Identification and Characterization of *Aspergillus nidulans* Mutants Defective in Cytokinesis

Steven D. Harris, Jennifer L. Morrell and John E. Hamer

Department of Biological Sciences, Hansen Life Science Research Building, Purdue University, West Lafayette, Indiana 47907-1392

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ABSTRACT

Filamentous fungi undergo cytokinesis by forming crosswalls termed septa. Here, we describe the genetic and physiological controls governing septation in *Aspergillus nidulans*. Germinating conidia do not form septa until the completion of their third nuclear division. The first septum is invariantly positioned at the basal end of the germ tube. Block-and-release experiments of nuclear division with benomyl or hydroxyurea, and analysis of various nuclear division mutants demonstrated that septum formation is dependent upon the third mitotic division. Block-and-release experiments with cytochalasin A and the localization of actin in germlings by indirect immunofluorescence showed that actin participated in septum formation. In addition to being concentrated at the growing hyphal tips, a band of actin was also apparent at the site of septum formation. Previous genetic analysis in *A. nidulans* identified four genes involved in septation (*sepA–D*). We have screened a new collection of temperature sensitive (ts) mutants of *A. nidulans* for strains that failed to form septa at the restrictive temperature but were able to complete early nuclear divisions. We identified five new genes designated *sepE*, *G*, *H*, *I* and *J*, along with one additional allele of a previously identified septation gene. On the basis of temperature shift experiments, nuclear counts and cell morphology, we sorted these cytokinesis mutants into three phenotypic classes. Interestingly, one class of mutants fails to form septa and fails to progress past the third nuclear division. This class of mutants suggests the existence of a regulatory mechanism in *A. nidulans* that ensures the continuation of nuclear division following the initiation of cytokinesis.

THREE species of fungi, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans* have served as useful genetic organisms for the study of structural and regulatory proteins essential for cell division (for reviews see FORSBURG and NURSE 1991; DOONAN 1992; MORRIS and ENOS 1992). In all three organisms mutations have been obtained in structural components of the mitotic apparatus (HAGAN and YANAGIDA 1990; OAKLEY *et al.* 1990; WINEY *et al.* 1991) and in regulatory proteins central to the control of mitosis (NURSE *et al.* 1976; REID and HARTWELL 1977; OSMANI *et al.* 1987). Most importantly, the regulatory pathways deduced from the study of cell division mutants in these organisms have been broadly conserved in all organisms (MURRAY and KIRSCHNER 1989; NURSE 1990). While the conservation of regulation and gene function between fungi and animals is apparent from the study of mitosis, it is less obvious at the last stage in cell division, cytokinesis.

Fungi complete cytokinesis by forming a crosswall termed a septum. Light and electron microscopy studies have found that septum formation in higher fungi is more similar to cytokinesis in animal cells than the phragmoplastic division process of higher plants (PATTON and MARCHANT 1978; GIRBARDT 1979; GABRIEL 1984). These studies identified a contracting microfilament band, referred to as the "septal band," that appears early in septum formation (GIRBARDT 1979). Fluorescent microscopy studies have now identified the septal band as a centripetal band of actin microfilaments (RAUDASKOSKI 1970; HUNSLEY and GOODAY 1974; HOCH and HOWARD 1980; MARKS and HYAMS 1985; ALFA and HYAMS 1990). This actin ring has been shown to contract in protoplasts of the fission yeast *Schizosaccharomyces japonicus*, and thus has been compared with the contractile ring of animal cells (GABRIEL 1984; JOCHOVÁ *et al.* 1991).

In fungi and slime molds, mutations affecting cytokinesis are well known. Gene disruptions have been used to demonstrate the requirement for myosin (WATTS *et al.* 1987; FUKUI *et al.* 1990; RODRIGUEZ and PATTERSON 1990; FUKUI and INQUE 1991), various phosphatases (HEALY *et al.* 1991; VAN ZYL *et al.* 1992), and an actin-associated protein, coronin, (DE HOSTOS *et al.* 1993) for cytokinesis. Temperature-sensitive (ts) mutations in several cell division cycle (cdc) genes (*CDC3*, *10*, *11* and *12*) of *S. cerevisiae* have a pleiotropic phenotype that includes defects in cytokinesis, resulting in the formation of multibudded and multinucleated cells (PRINGLE and HARTWELL 1981). More recent studies have suggested that these gene

products also participate in the early events of bud development (for review see CHANT and PRINGLE 1991). In *S. pombe*, mutations in nine *cdc* genes lead to blocks or alterations in septum formation (MINET *et al.* 1979; MARKS *et al.* 1992). Genetic studies of various double mutant combinations suggest that some of the products encoded by these genes physically interact (MARKS *et al.* 1992). Recently, the *cdc8* gene was found to encode a tropomyosin which localized to the actin (septal) band during cytokinesis (BALASUBRAMANIAN *et al.* 1992). Together these studies provide strong evidence that fungi can serve as useful model systems for the genetic analysis of cytokinesis.

