 10 min. The specimens were dehydrated through a graded series of ethanols and embedded in Spurr low-viscosity resin. Sections were cut with a diamond knife on a Sorvall MT-2B Ultramicrotome and were viewed and photographed by using a JEOL 100CX II TEM.

Preparation and Analysis of Cell Walls

Cell wall analysis was performed as previously described by Borgia (1992) and Borgia and Dodge (1992). Briefly, wild-type and Δ*nudC* strains were grown at 37°C for 8 h in liquid medium with agitation and cells were then collected by centrifugation. The pellets were washed three times with distilled water and lyophilized overnight. Dried cells were suspended in 1 M KOH, boiled for 20 min, and centrifuged at 14,000 rpm for 2 min. The supernatant and pellet were both retained. Pellets containing cell walls were washed three times and lyophilized for sugar analysis. The protein content in the supernatant was estimated by the Bradford colorimetric assay (Bradford, 1976) and used for standardization of the sugar determinations.

For the analysis of total KOH-insoluble GlcNAc and chitin content, dried pellets were digested at 37°C overnight in 50 mM sodium citrate-NaOH buffer (pH 5.8) containing 0.02% sodium azide and 4 mg/ml lysing enzyme (L2265, Sigma). After digestion, samples were centrifuged for 1 min in a microcentrifuge and the carbohydrate-containing supernatants were analyzed. Aliquots of the supernatants were assayed for both amino sugars (chitin) and neutral sugars (β-1,3 glucan) as described by Ghuysen *et al.* (1966) and Dubois *et al.* (1956), respectively.

Labeling of Chitin with N-[acetyl-^{3}H]Glucosamine

Wild-type and Δ*nudC* spores were grown at 37°C for 8 h in YG medium with shaking (150 rpm). Labeling of wall chitin was performed as described by Katz and Rosenberger (1971). Briefly, aliquots of cells were labeled with *N*-acetyl-D-[1-^{3}H]glucosamine (20 μCi/ml, Amersham, Arlington Heights, IL) for 7 min. Labeling was terminated by addition of 0.1 volume of 5 N NaOH. Samples were

then boiled for 10 min, collected by microcentrifugation, and resuspended in H_2O, followed by washing once with 0.5 N NaOH, 1 M NaCl, and 0.2% SDS and twice with H_2O. Aliquots (20 μl) were air-dried on slides, immersed in $CHCl_3$:methanol (2:1, vol/vol) for 1 h, and stained with Congo Red. Slides were coated with NTB-2 emulsion (Kodak, Rochester, NY) by the dipping method and exposed for 9 d before development.

RESULTS

The nudC3 Mutation Intensifies the Growth Defect of the ΔnudA and the ΔnudF Single Mutants

NudA, the gene for the cytoplasmic dynein heavy chain, and *nudF* are required for nuclear migration but are not essential genes. Although deletion of *nudA* or *nudF* blocks nuclear migration (Willins *et al.*, 1995; Xiang *et al.*, 1995a), the deletion strains germinate normally with a normal germ tube morphology and they grow slowly at 10–20% of the wild-type growth rate. Doubly mutant strains carrying a ts *nudF* mutation (*nudF6*) and Δ*nudA* have the same phenotype as the single ts and deletion mutants, as would be expected if NUDF protein functions on the same pathway as NUDA. To ascertain whether *nudC* also functions in this pathway, we constructed doubly mutant *nudC3*, Δ*nudA* and *nud3C*, Δ*nudF* strains. The double mutants produced colonies that were smaller than the singly deleted Δ*nudA* or Δ*nudF* strains at both permissive and restrictive temperatures (Figure 3). This suggested that the NUDC protein might have some function in addition to its role in nuclear migration. However, under the microscope these double mutants were morphologically similar to the singly mutant parental strains. They had a typical "nud" phenotype with nuclei clumped in the spore end of the germlings (data not shown). Otherwise the germ tube morphology was normal. To investigate further the possibility that *nudC* has a function in addition to nuclear migration and to learn what this might be, we deleted the *nudC* gene.

Deletion of nudC Is Lethal

Linearized pC-del (Figure 1A) was used to transform a GR5 ($pyrG^-$) strain to prototrophy ($pyrG^+$). Because this transformation failed to produce any slow-growing "nud-like" colonies, we considered the possibility that deletion of *nudC* might be lethal. We tested this by heterokaryon deletion, which is used in place of diploid transformation to characterize lethal mutations in *A. nidulans* (Osmani et al., 1988). The salient features of the heterokaryon deletion procedure are as follows. During transformation, fusion of protoplasts causes the formation of multinucleate protoplasts, which regenerate to form heterokaryotic mycelia. Because the *A. nidulans* mycelium is coenocytic, lethally transformed nuclei can survive in balanced heterokaryons with complementing untransformed nuclei (Figure 4). At conidiation the transformed and untransformed nuclei are segregated into uninucleate conidia (asexual spores). Thus lethally transformed heterokaryons can be identified by streaking their conidia to selective medium on which neither the spores containing lethally transformed nuclei nor the spores containing untransformed nuclei (which lack the *pyrG* selective marker) can make colonies. Strains carrying the *nudC* deletion mutation were identified by their inability to form colonies after restreaking to medium lacking uridine and uracil. After restreaking, 20% of $pyrG^+$

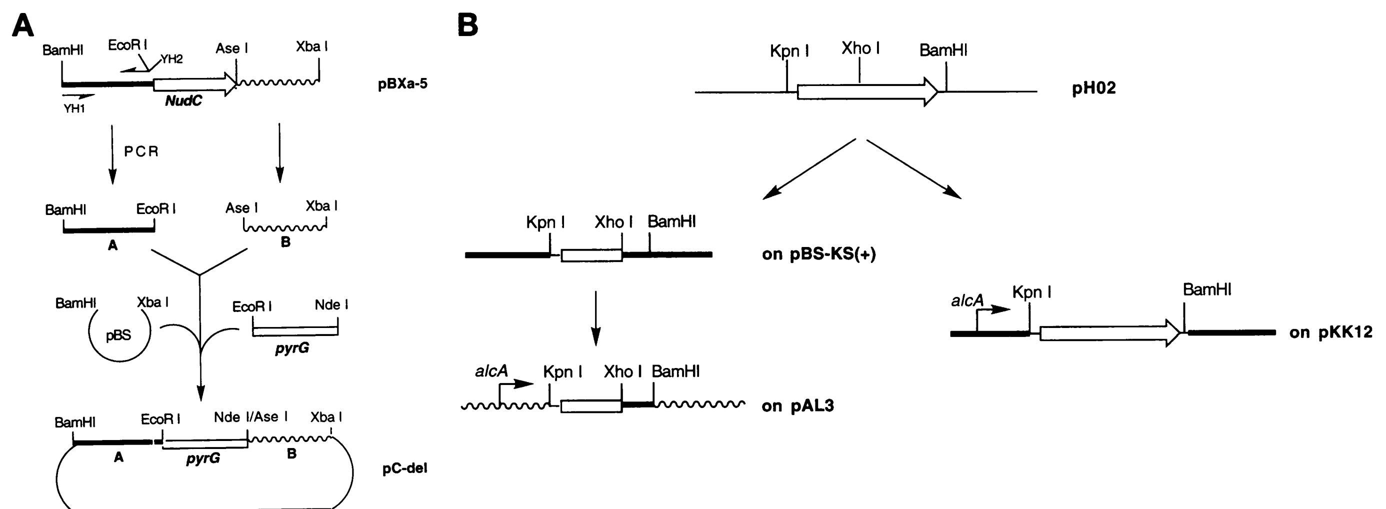


Figure 1. Construction of the pC-del, pKK-nc, and palcA(p)::nudC5′ plasmids. (A) Schematic illustrating construction of the *nudC* deletion plasmid pC-del. (B) Construction of the palcA(p)::nudC5′ (left) and pKK-nc (right) plasmids from pHO2. See MATERIALS AND METHODS for details.

