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Mitochondria and Nuclei Move by Different Mechanisms in *Aspergillus nidulans*

BERL R. OAKLEY and JANET E. RINEHART
Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

ABSTRACT We have examined the effects of the antimicrotubule agent benomyl and several mutations on nuclear and mitochondrial movement in germlings of the filamentous fungus *Aspergillus nidulans*. While, as previously reported, benomyl inhibited nuclear division and movement, it did not inhibit mitochondrial movement. To test the effects of benomyl more rigorously, we germinated two benomyl super-sensitive, β-tubulin mutants at a benomyl concentration 50–100 times greater than that required to inhibit colony formation completely. Again nuclear division and movement were inhibited, but mitochondrial movement was not. We also examined conditionally lethal β-tubulin mutations that disrupt microtubule function under restrictive conditions. Nuclear division and movement were inhibited but, again, mitochondrial movement was not. Finally we examined the effects of five heat-sensitive mutations that inhibit nuclear movement but not nuclear division at restrictive temperatures. These mutations strongly inhibited nuclear movement at a restrictive temperature but did not inhibit mitochondrial movement. These data demonstrate that the mechanisms of nuclear and mitochondrial movement in *Aspergillus nidulans* are not identical and suggest that mitochondrial movement does not require functional microtubules.

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Organellar translocation in its many forms is essential to the growth and maintenance of most, if not all, eukaryotic cells. In recent years organellar translocation has been studied extensively in many organisms (reviewed in reference 18) and while there is now good evidence that some forms of organellar translocation are microtubule-mediated (2, 19, 22, 23; and other data reviewed in reference 18) and others are mediated by actin microfilaments (reviewed in reference 18), the mechanisms of force production and the mechanisms by which force production is regulated are not known. We are studying organellar translocation in the filamentous fungus *Aspergillus nidulans* because this organism has a very good genetic system that makes it possible to apply the power of genetics to analyze the mechanisms of organellar translocation. Mutations in genes that encode the microtubule proteins α and β tubulin have been isolated in this organism (12, 15, 20) as have mutations that specifically inhibit nuclear movement (11, 14). These mutations have been used to demonstrate that nuclear movement is microtubule-mediated (14, 15). Our long-term goal is to identify the components of the organellar translocation apparatus(es) in *A. nidulans* and to determine how these components interact to produce organellar movement.

One of our initial goals has been to determine if there is but a single mechanism responsible for the translocation of organelles in *A. nidulans* or if there are two or more mechanisms as there apparently are in some organisms (reviewed in reference 18). We have, consequently, examined the movement of mitochondria from conidia (asexual spores) into germ tubes in the presence of the antimicrotubule agent benomyl and in strains that carry mutations that inhibit nuclear movement. We have found that while benomyl and these mutations inhibit the movement of nuclei from the conidium into the germ tube, mitochondrial movement occurs in virtually all germlings including those in which nuclear movement is blocked. These data demonstrate that there are at least two mechanisms of organellar translocation in *A. nidulans*.

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MATERIALS AND METHODS

Strains: Strains B3, BEN14, and BEN17 were originally constructed by Dr. J. M. van Tuyl (Agricultural University, Wageningen, The Netherlands) and were obtained by us from Dr. N. R. Morris (Rutgers Medical School, Piscataway, NJ). Each of these strains carries *bi*A1. BEN14 carries *ben*A16, and BEN17 carries *ben*A19. BRO2 was constructed by Dr. Berl R. Oakley in the

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laboratory of Dr. N. R. Morris and carries *ben*A33 and *y*A2. The cold-sensitive (cs⁻)[1] β-tubulin mutants are revertants of BRO2. Each carries *ben*A33 and each carries a different closely linked (recombination frequencies < 1.2×10^{-4}) intragenic suppressor that confers cold sensitivity. These revertants were isolated and characterized by Berl R. Oakley, C. Elizabeth Oakley, Kimberly S. Kniepkamp, and Janet E. Rinehart (17). FGSC154 was originally obtained from the Fungal Genetics Stock Center (Humboldt State University, Arcata, CA) and was obtained by us from Dr. N. R. Morris. It carries *ad*E20, *bi*A1, *w*A2, *cnx*E, *s*C12, *meth*G1, *nic*A2, *lac*A1, *cho*A1, and *cha*A1. The *nud* mutants were isolated by Morris (11) and carry mutations *nud*A1-*nud*E5 in a FGSC154 background. We obtained these mutants from Dr. N. R. Morris.