In contrast to the uninucleate and unicellular yeasts, little is known regarding the cytokinetic process in multicellular filamentous fungi. The vegetative mycelium of *A. nidulans* is composed of multinucleate cells partitioned at uniform intervals by septa (FIDDY and TRINCI 1976). The vegetative growth cycle is initiated by the germination of conidia, asexually produced, uninucleate spores arrested in the G_1 phase of the cell cycle (BERGEN and MORRIS 1983). The initial rounds of nuclear division that occur during germination are highly synchronous; however, this synchrony is lost once the germlings pass the 8–16 nuclei stage (ROSENBERGER and KESSEL 1967; FIDDY and TRINCI 1976). Subsequently, each multinucleate cellular compartment appears to divide in an autonomous manner. This complex pattern of cell growth and division has been referred to as a duplication cycle (FIDDY and TRINCI 1976). The hyphal tip cells grow by apical extension and are much longer than the uniformly sized intercalary cellular compartments, which must reorient their growth axis and branch to establish new apical tips. Additionally, the tip cells possess up to 40 nuclei, whereas intercalary cells contain 2–10 nuclei (FIDDY and TRINCI 1976; S. HARRIS and J. HAMER, unpublished data). Within the hyphal tip, the duplication cycle is initiated by a parasynchronous wave of nuclear division that extends basally. This mitotic wave is immediately followed by a wave of septum formation (KING and ALEXANDER 1969; CLUTTERBUCK 1970). Nuclear division cycles have been shown to be synchronous only within a cellular compartment delimited by septa (ROSENBERGER and KESSEL 1967; ROBINOW and CATEN 1969). Further back from the tip, older cells become competent for a variety of differentiation events, and are capable of forming both asexual and sexual reproductive structures (AXELROD *et al.* 1973; TIMBERLAKE 1991). It has been argued that the ability of higher fungi to partition cellular environments through septum formation imparts a unique ability to differentiate a multitude of cell types, while at the same time maintaining the complex homeostatic balance of filamentous growth (GULL 1978).

Septa formed by ascomycetes such as *A. nidulans* are composed of several electron dense layers that are perforated by a single septal pore (HUNSLEY and GOODAY 1974; RICHLE and ALEXANDER 1965; M. MOMANY and J. HAMER, unpublished data). Structural characterization of septa from the related fungus *Neurospora crassa* has identified chitin as the major component (HUNSLEY and GOODAY 1974). Several crystalline, electron dense vesicles called Woronin bodies are frequently found in the vicinity of the septal pore (BRACKER 1967; WERGIN 1973). Woronin bodies appear to plug the septal pore in older or injured hyphae (COLLINGE and MARKHAM 1985). During asexual sporulation in *A. nidulans*, changes in the pattern of cell division result in the formation of uninucleate cells which are bounded by thicker and more elaborate septa that lack pores (SEWALL *et al.* 1990).

A previous large-scale screen for *A. nidulans* ts mitotic mutants yielded five mutations that prevent septum formation and eventually lead to the cessation of hyphal growth at the nonpermissive temperature (MORRIS 1976). These five mutations defined four genes which were named *sepA*, *B*, *C* and *D*. In these cytokinesis mutants, the nuclear division cycle continues for a time at the nonpermissive temperature and cells accumulate multiple nuclei. Studies with the *sepA* mutant demonstrated that regularly spaced septa were rapidly formed throughout the hyphae following a shift from the nonpermissive to the permissive temperature, suggesting that sites for septum formation had already been established during growth at the nonpermissive temperature (TRINCI and MORRIS 1979).

We have initiated genetic and physiological studies of the process of cytokinesis in *A. nidulans*. Specifically, we have examined how the formation of the first septum is coordinated with the nuclear division cycle, demonstrated the involvement of actin in septum assembly and characterized a collection of cytokinesis mutants. The first septum is formed following the third nuclear division (eight nuclei stage) in germinating conidia. Although germlings form the first septum within a particular size or volume range, cell growth is not sufficient to trigger cytokinesis. We have used a variety of approaches to demonstrate that septum formation is dependent upon completion of the third nuclear division. Two independent approaches have been used to demonstrate that actin is associated with septum formation in *A. nidulans*. Finally, we have characterized a collection of ts cytokinesis mutants of *A. nidulans*. Single ts alleles were identified for five more genes that affect cytokinesis. Together with the *sepA–D* mutations, these nine mutants sort into three distinct phenotypic classes. Interestingly, one class of mutants fails to complete cytokinesis and demonstrates a concomitant nuclear divi-