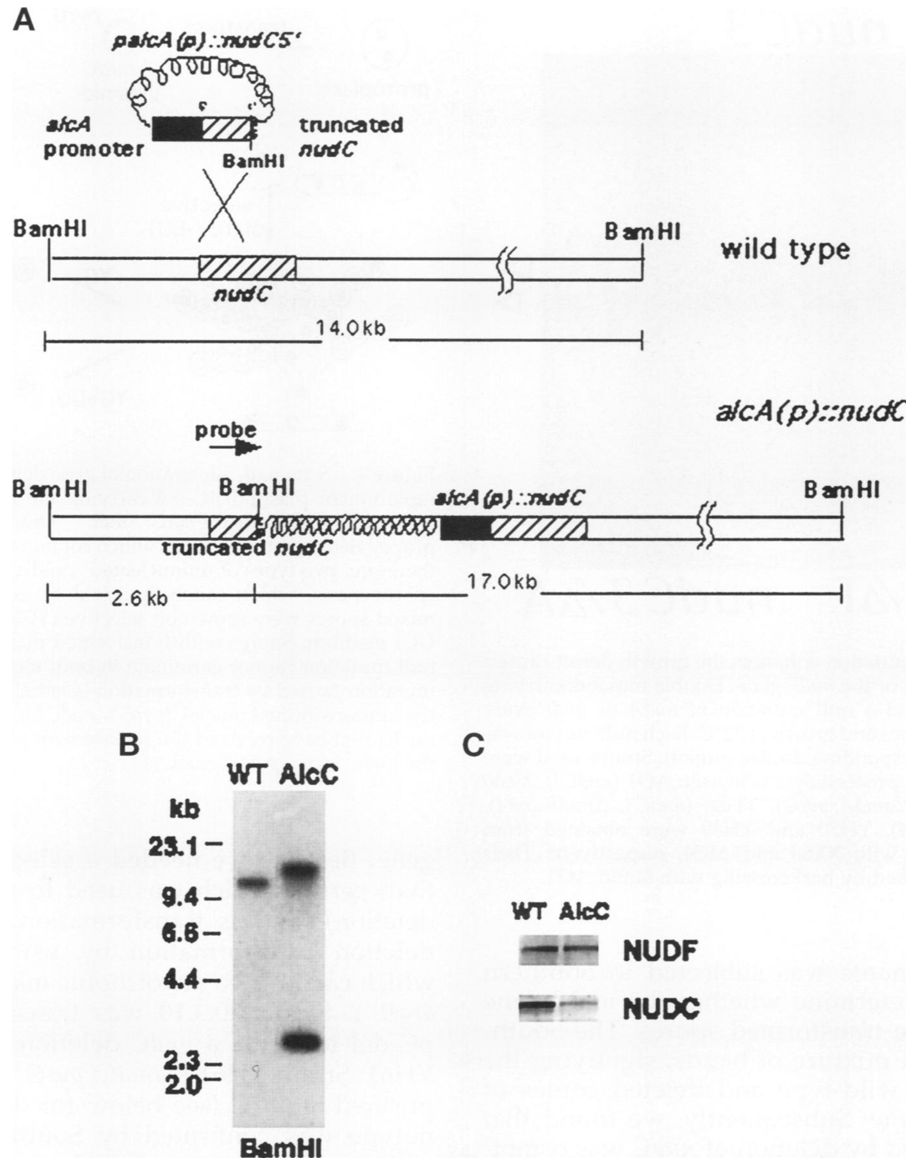


Figure 2. Construction of the *alcA(p)::nudC* strain. (A) Illustration of plasmid integration event that leads to a single copy of a full-length *nudC* gene under the control of the *alcA* promoter. (B) Southern blot confirming integration of the plasmid into the *nudC* locus. Genomic DNAs from the wild-type (GR5) and the *alcA(p)::nudC* strain were extracted and digested with *Bam*HI. The Southern blot was done with a 0.6-kb internal fragment of *nudC* as probe (shown as an arrow in A). As predicted in A, this probe detected a 14-kb signal in the wild-type and 2.6-kb and 17-kb signals in the mutant. (C) Western blot demonstrating that the NUDC protein in the *alcA(p)::nudC* strain is down-regulated in the repressing medium (glucose). Cells were inoculated into glycerol medium for 24 h and then shifted to YG medium for 7 h before protein isolation. Equal protein loading was ensured by Bradford assay and by Ponceau S staining of the blot. The blot was probed by anti-NUDF and anti-NUDC antibodies, respectively. WT, wild-type; Alc, *alcA(p)::nudC*.

transformants tested (81 of 433) failed to form colonies and, therefore, were potentially *nudC* null mutants. Conidia from these transformants were germinated and examined under the microscope. Two types of conidia could be distinguished by their growth patterns. Swollen nongerminating spores (the *pyrG*$^-$ phenotype) and germlings with growth-arrested germ tubes (the putative *nudC* deletion phenotype) were seen in YG; and a mixture of wild-type germlings and germlings with growth arrested germ tubes was observed in YG plus uracil and uridine (YG + UU). The common growth-arrested phenotype was ascribed to the deletion.