Growth Conditions: All experiments were carried out in complete medium (YG medium, 5 g/liter yeast extract, 20 g/liter dextrose) containing 0.1% agar to inhibit conidial clumping. All incubations were carried out under constant agitation on New Brunswick gyratory shakers (New Brunswick Scientific Co., Inc., Edison, NJ).

Microscopy: A variety of fixation and staining procedures were tested. We obtained consistently good staining of nuclear and mitochondrial DNA with the following procedure. 0.9-ml samples were taken and fixed with 0.1 ml of 10% glutaraldehyde for 10 min. The samples were then washed twice for 10 min in distilled water, once for 15 min in acetone, and twice for 10 min in distilled water and resuspended in a staining solution containing 0.015 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in distilled water. The acetone wash greatly improved the consistency of staining among germlings. DAPI was stored at 4°C as a 1.5 μg/ml solution in distilled water. Rhodamine 123 was dissolved immediately before use in distilled, deionized water to a concentration of 10 mg/ml and was added to samples at a ratio of 1 part rhodamine 123 solution to 99 parts sample, or 1 part rhodamine 123 to 199 parts sample. Samples were observed and photographed with a Zeiss standard microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence. Neofluor optics were used for all observations and photographs. DAPI staining was observed with a G365 filter set (365-nm exciter filter, 395-nm beam splitter, and 420-nm barrier filter) and rhodamine 123 with a H546 filter set (BP 546/12 [546-nm narrow band] exciter filter, 580-nm beam splitter, and 590-nm barrier filter). To inhibit loss of rhodamine 123 staining, a 0.5 neutral density filter was sometimes used to reduce the intensity of the exciting illumination. Specimens were photographed with Tri-X pan film which was developed with D-76 or Diafine developers (Acufine, Inc., Chicago, IL).

Chemicals: Benomyl (technical grade, 98% pure) was a generous gift from DuPont Co. (Wilmington, DE). DAPI was from Sigma Chemical Co. (St. Louis, MO). Rhodamine 123 was from Eastman Kodak Co. (Rochester, NY). Glutaraldehyde (10% solution, E. M. grade) and formaldehyde (16% solution, E. M. grade) were from Electron Microscopy Sciences (Fort Washington, PA).

RESULTS

Identification of Mitochondria in Germlings

We considered two fluorescent dyes for visualizing mitochondria: rhodamine 123, which specifically stains mitochondria (8, 9), and the DNA binding dye, DAPI (26), which should stain the DNA of mitochondrial nucleoids. Rhodamine 123 gave excellent staining of mitochondria when used as a vital dye at 50–100 μg/ml (Fig. 1) and was most effective when added immediately before observation. At lower concentrations, after long incubation and in fixed material, germlings exhibited substantial background fluorescence that made observation of mitochondria difficult. Even under optimal staining conditions the fluorescence localized in the mitochondria diffused in seconds into the cytoplasm when the specimen was illuminated.

DAPI was not effective as a vital dye because staining was inconsistent among germlings. In fixed material, however, DAPI stained nuclei and mitochondrial nucleoids very effectively (Fig. 1). A possible source of ambiguity with DAPI, however, is suggested by the work of Allan and Miller (1) who showed that polyphosphate granules in the vacuoles of several species of yeasts stain with DAPI. Polyphosphate granules are also found in vacuoles in filamentous fungi (reference 3 and references contained therein). These granules are only formed under certain growth conditions, however, and are only preserved with certain fixation procedures (1) that are quite different from those we have used. We are confident that the punctate fluorescence we observe is due to mitochondrial DNA because we observed no fluorescently stained particles in the vacuoles that were visible in many of the germlings and because in experiments where we stained germlings separately with DAPI and rhodamine 123 we obtained similar results with the two stains.