TABLE 1

***A. nidulans* strains**

Strain	Genotype
A28[a]	*pabaA6; biA1*
A104[a]	*yA2, adE20; ArcA1; phenA2; pyroA4; lysB5; sB3; nicB8; coA1*
A777[a]	*adE20, biA1; wA2, cnxE16; sC12; methG1; nicA2; bimC4, lacA1; choA1; chaA1*
A781[a]	*nimA5; wA2*
M3127[b]	*yA2, proA1, pyrG89; wA3; bimB3*
XX1[c]	*yA2; nicA2; chaA1; nudA1*
AH12	*argB2; chaA1*
ASH5	*sepA1; wA2*
ASH20	*sepA1; yA2; argB2*
ASH34	*sepA1; argB2*
ATS50	*sepA3 yA2; argB2*
ASH13	*sepB3; wA2*
ASH15	*sepB3; wA2; argB2*
AJM19	*sepC4; wA2; argB2*
AJM2	*sepD5; argB2*
AJM4	*sepD5; argB2*
ATS55	*sepE1; pabaA6*
ATS22	*sepG1; pabaA6; chaA1*
ATS44	*sepH1; pabaA6; chaA1*
ATS19	*sepI1; argB2; chaA1*
ATS18	*sepJ1; pabaA6; chaA1*

[a] Obtained from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420.

[b] Obtained from GREGORY MAY, Department of Cell Biology, Baylor College of Medicine, Houston, Texas.

[c] Obtained from RON MORRIS, Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey.

sion arrest at the eight nuclei stage. This phenotype is consistent with the presence of a regulatory mechanism in *A. nidulans* that prevents the progression of the nuclear division cycle beyond this stage unless cytokinesis has been initiated.

MATERIALS AND METHODS

Aspergillus strains and growth methods: All strains used in this study are derived from FGSC4 and are listed in Table 1. Media used were YEG (1% glucose, 0.5% yeast extract), CM (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, and 0.01% vitamins, pH 6.5) or MN (1% glucose, nitrate salts, trace elements, pH 6.5). Trace elements, vitamins and nitrate salts are described in the appendix to KAFER (1977). For solid media, 1.8% agar was added. When necessary, 0.01% Triton X-100 was added to solid media to promote colonial growth. Strains containing the *pyrG89* mutation were grown in YEG or MAG (2% malt extract, 2% glucose, 0.2% peptone, trace elements, and vitamins) supplemented with 5 mM uridine and 10 mM uracil. All other supplements were added at 1.0% (amino acids) or 0.01% (vitamins).

Standard genetic techniques for *A. nidulans* (KAFER 1977) were used for all strain constructions, except as described. Heterokaryons were constructed by inoculating conidia from strains containing complementary auxotrophic markers into 2 ml of appropriately supplemented CM in a microtiter plate. After 2 days, the mycelial mat was transferred to a MN plate and incubated at 30° until heterokaryotic growth was evident. Heterokaryons were subcultured on MN plates. Heterokaryons spontaneously form diploid conidia at a frequency of approximately 1×10^{-6} (KAFER 1977). Diploids were isolated by plating conidia harvested from subcultured heterokaryons in MN agar. Diploid strains were purified by streaking for single colonies on MN agar. Haploidization of diploid strains for mitotic mapping employed benlate as described (HASTIE 1970). For crosses, the original MN plate containing the heterokaryotic growth was sealed and incubated in the dark at room temperature for 2 wk to allow formation of cleistothecia. Cleistothecia were dissected out using fine tweezers and cleaned by rolling them on plates containing 4% agar. Ascospores were isolated by crushing cleistothecia in 200 μl of sterile water and plating dilutions onto appropriately supplemented media. Ascospores were stored in sterile water at 4°.

For experiments using light microscopy, strains were grown on coverslips in Petri dishes. Depending on the experiment, one to eight sterile glass cover slips were placed on the bottom of the Petri dish and gently overlaid with liquid YEG media containing $1-5 \times 10^4$ conidia/ml of the relevant strain. The conidia settled to the bottom of the Petri dish and adhered tightly to the coverslips. For experiments monitoring the appearance of septa during conidial germination, cultures of A28 were grown at 37° and cover slips were removed at hourly intervals and processed for microscopy. For all time points, 200 germlings were scored for the presence of septa and for the number of nuclei that they contained. All experiments were repeated three times with essentially identical results. For the data presented in Figures 1 and 2, a data point represents the modal average of the distribution of values for the corresponding time point. For example, if the majority of the 200 germlings counted at a particular time point contained four nuclei, the data point was plotted at four. However, it should be noted that a minority fraction of the counted germlings at that time point could have contained two or eight nuclei.