We were initially unable to purify the putative lethally transformed Δ*nudC* spores from the *pyrG*$^-$ spores. Therefore, DNA from the primary hetero-

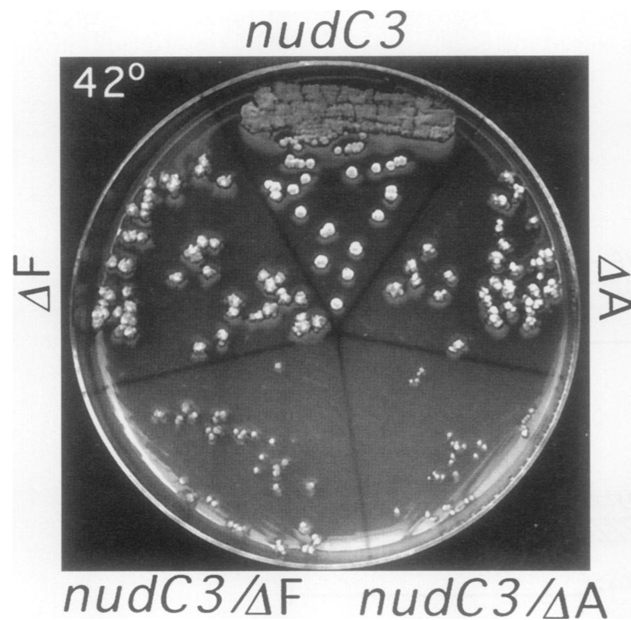


Figure 3. The *nudC3* mutation enhances the growth defect caused by deletion of the *nudA* or the *nudF* gene. Double mutants carrying the *nudC3* mutation and a null mutation of *nudA* or *nudF* were streaked to single colonies and grown at 32°C. Each null mutant was compared with its corresponding double mutant. Strains used were (starting at the top and proceeding clockwise): AO1 (*nudC3*), XX60 (Δ*nudA::pyrG*), YH70 (Δ*nudA::pyrG*), YH80 (*nudC3*, Δ*nudF::pyr4*), and ΔF54 (Δ*nudF::pyr4*). YH70 and YH80 were obtained from crosses of strain AO1 with XX60 and ΔF54, respectively. Their genotypes were confirmed by backcrossing with strain AO1.

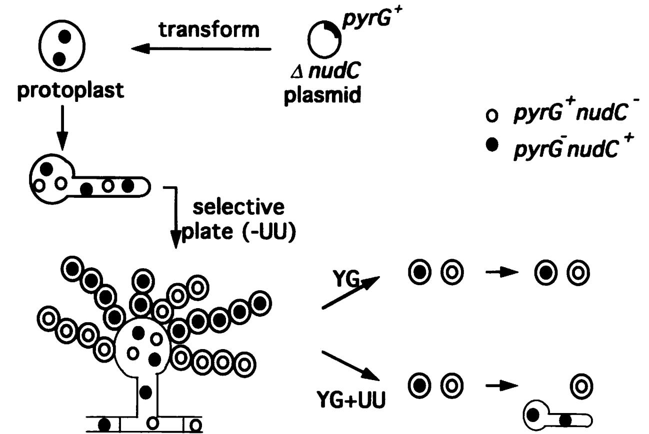


Figure 4. Schematic illustration of gene deletion in *A. nidulans*. The replacement plasmid pC-del carrying the *pyrG* selectable marker was transformed into a *pyrG*$^-$ *nudC*$^+$ strain. Transformants were propagated as heterokaryons which contain two types of nuclei and, therefore, two types of uninucleated conidia were produced during sporogenesis, which can be purified by repeated streaking. The mixed spores were grown on selective (YG) or nonselective (YG + UU) medium. Spores with transformed nuclei that carry the *nudC* null mutation cannot germinate in both media, indicating that the mutation caused by transformation is lethal. Solid circles represent the untransformed nuclei (*pyrG*$^-$ *nudC*$^+$); open circles represent nuclei that have received the replacement plasmid with deletion in the *nudC* locus (*pyrG*$^+$ *nudC*$^-$).

karyotic transformants was subjected to Southern blot analysis to determine whether the *nudC* gene was deleted in the transformed spores. The Southern blot showed a mixture of bands, signifying the presence of both wild-type and deleted copies of *nudC* in the genome. Subsequently, we found that the lethality caused by deletion of *nudC* was osmotically remediable at low temperature. This permitted the isolation of a pure Δ*nudC* strain, designated YH60 (see below for details; Table 1). DNA from this strain was subjected to Southern blot analysis, which verified that the *nudC* gene had been deleted (Figure 5).

Complementation of ΔnudC by the Wild-Type nudC Gene

Because the effect of deleting *nudC* was more severe than that caused by the *nudC3* mutation or by deletion of either *nudA* or *nudF*, we considered the possibility that a nonspecific genomic event undetected by our Southern blot analysis could be responsible for the lethality of the Δ*nudC*. We ruled this out by showing that the growth abnormality of Δ*nudC* could be fully complemented by the wild-type *nudC* gene. Because we needed a selective marker other than *pyrG*$^+$ (which was used to produce the *nudC* deletion) for this transformation, we repeated the deletion transformation by using strain 20.3.10, which carries two auxotrophic markers (*pyrG89* and *argB*$^-$). Strain 20.3.10 was transformed first with pC-del to create a *nudC* deletion strain designated YH61. Strain YH61 (Δ*nudC::pyrG*$^+$, *argB*$^-$) was then purified at 18°C (see below for details) and its genotype was confirmed by Southern blotting. The pKK-nc plasmid carrying the *argB* gene and the wild-type *nudC* gene under the control of the *alcA* promoter (Figure 1B) was then transformed into strain YH61. The wild-type *nudC* gene was placed under the control of the inducible/repressible *alcA* promoter to demonstrate that any rescue observed was specifically caused by the presence of the *nudC* gene product. Transformants were selected for both *pyrG* and *argB* prototrophy and their growth was examined on minimal medium containing various carbon sources. Almost all of the transformants that grew well on glycerol or ethanol medium grew poorly on medium containing glucose, confirming that the expression of *alcA::nudC* was responsible for the growth seen on nonrepressive medium. This result ruled out the possibility that the lethality of Δ*nudC* was caused by a nonspecific genomic event.