Benomyl and Conditionally Lethal β-Tubulin Mutations Inhibit Nuclear Division and Movement but Not Mitochondrial Movement

Oakley and Morris (14) have shown that the antifungal, antimicrotubule agent benomyl inhibits nuclear migration in *A. nidulans* by acting on the β-tubulin encoded by the *ben*A gene. To determine if benomyl also inhibited mitochondrial movement, we allowed a wild-type strain to germinate in medium with 10 μg/ml benomyl. As Fig. 1 and Table I show, benomyl strongly inhibited nuclear division and nuclear movement but not mitochondrial movement.

To test the effects of benomyl on nuclear migration more rigorously, we used *ben*A16 and *ben*A19, β-tubulin mutations that confer super-sensitivity to benomyl (24). Oakley and Morris (14) have shown that nuclear division and movement are inhibited at lower concentrations of benomyl in a strain carrying one of these mutations than in a wt strain. Growth is completely inhibited in these mutants at benomyl concentrations of 0.2 μg/ml, and at a concentration of 10 μg/ml nuclear movement and division were strongly inhibited, as expected, but mitochondrial movement was, again, not significantly inhibited (Fig. 1, Table I).

To verify these data by rhodamine staining, we germinated the strain carrying *ben*A16 in medium containing 10 μg/ml benomyl for 10 h. We then took two samples, adding rhodamine 123 to one and fixing the other for DAPI staining. In rhodamine-stained material, observed in the incubation medium within 30 min of sampling, 96 of 100 germlings exhibited mitochondrial movement. There was evidence of clumping of mitochondria in a few germlings but in no case was a clear, mitochondria-free zone visible near the hyphal tip. Even in germlings with very short germ tubes (2–3 μm), mitochondria had moved from the conidium into the germ tube. In the DAPI-stained sample, 12 of 100 germlings exhibited nuclear movement.

Oakley and Morris (14) have isolated a heat-sensitive (hs⁻) β-tubulin mutation, *ben*A33, that inhibits nuclear division and movement at a restrictive temperature, and Oakley et al. have isolated five cs⁻ mutants that carry *ben*A mutations that also inhibit nuclear division and movement at a restrictive temperature (16, 17). We tested each of these mutants for inhibition of mitochondrial movement at restrictive temperatures and found that, in each, mitochondrial movement occurred to a normal extent, while nuclear division and movement were inhibited (Fig. 2, Table II).

Nud Mutations Inhibit Nuclear Movement but Not Nuclear Division or Mitochondrial Movement

Morris (11) has isolated five non-allelic mutations (desig-

[1] *Abbreviations used in this paper:* cs⁻, cold-sensitive; DAPI, 4′,6′-diamidino-2-phenylindole; hs⁻, heat-sensitive.