Block-and-release experiments: Conidia of strain A28 were grown on coverslips at 37° in YEG and germination, nuclear division and septation were monitored by taking time points every hour. At 6 hr, several coverslips with adherent germlings were transferred to fresh prewarmed YEG containing 20 mM hydroxyurea (HU) (Sigma Chem. Co., St. Louis) and maintained at 37°. Germlings were incubated in the presence of HU for 4 hr after which they were released from the block by subjecting them to three brief rinses in prewarmed YEG and transferring them to fresh prewarmed YEG for continued growth. An untreated control culture was followed under identical conditions. Two hundred germlings were scored at each time point, and in addition, at selected time points, the total length of 100–200 germlings was determined using a calibrated stage micrometer. The values presented in Figure 2, B and C are, respectively, for untreated germlings following 8-hr growth and for germlings treated with HU for 4 hr. Similar block-and-release experiments were done using cytochalasin A (CA). CA (Sigma Chem. Co.) was prepared as a 20 mg/ml stock solution in dimethyl sulfoxide (DMSO) and was added to prewarmed YEG at final concentrations ranging from 1–20 μg/ml. The concentration of DMSO was adjusted to 0.1% in all control cultures for CA block-and-release experiments. When added at concentrations above 2 μg/ml, CA rapidly arrested growth and nuclear division. At each time point, 200 germlings were scored for the number of septa they contained and 100–200 were subjected to nuclear counts.

Mitotic block experiments with Benomyl: Conidia of strain A28 were grown on coverslips at 37° in YEG and germination, nuclear division and septation were monitored

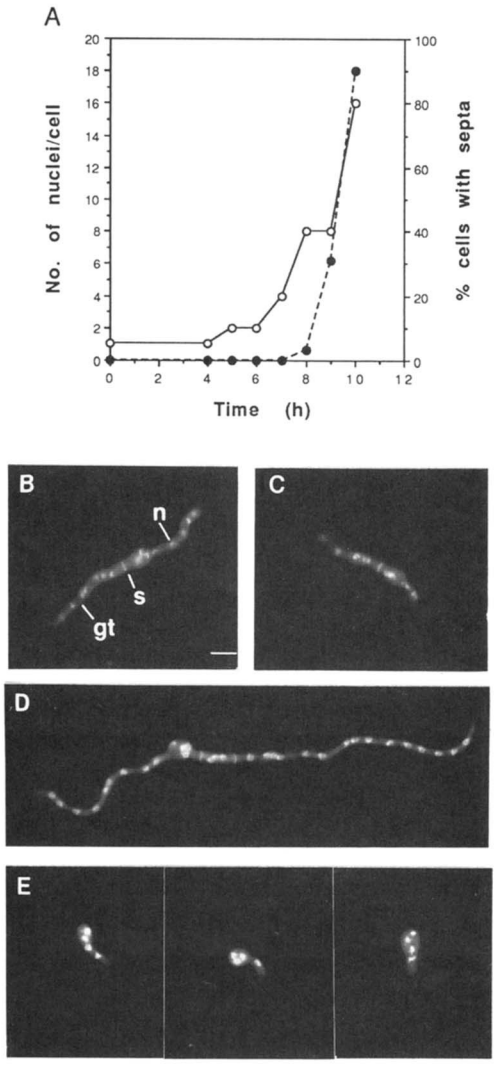

FIGURE 1.—Septation in germlings of *A. nidulans*. (A) Kinetics of septum formation and nuclear division. Conidia of strain A28 were germinated on cover slips in YEG at 37°. Coverslips were removed at various intervals, fixed and stained with a combination of Calcofluor and DAPI (B–E) to count septa and nuclei, respectively. (○) number of nuclei; (●) percentage of germlings with septa. Septa form following the third round of nuclear division (eight nuclei stage) at the basal end of the germ tube (B and C). Thereafter each round of nuclear division is followed by a round of septation so that a germling with 32 nuclei contains three septa (D). Prior to the third nuclear division septa are not seen (E). Abbreviations; n, nuclei; gt, germ tube; s, septum. Bar, 3 μm.

by taking time points every hour. Additionally, at each hour, a single coverslip was transferred to prewarmed YEG media containing Benomyl (BEN, a gift of the Dupont Company) at a concentration of 5 μg/ml. Germlings were incubated in the presence of BEN for 3 hr before being processed for microscopy. Two hundred germlings were scored at each time point.

Temperature shift experiments: Temperature-sensitive *sep*, *nud*, *nim* and *bim* mutants were grown on coverslips at 42° in YEG for 14–16 hr prior to being processed for microscopy. Shift-down experiments with septation mutants were initiated by growing strains on coverslips at 42° in YEG for 12 hr. At this time one coverslip was removed and processed for microscopy while the remainder were transferred to fresh YEG prewarmed at 28°. During the subse-