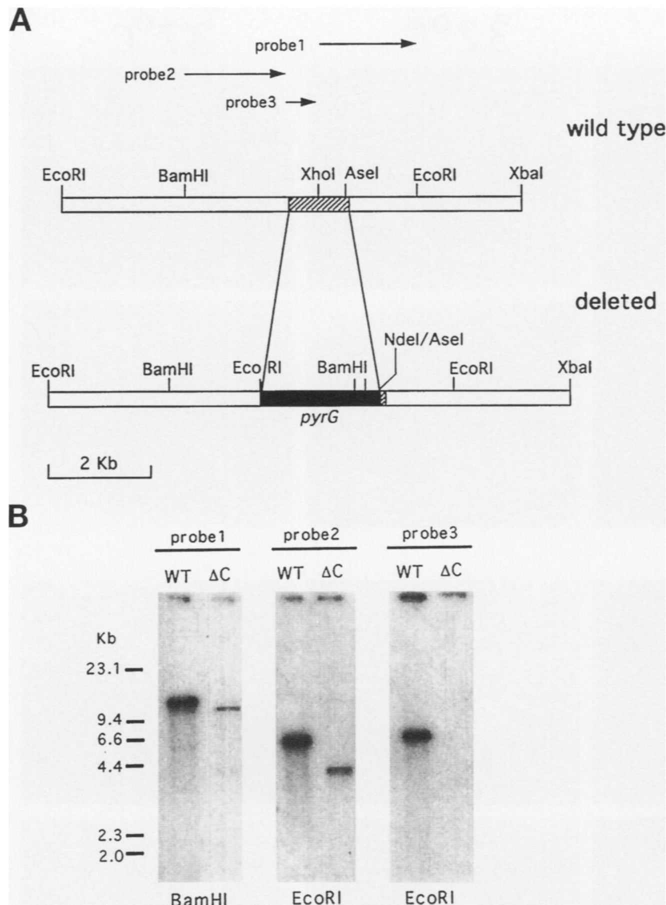


Figure 5. Construction of the Δ*nudC* strain. (A) The wild-type *nudC* locus and the locus after gene replacement. Hatching indicates genomic sequence of *nudC*. Radiolabeled probes used in Southern blot analysis are indicated by arrows. (B) Genomic DNA was isolated from a control strain, GR5 (WT), and from YH60 (Δ*nudC::pyrG, pyrG89*), which had specifically integrated pC-del plasmid, resulting in a deletion of *nudC*. The DNA was digested with *Bam*HI or *Eco*RI and subjected to Southern blot analysis using probes 1, 2, or 3. With probe 1 (lanes 1 and 2), a 14-kb signal and a 11-kb signal were detected in the wild-type and in the ΔC mutant, respectively. Probe 2, which contains the 5′ upstream flanking region of *nudC*, detected a 6.0-kb fragment in wild-type and a 4.3-kb fragment in the ΔC strain, as predicted in A. Probe 3, which corresponds to the 5′ deleted coding sequence of *nudC*, only detected a 6.0-kb signal in wild-type, indicating that this sequence is no longer present in the ΔC strain.

Deletion of nudC Causes Cell Swelling and Lysis That Is Partially Remediable at Low Temperature by Osmotic Stabilizers

Deletion of *nudC* is lethal under normal growth conditions on YAG at 37°C, yet the Δ*nudC* strain forms essentially normal colonies at 18°C on hyperosmotic media (Figure 6). Thus, Δ*nudC* is a temperature- and osmotic pressure-dependent conditionally lethal mutant. We were able to isolate a pure Δ*nudC* strain by streaking the conidial mixture from a heterokaryotic primary transformant onto solid selective medium (lacking uridine and uracil) at 18°C in the presence of osmotic stabilizers (sorbitol, KCl, or NaCl). Under these conditions, viable Δ*nudC* colonies were produced that generated asexual spores. The spores were streaked to single colony three times on osmotically stabilized medium at 18°C to produce a pure Δ*nudC* strain (YH60). Deletion of the *nudC* gene was verified by Southern blotting of DNA from this strain (Figure 5B).

KCl, NaCl, and sorbitol were equally effective in supporting Δ*nudC* colony formation at 18°C. Under these conditions the purified Δ*nudC* spores produced phenotypically wild-type germ tubes in which nuclei were normally distributed. Under the same conditions of low temperature and hyperosmotic media Δ*nudA* exhibits a nud phenotype. This indicates either that *nudC* is not needed or that another protein assumes the dynein-mediated nuclear migration and cell wall functions of *nudC* under these conditions. At low temperature without osmotic stabilization and at higher temperatures even with osmotic stabilization, the purified Δ*nudC* spores did not produce either germ tubes or colonies. Above 32°C on YAG medium the spores grew spherically and lysed. The germination of the Δ*nudC* mutant under different conditions is summarized in Table 2. Under permissive conditions (marked as ++ in Table 2), the morphology of Δ*nudC* germlings was essentially normal except that their tips occasionally lysed (Figure 6, 18° plus KC1 or sorbitol). Wild-type cells never exhibited tip swelling or lysis at any temperature tested (18, 26, 32, or 37°C). Addition of sorbitol, KCl, or NaCl to the growth medium only partially prevented cell lysis in a temperature-dependent manner. For example, 0.6 M KCl partially prevented cell lysis at 18°C but did not prevent cell lysis at higher temperatures such as 26°C or 32°C. Under all growth conditions, the morphology of the Δ*nudC* strain was heterogeneous. The temperature sensitivity of *nudC3* and the ts *nudA* and *nudF* mutations was slightly remediated by osmotically stabilized medium, but the nud phenotypes of *nudA* and *nudF* deletions were not affected by osmotic stabilization.

We also constructed a strain in which *nudC* was placed under the control of the inducible/repressible *alcA* promoter. This strain failed to produce healthy colonies on glucose repression medium, confirming that *nudC* is an essential gene. However, the *AlcA(p)::nudC* spores germinated and produced short swollen germ tubes that lysed at the tip and resembled Δ*nudC* germlings produced by spores from the transformed heterokaryon before purification. The fact that the *AlcA(p)::nudC* and heterokaryon-derived Δ*nudC* spores produce a short germ tube before the tips swell and lyse, whereas spores from the purified Δ*nudC* strain swell immediately on germination may be due to the former containing a "dowry" of wild-type