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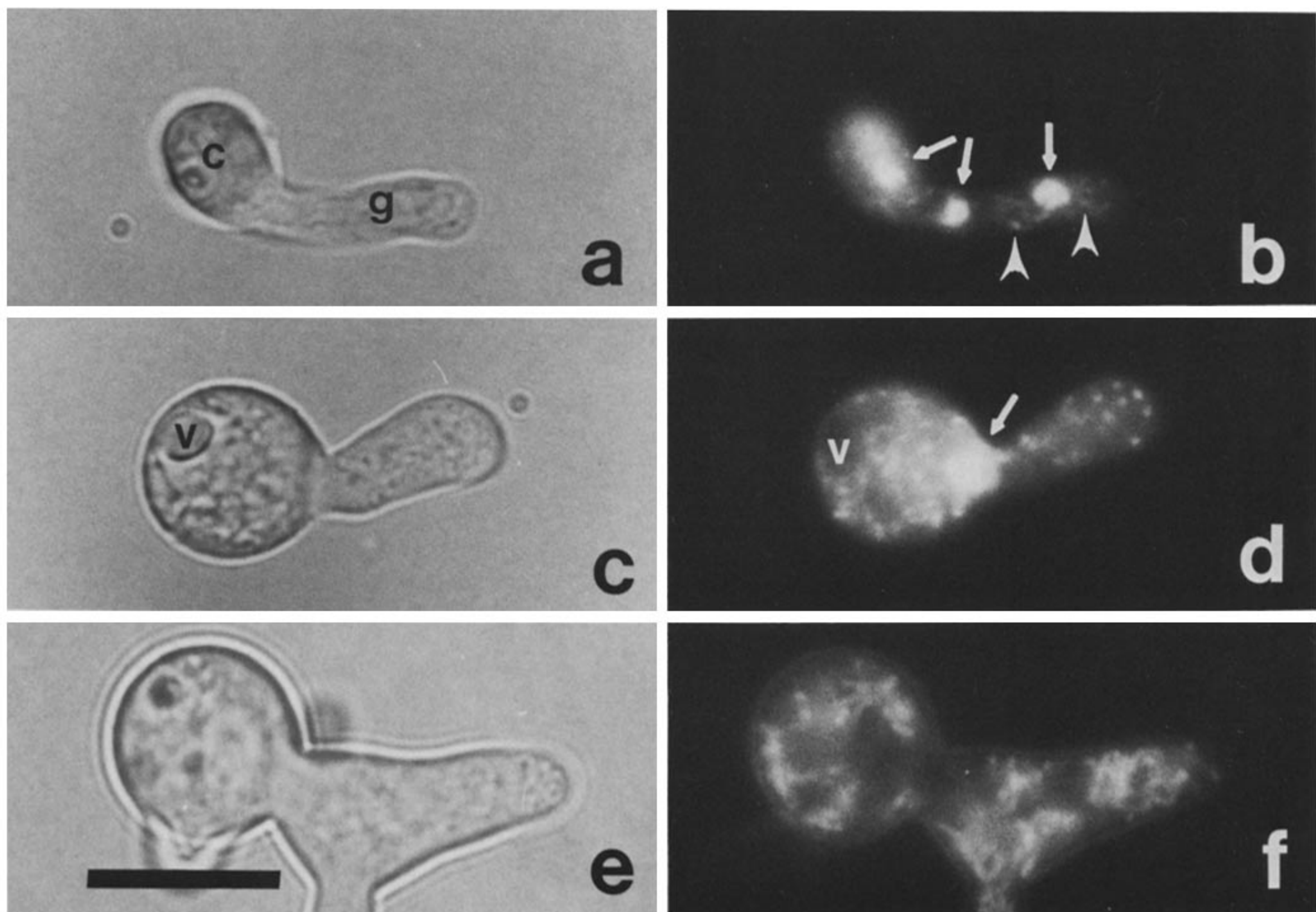

FIGURE 1 Nuclear division and movement and mitochondrial movement in a benomyl super-sensitive β-tubulin mutant. All figures show a strain carrying *ben*A16, a β-tubulin mutation that confers super-sensitivity to benomyl. *a* and *b* show a germling incubated for 8.3 h in the absence of benomyl. *a* shows a germ tube (*g*) extending from the conidial swelling (*c*). *b* is a fluorescence image of the same field. The germling has been stained with DAPI, and nuclei are indicated by arrows (a fourth nucleus was present out of the plane of focus). Mitochondrial nucleoids are visible as faint punctate fluorescence, and two of them are indicated by arrowheads. Two nuclei and several mitochondrial nucleoids have moved out of the conidial swelling. *c* and *d* show a germling of the same strain incubated for 12 h in the presence of 10 μg/ml benomyl. DAPI staining (*d*) reveals that a single nucleus (arrow) remains in the conidial swelling, and several mitochondrial nucleoids are present in the germ tube. Fluorescent particles are absent from the vacuole (*v*). *e* and *f* also show a germling incubated in 10 μg/ml benomyl for 12 h. This germling has been stained with rhodamine 123, and the fluorescent structures are mitochondria. Mitochondria are visible all the way to the tip of the germling. Bar, 10 μm. × 2,600.