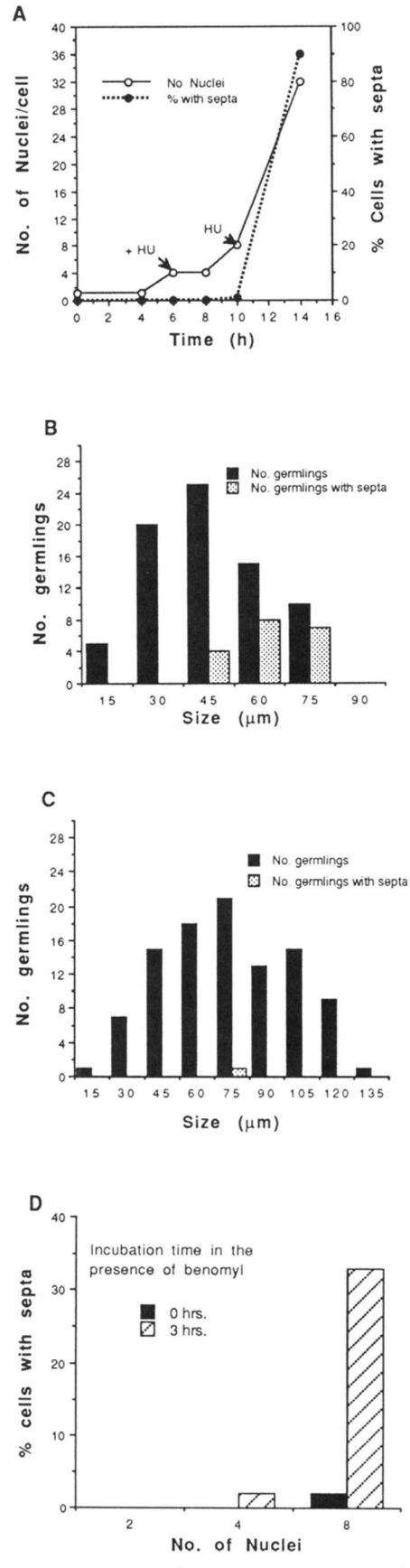

FIGURE 2.—Septation is dependent upon the completion of the third nuclear division cycle. (A) HU was used to perform block-and-release experiments on germinating conidia adhering to glass

quent incubation, coverslips were removed at 30, 60 and 90 min intervals and processed for microscopy. At each time point, 100–200 germlings were scored for the number of septa and nuclei they possessed.

Staining and microscopy: Coverslips with adherent germlings were transferred to 3.7% formaldehyde, 50 mM phosphate buffer (pH 7.0), 0.2% Triton X-100 for fixation and incubated at room temperature for 30–45 min. The coverslips were rinsed in distilled water prior to staining. For staining, the coverslips were incubated for 5 min at room temperature in a solution containing 10 μg/ml Calcofluor (a gift of American Cyanamid) and either 800 ng/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.) or 100 ng/ml Hoechst 33258 in distilled water. The coverslips were then rinsed in distilled water and mounted in 10% phosphate buffer (pH 7.0), 50% glycerol, 0.1% n-propyl-gallate.

For immunofluorescence, coverslips were transferred to 3.7% formaldehyde, 50 mM Pipes, pH 6.7, 5 mM $MgSO_4$, 25 mM EGTA, pH 7.0, for fixation and incubated at room temperature for 45 min. The coverslips were washed three successive times for 5 min each in PEM (50 mM Pipes, 25 mM EGTA, 5 mM $MgSO_4$). Walls were removed from hyphal cells by inverting the coverslips over 200 μl drops of digestion solution [50 mg/ml Novozym 234 (batch 3897, a gift from Novo Nordisk Biotech Inc.) containing 5 mM EGTA and diluted 1:1 with egg white]. Optimal staining was achieved with digestion times ranging from 45–75 min. Coverslips were subjected to three successive washes for 10 min each in PEM and then a 5 min incubation in extraction solution (100 mM Pipes, 25 mM EGTA, 0.1% Nonidet P-40). Following a 5 min wash in PEM, coverslips were immersed in absolute methanol at −20° for 10 min and then subjected to two additional 5 min washes in PEM. Prior to treatment with the primary antibody, the coverslips were incubated in PBS/BSA for 5 min. Hyphal cells were exposed to the anti-actin N.350 monoclonal antibody (Amersham) diluted 1:500 in PBS/BSA for 1 hr. Coverslips were then washed three successive times for 10 min each in PBS/BSA prior to being incubated in FITC-conjugated sheep anti-mouse IgG (Sigma) diluted 1:128 in PBS/BSA for 1 hr in the dark. Coverslips were subjected to three successive 10 min washes in PBS/BSA before being stained with Hoechst 33258 and Calcofluor.

Coverslips were mounted on clean glass slides and observed using an Olympus BH-2 and DPLANAPO 40× and 100× objectives (oil immersion). Photographs were taken with Kodak Technical-Pan or Kodak T-Max 400 film at ASA 400–800 and developed in Kodak HC-110 developer.

Mutagenesis: Conidia from strain A28 were mutagenized at a concentration of 2×10^6/ml in sterile distilled water employing 4-nitroquinoline 1-oxide [4 μg/ml (Aldrich Chem. Co., St. Louis)] as mutagen. Conidia were mutagenized for 30 min at 37° with constant shaking. The 4-nitroquinoline 1-oxide was inactivated by diluting the mutagenized conidia with an equal volume of 5% sodium thiosulfate. Mutagenized conidia were diluted and plated onto CM + Triton X-100 plates and incubated at 28° for 3 days. Colonies were then replica plated and the replica plates were incubated at 28° and 42°. Putative ts mutants were picked and retested. Ts mutants were stored as a colony plug in 15% glycerol at −70°. A total of eleven independent mutagenic treatments were used to generate a collection of 1156 ts mutants.