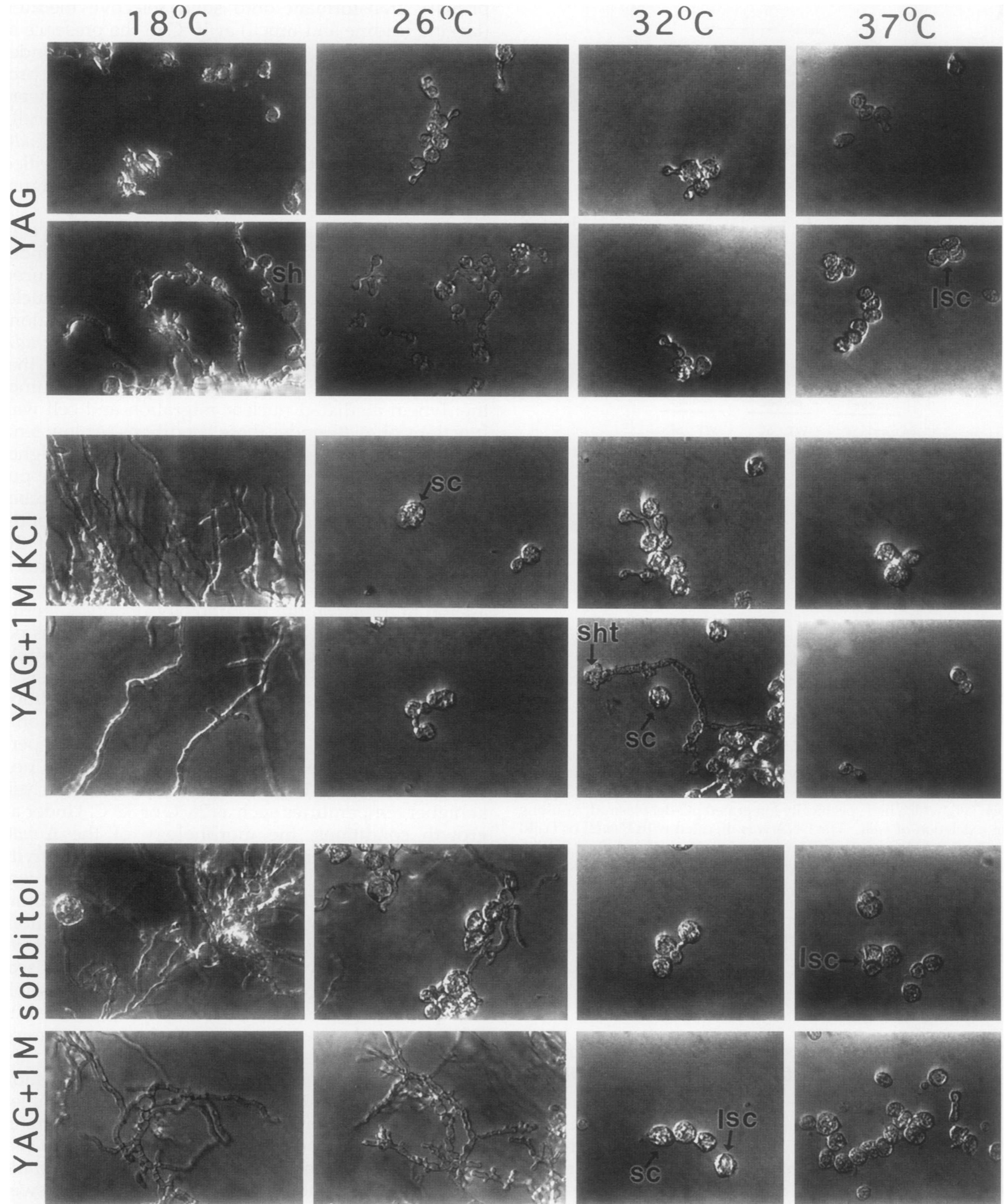


Figure 6. Morphological phenotypes of the Δ*nudC* mutant. Conidia of YH60 (Δ*nudC*) were streaked on YAG, YAG+1 M KCl, or YAG+1 M sorbitol medium and grown at 18°C, 26°C, 32°C, and 37°C for 4 d. Cells were photographed directly on the solid medium covered with

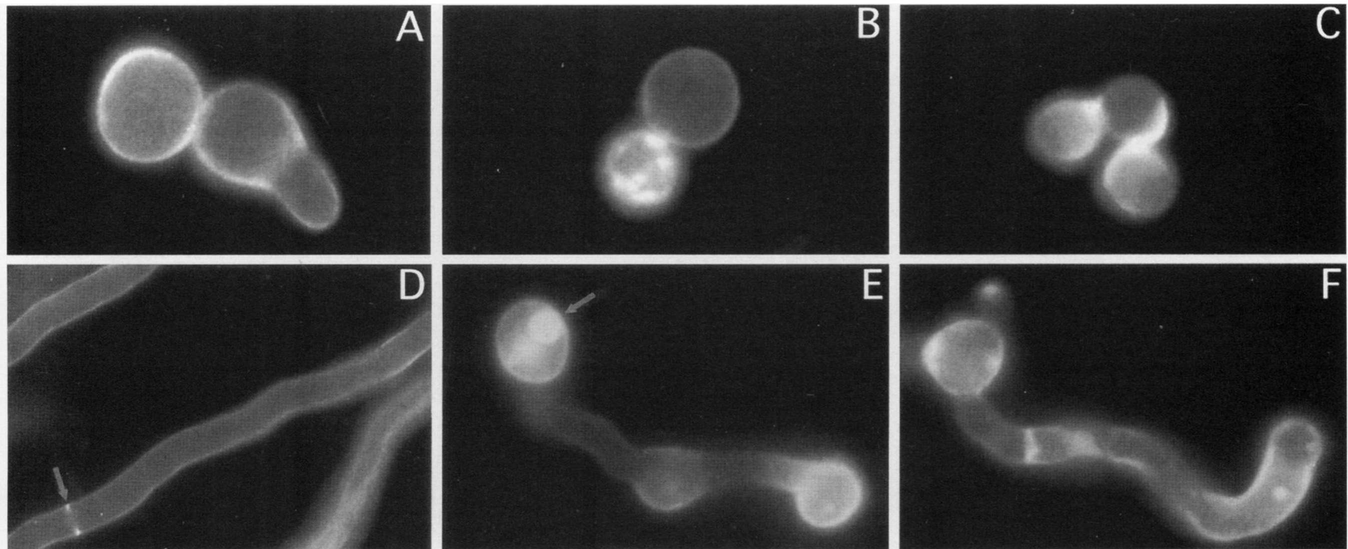


Figure 7. Distribution of chitin in the Δ*nudC* mutant is abnormal, as revealed by Calcofluor staining. (A–C) Cells grown at 37°C for 7 h. (D–F) Cells grown at 18°C for 60 h. (A and D) Wild-type cells (R153). (B, C, E, and F) Δ*nudC* mutant (YH60). Cells were all stained by Calcofluor. Chitin staining was mainly seen at the tips and in septal regions of wild-type cells (indicated by an arrow in D). Unequal or spotted Calcofluor stainings were seen in the Δ*nudC* mutant in both growth conditions. Chitin was abnormally deposited in vacuoles in Δ*nudC* mutant grown at 18°C (indicated by an arrow in E).

NUDC protein from their NUDC-containing parental strains that must be used up before the absence of NUDC can produce a phenotype. The delayed swelling and lysis of the dowry-carrying spores demonstrates that NUDC deprivation affects the integrity of the spore wall rather than affecting cell polarity and/or germ tube emergence.

The Cell Wall of the ΔnudC Mutant Is Abnormal

Some *A. nidulans* mutations that affect the synthesis of the chitin precursor GlcNAc cause morphological abnormalities similar to those caused by deletion of *nudC* but can be remediated by the addition of GlcNAc to the medium (Borgia and Dodge, 1992). We therefore asked whether GlcNAc could reverse the Δ*nudC* phenotype. The growth of Δ*nudC* cells was unchanged by the addition of GlcNAc to the medium, indicating that the effect of Δ*nudC* is not on an early step of chitin synthesis. We also used the chitinophilic dye Calcofluor to ask whether cell wall chitin was abnormal in the Δ*nudC* mutant. Calcofluor staining of wild-type cells is most intense at the tip of the germ tube and at septa (Figure 7). Δ*nudC* cells exhibited increased Calcofluor staining of the germling wall at swollen regions of the wall at lower temperatures and over the whole surface of spheres formed at the higher temperature. Many cells also showed bright dots or bars of more intense staining (Figure 7). At 18°C on medium containing salt Calcofluor staining was heterogeneous (Figure 7F) and sometimes was seen inside vacuoles of Δ*nudC* cells (Figure 7E). Calcofluor staining inside vacuoles was not seen in wild-type strains.