TABLE I. *The Effects of Benomyl on Nuclear Division, Nuclear Movement, and Mitochondrial Movement*

	0 μg/ml Benomyl			10 μg/ml Benomyl		
Mutation	Nuclear division	Nuclear movement	Mitochondrial movement	Nuclear division	Nuclear movement	Mitochondrial movement
wt	100	93	97	9	23	96
*ben*A16	100	97	100	4	17	99
*ben*A19	100	95	100	10	16	96

Values shown are the percentages of germlings exhibiting nuclear division, nuclear movement, and mitochondrial movement. Germlings were scored as exhibiting nuclear division if two or more nuclei were present, as exhibiting nuclear movement if at least one nucleus had moved completely out of the conidial swelling, and as exhibiting mitochondrial movement if mitochondria had moved out of the conidial swelling as judged by the positions of mitochondrial nucleoids. 100 germlings were scored for each mutant under each condition. All germlings were incubated at 33°C. Because benomyl at high concentrations delays germination, germlings were incubated longer (12 h) in medium with benomyl than without (8.3 h). To eliminate any effects due to variation in germination times for the strains, only germlings 15–25-μm long were scored. In medium with benomyl, the nuclei of many of the germlings in which nuclear division had occurred were abnormal in size, suggesting that chromosomes had not been distributed evenly to the daughter nuclei. The nuclear movement rates in the benomyl-treated material may be slightly inflated due to the fact that a vacuole often forms in the conidial swelling that displaces the nucleus into the germ tube. The wild-type strain was B3, the parental strain in which *ben*A16 and *ben*A19 were isolated.

nated *nud*) that are hs$^-$ for growth and in which nuclear movement but not nuclear division is inhibited at a restrictive temperature (14). To test the effects of these mutations on mitochondrial movement, we incubated conidia of strains carrying each of these mutations at a restrictive temperature. As expected, nuclear movement was inhibited but nuclear division was not and, as in our previous tests, mitochondrial movement was not inhibited significantly (Fig. 2, Table III).