The collection was screened for mutants exhibiting defects in septation by employing a Calcofluor plate assay. *A. nidulans* septa can be readily visualized using the dye Calcofluor (see text). Conidia from each ts mutant were spotted in duplicate onto thin YEG plates [solidified with 0.8% agarose (electrophoresis grade)] and incubated for 16 hr at either 28° or 42°. Each plate was then flooded with a 5-ml solution of 2% Calcofluor in sterile distilled water and incubated at room temperature in the dark for 5 min. The staining solution was poured off and the plates were briefly allowed to air dry. The plates were screened by fluorescence microscopy using a 20× objective. All putative *sep* mutants (as well as other interesting classes of mutants) were subsequently screened by growing them on coverslips in liquid culture and staining with DAPI and Calcofluor as described above.

RESULTS

Temporal and spatial control of septum formation in germinating conidia: The dormant conidia of *A. nidulans* contain a single nucleus arrested in G_1 of the nuclear division cycle (BERGEN and MORRIS 1983). Germination involves the hydration and swelling of the conidium and a lag of approximately 3–4 hr prior to the first nuclear division and the emergence of a germ tube (Figure 1A). The first nuclear division occurs prior to germ tube emergence (data not shown). To determine the spatial and temporal relationship between septation and the nuclear division cycle, we monitored nuclear division and septation in germlings growing at 37° on glass coverslips. Coverslips with adherent germlings were removed at various times, fixed and stained with a combination of DAPI and Calcofluor. Figure 1A shows a typical time course for nuclear division and septation events in germinating conidia of strain A28. The first three nuclear divisions occur synchronously at intervals of approxi-

cover slips as described in the MATERIALS AND METHODS. Arrows indicate the time of addition and removal of HU. HU was added for 4 hr following the second nuclear division, and septum formation was delayed until release from the HU block (compare with Figure 1A). (○) number of nuclei; (●) percentage of germlings with septa. During these experiments, the length of individual germlings was measured to determine the relationship between septum formation and germling size. (B) Germlings from an untreated population following 8 hr of growth (the time when septa are forming, see Figure 1A) and (C) from an HU-treated population at 10 hr (the time of release from the HU block shown in panel A) were measured using a calibrated stage micrometer. To plot the data, individual germling lengths were sorted into size classes of <15 μm, 15–30 μm, 30–45 μm, etc. Untreated germlings that form septa were found to be 30 μm or longer (B). In contrast, HU treated germlings at the 10-hr time point grew well beyond 30 μm but failed to form septa (C). Mitotic blocks with benomyl also arrest septation (D). Conidia were germinated and benomyl was used to block the progression of nuclear division at both the four and eight nuclei stages as described in the Materials and Methods. The data presented represent time points where the majority of germlings possessed four or eight nuclei. The percentage of cells containing septa was determined at the time of addition of benomyl (0 hr) and 3 hr later. Cells with four nuclei fail to form septa following 3 hr incubation in benomyl. In contrast, a significant proportion of germlings with eight nuclei were capable of forming septa in the presence of benomyl. DAPI staining demonstrated that nuclear division was arrested in cells treated with benomyl at all time points (data not shown).

mately 100 min (BERGEN and MORRIS 1983) as the nuclei migrate into the extending hyphal cell. The first septation event occurs upon completion of the third nuclear division (8 nuclei stage). Approximately 40–50% of the germlings in any given population make the first septum at this stage and the remainder do so following the next nuclear division.

Figure 1, B, C and D, show fluorescent light micrographs of germinated conidia that have completed at least three rounds of nuclear division. Calcofluor staining shows that the first septum forms invariantly at the basal end of the extending germ tube. A second germ tube often emerges from the conidium at the time the first septum is formed (Figure 1, B and C). Following the first septation event, each subsequent nuclear division is followed by a round of septum formation (CLUTTERBUCK 1970). Figure 1D shows a germling containing approximately 32 nuclei where at least one additional round of septation has occurred to yield three septa. We have never observed septa in germlings that have completed only two rounds of nuclear division (Figure 1E). It should be noted that since Calcofluor stains chitin-containing wall material that persists in fully formed septa, we surmise that the appearance of Calcofluor staining is likely to indicate a late event in the process of septum assembly.

Dependency relationships between nuclear division and septation: The formation of the first septum is delayed until at least the third nuclear division has been completed. Thus, a critical product for septation may not be synthesized until this time (7–8 hr) in conidial germination. Alternatively, the formation of the first septum may be delayed until a critical cell size (volume) has been attained. Finally, the formation of the first septum may require a specific event intimately associated with the third mitotic division (dependency). To assess the degree to which the latter two factors influence the temporal controls upon the formation of the first septum, we employed two inhibitors of the nuclear division cycle in a series of experiments.