The ultrastructure of the Δ*nudC* strain was examined by electron microscopy (Figure 8). Cells grown under semirestrictive conditions (60 h at 18°C in YG) had walls twice as thick as those in wild-type cells (175 nm versus 92 nm, average). More severely affected spherical cells grown under fully restrictive conditions (8 h at 37°C in YG) had walls five times thicker than wild-type (Figure 8). Many spherical cells also had wall material extending inward toward the center of the cell (Figure 8, C–F). Determination of the chitin and glucan contents of walls of these spherical cells confirmed our electron microscopy observations. As shown in Table 3, the chitin content of Δ*nudC* cells was five times greater and the glucan content was twice greater than the respective chitin and glucan contents of the parental strain (GR5) under the same growth conditions (37 for 7 h in YUU).

Figure 6 (cont). a coverslip. Excessively swollen conidia (SC), lysed swollen conidia (LSC), swollen hyphae (SH), and swollen hyphae tip (SHT) are indicated by arrows.

Deposition of Chitin Is Disturbed in the ΔnudC Mutant

Many mutations that affect the chemical composition of the cell wall cause spherical growth, presumably by affecting the strength of the wall. However, spherical

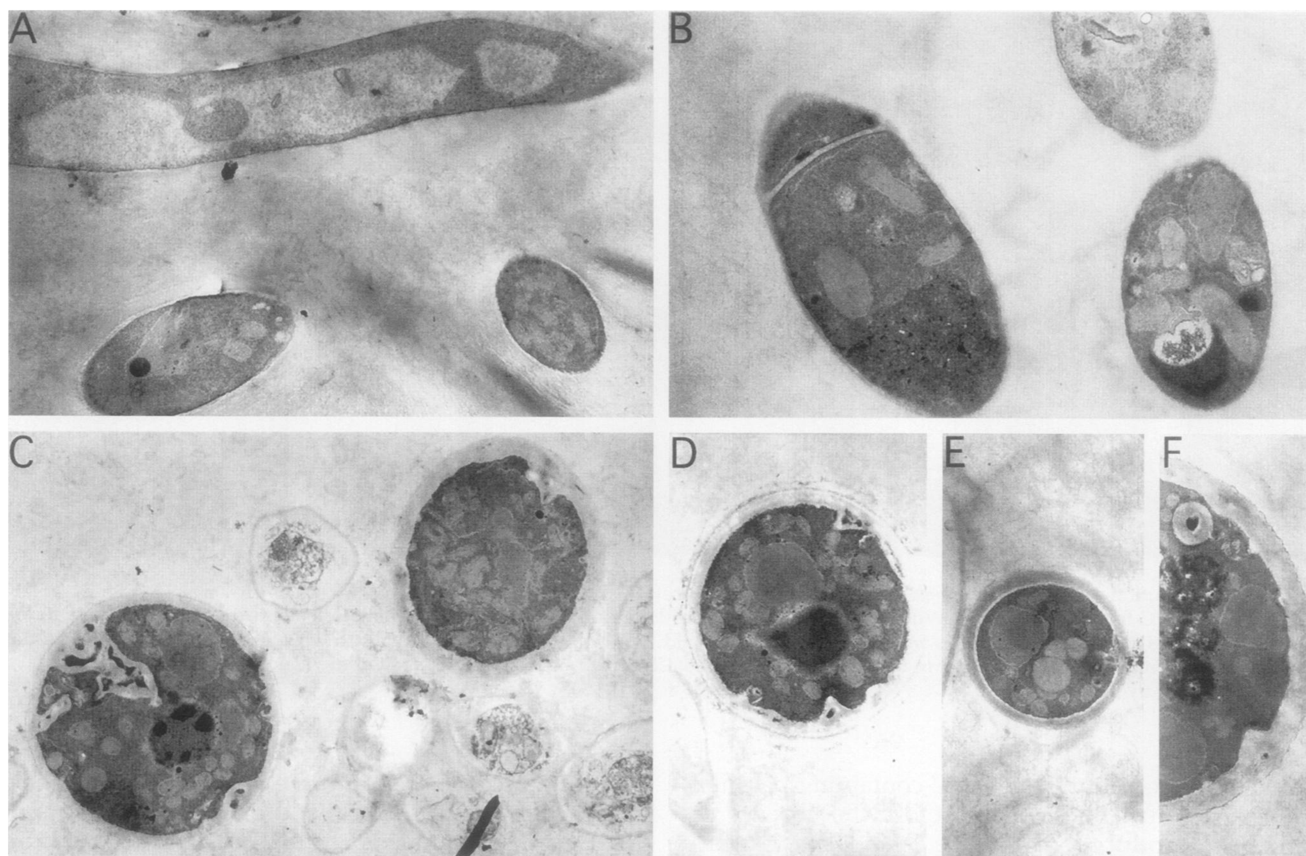


Figure 8. The Δ*nudC* mutant has a cell wall that is much thicker than the wild-type cell wall and also exhibits inward extensions of the wall that are not seen in wild-type cells. Cells were grown at 37°C for 9 h. Under this condition, Δ*nudC* cells swelled extensively and grew spherically. All panels are in the same magnification. (A and B) Wild-type cells (GR5). (C–F) Δ*nudC* cells.

growth would also result if the normally focal deposition of wall material were disseminated over the whole surface of the cell. We distinguished between these causes of spherical growth by using radioautography to determine where chitin was deposited in the Δ*nudC* mutant. Wild-type and Δ*nudC* spores were germinated for 8 h at 37°C. Radioactive *N*-acetyl-D-[1-H^3]glucosamine was added to the medium, and the incubation was continued for 15 min before the cells were fixed, stained, and processed for radioautography. In wild-type cells the distribution of grains representing the incorporation of radioactive label into the cell wall was predominantly at the tip of the mycelium (Figure 9A). This was also true of the *nudC3* strain grown at either permissive or restrictive temperature. However, in spherically growing cells of the Δ*nudC* mutant at higher temperatures the grains were distributed over the whole cell surface (Figure 9B), indicating that chitin deposition was disseminated.

Table 3. Chitin and glucan are both overexpressed in the Δ*nudC* mutant

Strain	Glucan	Chitin
Wild-type (GR5)	1.9 ± 0.33	0.68 ± 0.035
Δ*nudC* (YH60)	3.6 ± 0.14	3.4 ± 0.027

Glucan and chitin values are listed as μg of sugar/μg of protein.