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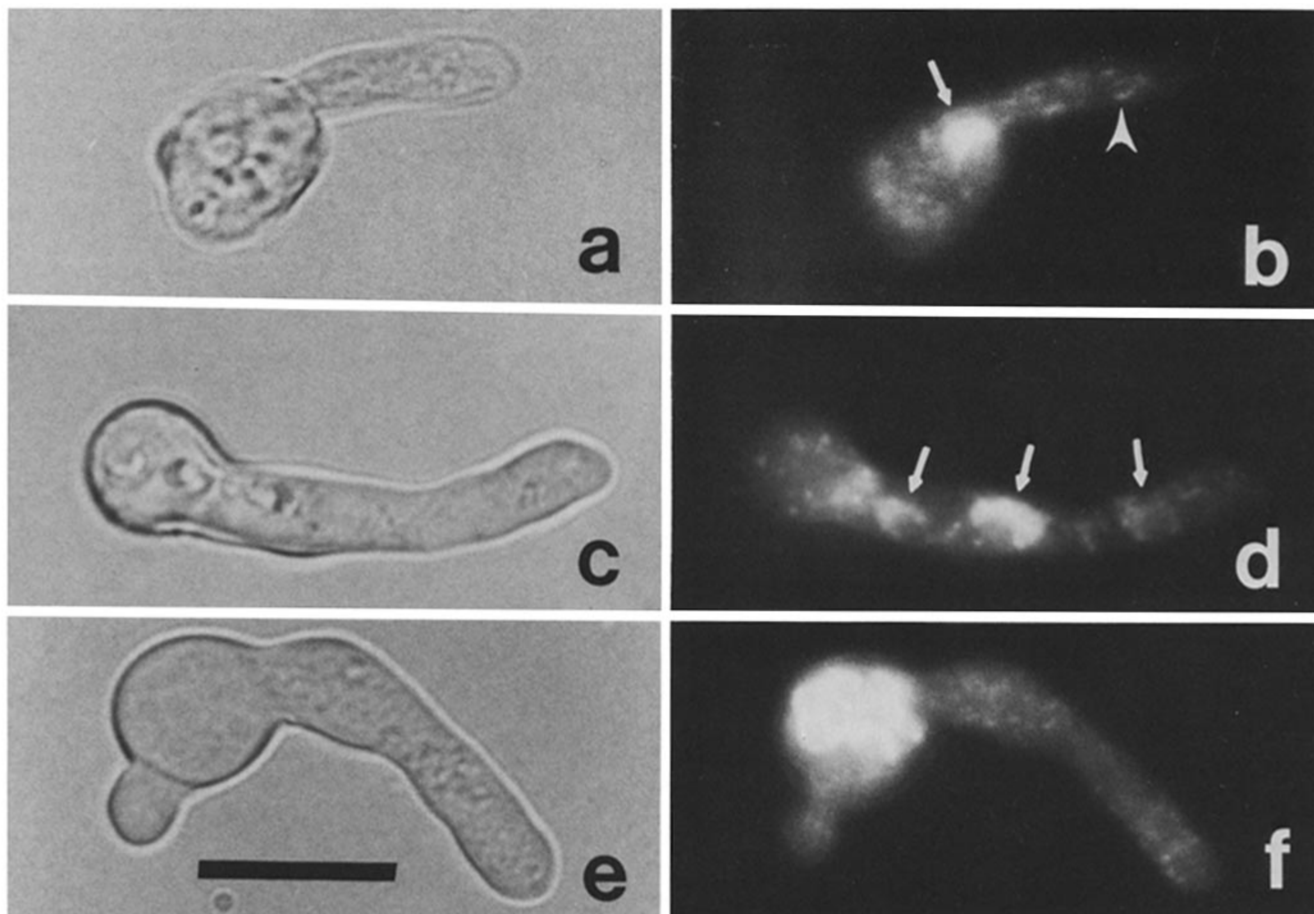

FIGURE 2 The effects of β-tubulin and *nud* mutations on nuclear division and movement and mitochondrial movement. All germlings have been stained with DAPI, and the figures are arranged in pairs with a transmitted light micrograph at the left and a fluorescence micrograph of the same field at the right. *a–d* show a cs⁻ *ben*A mutant carrying *ben*A33 and *ben*A71. The germling shown in *a* and *b* was incubated for 15 h at a restrictive temperature of 25°C. A single nucleus (arrow) is present inside the conidial swelling, but mitochondrial nucleoids, one of which is indicated by an arrowhead, are present in the germ tube. *c* and *d* show the same strain germinated for 8 h at 37°C. Three nuclei (arrows) are present in the germ tube as are numerous mitochondrial nucleoids. The difference in nuclear size and staining intensity is typical for this strain and may reflect differences in ploidy due to failure of nuclear division. *e* and *f* show a germling of *nud*A1 incubated for 8 h at 44°C. *f* shows several nuclei composing a fluorescent mass inside the conidial swelling and, numerous mitochondrial nucleoids in the germ tube. Bar, 10 μm. × 2,600.

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TABLE II. *The Effects of Conditionally Lethal β-Tubulin Mutations on Nuclear Division, Nuclear Movement, and Mitochondrial Movement*

	Restrictive temperature			Permissive temperature		
Mutation(s)	Nuclear division	Nuclear movement	Mitochondrial movement	Nuclear division	Nuclear movement	Mitochondrial movement
wt	97	98	86	100	99	97
*ben*A33	50	46	95	94	88	99
*ben*A33, *ben*A71	5	54	92	71	62	98
*ben*A33, *ben*A74	11	60	89	67	65	99
*ben*A33, *ben*A75	12	55	88	84	71	99
*ben*A33, *ben*A80	10	60	88	73	64	97
*ben*A33, *ben*A58	17	67	90	69	57	95