HU has been shown to rapidly and specifically inhibit DNA but not RNA synthesis in germinating conidia of *A. nidulans* (BERGEN and MORRIS 1983). We used HU (final concentration 20 mM) to block cells in S phase for up to 4 hr following the second nuclear division (Figure 2A). During the HU block cells failed to form septa, yet germlings continued to grow beyond the size at which untreated cells formed septa (Figure 2, B and C). These results are in agreement with earlier work which showed that HU had no effect on germ tube extension (BERGEN and MORRIS 1983). We observed, as has been previously noted (OSMANI *et al.* 1988), that nuclei in HU-treated germlings became elongated and multilobed. In addition, when stained with DAPI, background fluorescence increased considerably. This could account for the apparent rise in the number of nuclei in germlings treated with HU for 4 hr. It is also possible that a subpopulation of germlings were beginning to overcome the effects of the HU block and had undergone nuclear division, even though their nuclei appeared abnormal. Release from the HU block resulted in an immediate resumption of nuclear division and a concomitant round of septation (Figure 2A). Thus, in the presence of HU, cells with four nuclei were able to continue to grow and presumably synthesize RNA and protein; however, septation remained blocked until HU was removed and progression through the third nuclear division was permitted.

Identical block-and-release experiments were performed using the antimicrotubule drug BEN. In *A. nidulans* BEN causes depolymerization of microtubules and germlings become blocked in M phase while they continue to increase in size (ORR and ROSENBERGER 1976; SHEIR-NEISS *et al.* 1978; OAKLEY and MORRIS 1980). As was observed with the HU block-and-release experiments, septum formation could not occur until germlings completed the third round of nuclear division (data not shown). BEN was also used to arrest nuclear division in germlings containing either four or eight nuclei for 3 hr as described in the MATERIALS AND METHODS. During the incubation period in BEN-containing media, nuclei appeared as small masses of highly condensed chromatin suggestive of a block in mitosis (data not shown). In germlings blocked with predominantly four nuclei, septum formation was restricted to a few germlings (Figure 2D). In contrast, a much higher percentage (35%) of germlings blocked at the eight nuclei stage were able to form septa during the treatment with BEN. This result suggests that completion of the third nuclear division is sufficient to allow septum formation in the absence of further nuclear division. The observation that septa can form in the presence of a microtubule depolymerizing agent suggests that microtubules are not directly involved in septum assembly.

To elucidate other requirements for septum assembly, we examined several different types of nuclear and cell division mutants of *A. nidulans*. The *nimA* gene is required for nuclei to traverse the boundary between G_2 and M phases (OSMANI *et al.* 1987); thus, *nimA* mutants arrest with a single nucleus and are not capable of forming septa (Figure 3). Germlings may require a specific number of nuclei (*i.e.*, 8), or a specific amount of DNA per cell (*i.e.*, 8N) to trigger septation. Both the *bimB* and the *bimC* mutants of *A. nidulans* undergo multiple rounds of DNA replication producing polyploid nuclei without successfully completing a single mitosis (MAY *et al.* 1992; ENOS and MORRIS 1990). Following incubation for 16 hr at the restrictive temperature, neither of these mutants were

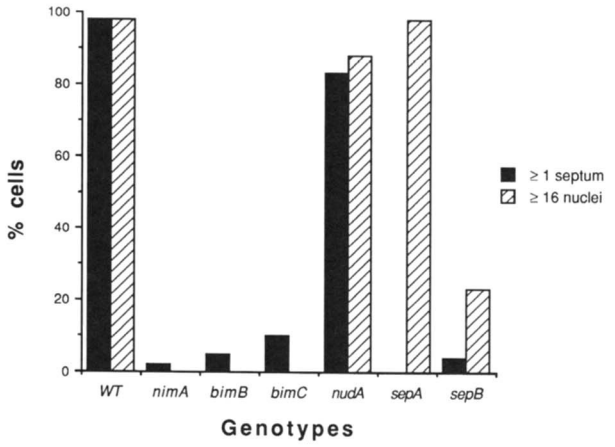

FIGURE 3.—Septation in cell division mutants of *A. nidulans*. Wild-type (A28) and a collection of ts cell division mutants of *A. nidulans* were germinated at 42° for 14–16 hr and then fixed and stained with DAPI and Calcofluor. The percentage of germlings with 16 or more nuclei and the percentage with at least one septum are presented. Nuclear division mutants *nimA*, *bimB* and *bimC* are impaired in their ability to form septa. Septation mutants such as *sepA* and *sepB* undergo multiple rounds of nuclear division but fail to form septa. When tested at 28°, all wild-type and mutant germlings formed septa and possessed greater than 16 nuclei (data not shown).