Actin Distribution Is Abnormal in the Absence of NUDC

Since actin is involved in chitin deposition (Novick and Botstein, 1985), we stained cells with an anti-actin antibody to look for abnormalities in the actin cytoskeleton of the Δ*nudC* mutant. Actin staining was most intense at the growing tips and at the septa of wild-type germlings. A similar staining pattern was seen in Δ*nudC* cells grown at 18°C, where the Δ*nudC* mutant is morphologically normal. At those elevated temperatures at which Δ*nudC* was morphologically abnormal, actin localization was also abnormal. In moderately affected cells at intermediate temperatures, actin staining extended over the swollen hyphal

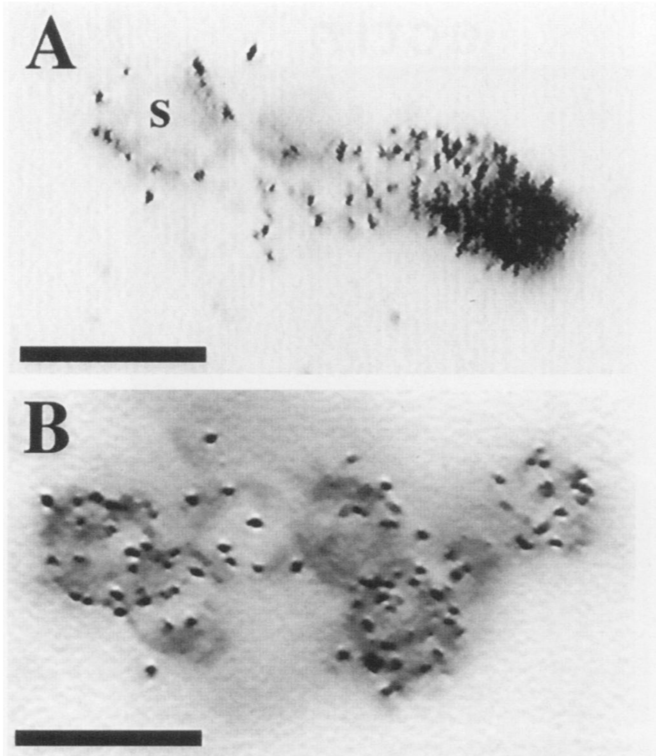


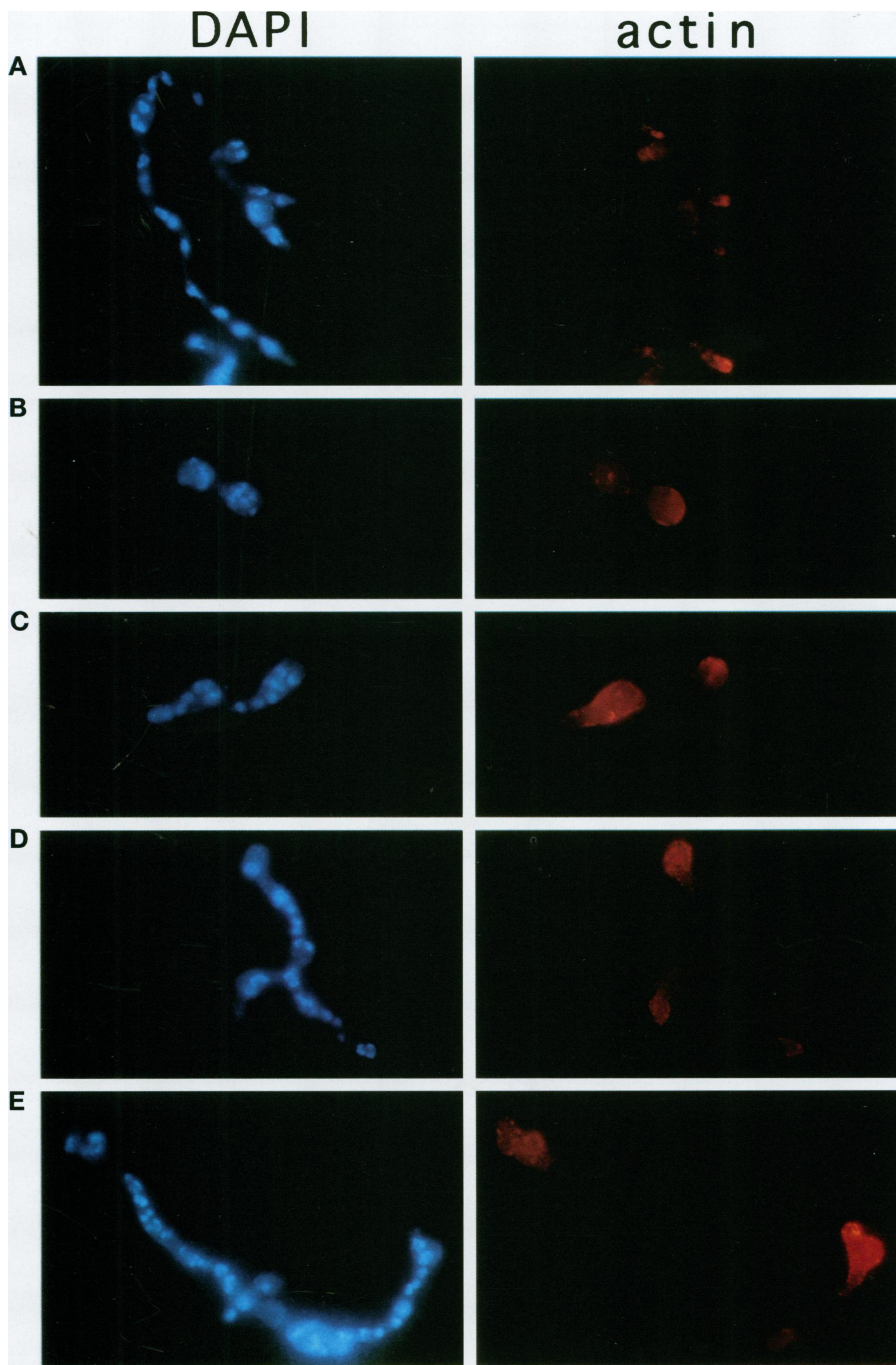
Figure 9. Polarized chitin deposition is disturbed in Δ*nudC* cells. Cells were grown at 37°C for 8 h, labeled with *N*-acetyl-D-[1-^{3}H]glucosamine, and autoradiographed. Compared with wild-type (GR5) cells, which showed polarized stainings at the hyphal tip (A), a random distribution of silver grains was seen in the Δ*nudC* cells (B). (A) Single wild-type germling. (B) Cluster of swollen Δ*nudC* cells. S, spore head. Bar, 10 μm.

regions. At higher temperatures, where the abnormalities of cell shape were most extreme, actin staining extended over the whole surface of the ovoids and spheres (Figure 10).

DISCUSSION

The observations presented in this article demonstrate that *nudC* in addition to its involvement in nuclear migration has an important role in cell wall synthesis. In the complete absence of NUDC, cell wall production is grossly abnormal. Wall synthesis moves from its normal focus at the growing tip of the germling to become uniformly distributed over the whole expanse of the cell membrane, as does the distribution of actin. Consequently, growth is spherical rather than polar. In addition the composition of the Δ*nudC* wall is atypical. The chitin to glucan ratio is abnormal, and both components are overproduced. The overproduction of the wall components correlates with an unusually thick wall. That these abnormalities are caused by the absence of NUDC protein is demonstrated by the fact that all were reversed by reintroduction of the wild-type *nudC* sequence into the genome. They were also reversed by low temperature in the presence of osmotic stabilizers. Thus, although *nudC* is an essential gene at high temperatures, it is dispensable at low temperature in the presence of osmotic stabilizers. Nuclear migration is also essentially normal in the Δ*nudC* deletion strain under these conditions; whereas under the same conditions the Δ*nudA* deletion mutant exhibits a "nud" phenotype. This suggests that either NUDC protein is dispensable or that some other protein assumes the NUDC nuclear migration function as well as its function in cell wall production under conditions of low temperature and high osmotic pressure.