Values shown are the percentages of germlings exhibiting nuclear division, nuclear movement, and mitochondrial movement. Germlings were scored by the criteria listed in the caption for Table I. Restrictive temperature for *ben*A33 was 44°C, and permissive temperature was 25°C. For the remainder of the mutants, which are cold-sensitive, and for the wild type, restrictive temperature was 25°C and permissive temperature was 37°C. Each of the cs⁻ mutants carries *ben*A33 and an intragenic suppressor of *ben*A33 as specified, and the combined effect of the two mutations in each case is to render the mutant cold-sensitive. Incubation at 25°C was for 15 h, at 37°C for 8 h, and at 44°C for 8 h. 100 germlings were scored at permissive and restrictive temperatures for each strain and only germlings 15–25-μm long were scored. Although nuclear division and movement are inhibited at the restrictive temperature in the cs⁻ *ben*A mutants, the two processes are not inhibited to the same extent. Growth is partially inhibited at 37°C in these mutants relative to the wt, and the partial inhibition of nuclear division and movement at 37°C is consistent with this observation. Nuclear division and movement values for the cs⁻ *ben*A mutants have been reported elsewhere (17).

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TABLE III. *The Effects of nud Mutations on Nuclear Division, Nuclear Movement, and Mitochondrial Movement*

Mutation	Nuclear division	Nuclear movement	Mitochondrial movement
wt	100	97	100
*nud*A1	100	14	99
*nud*B2	100	22	100
*nud*C3	100	9	100
*nud*D4	100	24	97
*nud*E5	100	26	97

Values and scoring criteria are as listed in the captions for Tables I and II. Incubation was for 8 h at 44°C, a restrictive temperature for the *nud* mutants. Values for nuclear division and movement under permissive conditions have been published (15). Only germlings 15–25-μm long were scored for each strain. FGSC 154, the parental strain for the *nud* mutants, was used as the wt control.

Verifying results obtained with the *nud* mutants or other temperature-sensitive mutants by rhodamine 123 staining was difficult because it was not possible to maintain the mutants at an accurate restrictive temperature on the microscope stage. We did, however, attempt to verify our results with *nud*A1 by staining germlings, that had been incubated at a restrictive temperature of 44° C for 8 h, with rhodamine 123 for 5 min at the restrictive temperature and rapidly transferring them to the microscope for observation. We were able to observe approximately 20 germlings less than 1 min after their removal from the restrictive temperature and in all cases mitochondria were found throughout the germ tube all the way to the hyphal tip.

DISCUSSION

We have shown that benomyl, conditionally lethal β-tubulin mutations, and *nud* mutations inhibit nuclear migration but not mitochondrial migration. These results reveal a simple but fundamental fact that the mechanisms of nuclear and mitochondrial movement are not identical in *A. nidulans*. In a sense these data are negative in that we have been unable to inhibit mitochondrial movement. In each of our experiments, however, we have a strong internal control, the inhibition of nuclear movement, that makes the lack of inhibition of mitochondrial movement significant. It is clear, for example, that benomyl inhibits microtubule function because nuclear division and movement are inhibited. Likewise it is clear that the *ben*A and *nud* mutations have an effect because they inhibit nuclear movement in the same germlings in which mitochondrial movement occurs. Our data argue, moreover, that the β-tubulin encoded by the *ben*A gene and the products of the five *nud* genes function in nuclear movement but are not required for mitochondrial movement.

One could argue that mitochondrial movement is not an active process, that mitochondria are simply caught in a flow of cytoplasm toward the hyphal tip. This hypothesis, while consistent with our central finding that nuclei and mitochondria move by different mechanisms, is not likely to be correct because if such a bulk cytoplasmic flow occurred, it should carry not only mitochondria toward the hyphal tip but nuclei as well, and nuclear movement was inhibited in each of our experiments.