The shape of the fungal germ tube is defined by the site of new wall synthesis, the tensile strength of the wall, and by internal tissue turgor. Cell wall polysaccharides are normally added at the hyphal tip (Katz and Rosenberger, 1971) with the expansive force for tip extension driven by internal tissue turgor (Cosgrove, 1986; Ortega *et al.*, 1989). The tensile strength of the wall depends on both the cell wall composition and on a poorly characterized set of biochemical modifications that rigidify the wall as it passes backward over the mycelium (Wessels, 1986). We found that cell wall deposition is aberrant in the Δ*nudC* mutant strain, as demonstrated by incorporation of radioactive GlcNAc over the whole surface of the wall rather than focally at the tip, as in the wild-type. However, we have not been able to determine whether loss of polarity is the primary cause of the Δ*nudC* phenotype or whether this is a response to some other factor such as weakening of the cell wall. Numerous mutations in *A. nidulans* and other fungi produce hyphal swelling and lysis similar to that caused by Δ*nudC*. Some affect wall components. Others affect the cytoskeleton. Examples of mutations that affect wall components include *orlA*, *orlB*, *tsE*, and *bimG*, which inhibit chitin synthesis (Shaw *et al.*, 1991; Borgia, 1992; Borgia and Dodge, 1992), and *orlC*, *orlD*, and *manA*, which affect glucan and mannan synthesis (Borgia and Dodge, 1992; Markham and Bainbridge, 1992; Smith and Payton, 1994). All of these cause spherical cell swelling and lysis. The dyes Calcofluor and Congo Red, which inhibit chitin maturation and crystallization (Kopecka and Gabriel, 1992; Bartnicki-Garcia *et al.*, 1994; Engle *et al.*, 1994), similarly cause cell tip swelling, spherical growth, a thickened cell wall, and lysis (Pancaldi *et al.*, 1984; Roncero and Durán, 1985; Dawe and Morris, unpublished data). Actin colocalizes with the site of new cell wall deposition at the growing hyphal tip in filamentous fungi and at the bud tip in yeast. In yeast the small GTP protein Rho1p that activates glucan synthesis colocalizes with actin at the bud tip (Yamochi *et al.*, 1994; Bussey, 1996; Drgonová *et al.*, 1996; Quadota *et al.*, 1996). Mutations affecting the actin cytoskeleton and myosin cause cell rounding and


DAPI
actin
A
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swelling and in some cases thickening of the cell wall (Read *et al.*, 1992; McGoldrick *et al.*, 1995; Goodson *et al.*, 1996). Presumably, the actin cytoskeleton is involved in delivery of components to the cell wall, because secretory vesicles accumulate in yeast actin mutants (Novick and Botstein, 1985). Deletion of *FAB1*, which affects normal vacuole function in yeast, also causes cell swelling and lysis (Yamamoto *et al.*, 1995).

We have no convincing evidence that favors any one possible mechanism of action for NUDC over any other mechanism. Suppression of the lethality of Δ*nudC* by high osmotic pressure is consistent with either a weak wall or increased tissue turgor as a cause of cell swelling. High external osmotic pressure should diminish effective cell turgor and reduce the stress on the wall in either case. However, increased osmotic pressure also can affect actin filament organization (Chowdhury *et al.*, 1992) and gene expression (Varela *et al.*, 1992). The mislocalization of actin and chitin deposition in the Δ*nudC* mutant might suggest that NUDC is required for cell polarity. However, a weak wall might itself affect the positioning of the actin cytoskeleton and, consequently, wall deposition, if there were a compensatory mechanism to target actin and new wall deposition to regions of weakness. The cell wall hypertrophy observed in many of the above-mentioned examples of conditions in which cells swell suggests such a mechanism may exist. Because cytoplasmic dynein is involved in vesicle migration in other organisms, it would not be unreasonable to suppose that *nudC* exerts its wall effect by affecting a dynein-dependent migration of vesicles containing essential materials to the wall. This is unlikely (unless *A. nidulans* has an as yet uncharacterized dynein heavy chain) because the *nudA* disruption/deletion mutants (and all other known *nud* mutants) have no effect on cell shape or wall morphology (Beckwith *et al.*, 1995b; Xiang *et al.*, 1995b). Nor did we observe any abnormality of vesicle or vacuole distribution in the spherical Δ*nudC* cells. We did, however, observe one vesicle abnormality, which was the staining of the major spore head vesicle with Calcofluor. This might represent some mistargetting of wall components but was seen only at low temperature in the absence of osmotic stabilization and not under more restrictive conditions for cell wall synthesis.

Could the *nudC3* inhibition of nuclear migration and the effect of *nudC* down-regulation and deletion on the cell wall be connected? Cytoplasmic dynein, the main motor for nuclear migration, is a minus-end-directed microtubule-dependent motor (Holzbaur and Vallee, 1994). It has been suggested that translocation of cortically anchored cytoplasmic dynein along nuclear spindle pole microtubules pulls nuclei toward the bud tip in yeast (Eshel *et al.*, 1993; Carminati and Stearns, 1996) and toward the hyphal tip in filamentous fungi (Xiang *et al.*, 1994). Evidence for physical interactions between dynein and dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995) and between spectrin and dynactin (Holleran *et al.*, 1996) has suggested that dynactin and spectrin may be involved in dynein binding to the cell cortex. Although the ts *nudC3* nuclear migration mutation doesn't cause a visible wall defect either by itself or in combination with *nudA* or *nudF* deletions, the possibility can't be ignored that it could subtly affect dynein attachment to the wall and thereby affect nuclear migration. In fact many mutations that affect cell wall synthesis affect nuclear migration from the mother cell into the bud in yeast (Bulawa and Osmond, 1990; Shaw *et al.*, 1991; see also Beckwith *et al.*, 1995b). Similar mutations in filamentous fungi cause growth to be spherical, making it difficult to study nuclear movement by the conventional method of directly observing nuclear distribution through the mycelium. Steinberg and Schliwa (1993) have shown that a cell wall is not absolutely necessary for nuclear movement by demonstrating that nuclear movement occurs in the wall deficient slime mutant and protoplasts and cytoplasts of *N. crassa* attached to the protamine-coated surface of a glass slide. However, because the glass slide may provide the proposed dynein-anchoring function of the cell wall, it is still not clear whether nuclear migration can occur in the absence of an attachment to a substrate. Whether or not the cell wall is required for dynein anchoring and for nuclear migration, the fact that NUDC plays a role in both cell wall morphogenesis and in nuclear migration makes it a particularly interesting protein.

Figure 10 (facing page). Localization of actin in the Δ*nudC* mutant. Cells were grown at 32°C for 15 h in YG and stained with an anti-actin monoclonal antibody. (Left) DAPI staining. (Right) Actin staining of the same field. (A) Wild-type (GR5). (B–E) Δ*nudC* mutant.

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