The simplest and most likely explanation for our findings that β-tubulin mutations and the anti-microtubule agent benomyl do not inhibit mitochondrial movement is that mitochondrial movement is not microtubule-mediated. There are two other possible explanations, however. One is that mitochondrial movement is microtubule-mediated but involves a set of microtubules that are benomyl-resistant and do not contain the β-tubulin encoded by the *ben*A gene. This possibility gains some credence from the recent demonstration by Weatherbee and Morris (25) that a β-tubulin gene in addition to the *ben*A gene is expressed in *A. nidulans*. The β-tubulin encoded by the second gene appears, however, to function specifically in conidiation (10). The second is that the requirements for microtubules in the two forms of movement are different. Nuclear movement could, for example, require long, intact microtubules, whereas mitochondrial movement might require only short fragments of microtubules to bridge between mitochondria and some other cytoskeletal element such as actin microfilaments.

Our results were initially surprising to us in view of the demonstration that mitochondria can move along single microtubules in squid axoplasm (19, 22), that antimicrotubule agents cause the displacement of mitochondria from the hyphal tips of *Fusarium* (6, 7) and *Uromyces* (5), and that in *Uromyces* microtubules are associated with mitochondria at a significantly higher than random frequency (4). Three possible explanations for the differences between our results and those obtained with *Fusarium* and *Uromyces* have occurred to us. The first is that the effects of antimicrotubule agents on mitochondrial positioning in *Fusarium* and *Uromyces* might not be due to the antimicrotubule activities of the agents used but to actions at other, as yet unknown, sites. Several antimicrotubule agents have been shown to have significant activities, including effects on motility, that are unrelated to their antimicrotubule activities (discussed in reference 14). In the absence of tubulin mutants such as have been used to demonstrate the specificity of benomyl in *Aspergillus nidulans*, or other means of demonstrating specificity, this possibility cannot be ruled out. The second possibility is that the differences might be due to the different assays used. We have scored a germling as exhibiting mitochondrial movement if any mitochondria have moved out of the condidial swelling. Howard and Aist (6) and Herr and Heath (5) assayed displacement of mitochondria from the hyphal tip, and their assays might reveal smaller effects of antimicrotubule agents on mitochondria than ours. We do not believe that this explanation is correct, however, because we have not found a mitochondria-free zone near the hyphal tip in any of our experiments. The third possibility is more interesting and, in our view, more likely. It is that microtubules and another cytoskeletal element function in mitochondrial movement in *Fusarium* and *Uromyces*, and that only one of these two elements, the nonmicrotubule element, is required for mitochondrial movement in *A. nidulans*. A close examination of the results of Howard and Aist (6) and Herr and Heath (5) suggests that microtubules are not the sole structures that function in the positioning and movement of mitochondria in these organisms. In *Uromyces* treated with antimicrotubule agents, although the mitochondria are displaced from the germling tips, they do move out of the urediospores (5). In *Fusarium*, mitochondria apparently are shifted back from hyphal tips when MBC is added (6), demonstrating that movement occurs in the presence of this antimicrotubule agent and thus suggesting that mitochondria are interacting with some structure in addition to microtubules to produce movement. In addition, Heath and Heath

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(4) have analyzed microtubule lengths and distribution in *Uromyces* and have concluded that unless microtubules are reduced in length in fixation, they are too short to account for organellar movement unless they are linked to a more extensive cytoskeletal system.

While our data do not cast any light on the identity of a second cytoskeletal element, actin microfilaments are an obvious candidate. Novick and Botstein (13) have provided excellent evidence using conditionally lethal actin mutations that vesicle transport in *Saccharomyces cerevisiae* is microfilament-mediated. Herr and Heath (5) have shown that cytochalasin B causes a striking disruption of tip growth in *Uromyces*, a phenomenon that has also been observed in other fungi (21). As Herr and Heath point out, this result suggests that the transport of vesicles to the point of growth of the germ tube is microfilament-mediated.

In any case, our results suggest that we must be cautious in looking for universal mechanisms of organellar motility. The work that suggests that a single mechanism cannot be responsible for all organellar translocation is well documented (18), and our data in combination with others suggest that a single type of organelle may move by different mechanisms in different organisms.

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