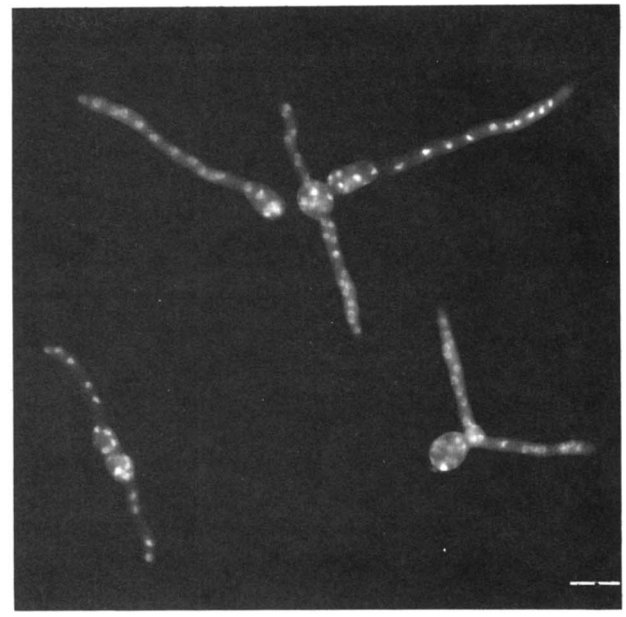

FIGURE 4.—Cytochalasin treated germlings of *A. nidulans*. Conidia of strain A28 were germinated on cover slips for 6 hr in YEG (when the majority of germlings possessed either 2 or 4 nuclei) and then shifted to YEG containing 1 μg/ml CA for 4 hr. Germlings were fixed and stained with DAPI and Calcofluor. Although nuclear division proceeded in the presence of CA, septum formation did not occur. Bar, 5 μm.

able to make septa (Figure 3). As expected, DAPI staining revealed that for both mutants each germling contained a single large mass of chromatin (data not shown). We conclude that net DNA content per cell is not a trigger for septum formation. Our results are consistent with a requirement for progression through the third nuclear division to trigger septum formation.

In contrast to the mitotic mutants, the nuclear distribution mutant *nudA* was able to form one basally located septum per germ tube (data not shown). In this mutant, nuclear division is not blocked but nuclei fail to migrate into the extending germ tube (MORRIS 1976). After 16 hr at the restrictive temperature, multiple germ tubes emerge from the multinucleate conidial cell of *nudA* mutants. Septa were observed only at the basal end of the emergent germ tubes (data not shown). This result suggests that orderly partitioning of *A. nidulans* cells requires correctly positioned nuclei. For comparison with *nim*, *bim* and *nud* mutants, the phenotypes of two *sep* mutants are shown (Figure 3). These *sep* mutants arrest with multiple nuclei but fail to form septa (MORRIS 1976). Germlings containing the *sepA* mutation arrest with as many nuclei as wild-type cells suggesting that nuclear division is unaffected by this mutation. In contrast, *sepB* mutants arrest with substantially fewer numbers of nuclei. The phenotypes of various septation mutants are presented and discussed in detail in a later section.

Cytochalasin A blocks septum formation: Actin is the prominent component of the contractile ring in animal cells. An actin ring or band has also been demonstrated at the site of septum formation in *S. cerevisiae*, *S. pombe* and a number of filamentous fungi (KILMARTIN and ADAMS 1984; MARKS and HYAMS 1985; RUNEBERG *et al.* 1986; ROBERSON 1992), but not in *A. nidulans*. *A. nidulans* contains a single actin gene that encodes a γ-actin (FIDEL *et al.* 1988). We have demonstrated the involvement of actin in septum formation by two independent methods. First, we examined the effect of CA on septum formation. Cytochalasins are known for their ability to block actin-dependent processes, such as locomotion and cytokinesis (BROWN and SPUDICH 1981; COOPER 1987). Furthermore, CA has been shown to cause abnormal patterns of tip growth in *N. crassa* (ALLEN *et al.* 1980). At 1 μg/ml, CA blocks septum formation in germinating conidia of *A. nidulans* but permits nuclear division to continue (Figure 4). Additionally, germlings exhibit pronounced regions of swelling, suggesting that tip growth is transiently perturbed (data not shown).

Table 2 shows results from block-and-release experiments with CA. Conidia were germinated on coverslips for 6 hr and shifted to media containing 1 μg/ml CA for 4 hr. Although nuclear division appeared to be slightly delayed when compared with untreated control cultures, the majority of germlings (81%) progressed beyond the third mitotic division and failed to form septa. Upon release from the CA block, germlings underwent multiple rounds of septation within 90 min. Moreover, septa were formed in the

TABLE 2

Cytochalasin A reversibly inhibits septum formation

Time (hr)	No. of septa				No. of nuclei					
	0	1	2	>2	1	2	4	8	9–16	>16
6	200				4	103	93			
10 Control	2	19	55	124				1	4	95
CA + 4	199	1						19	79	2
Release from CA block										
+15 min	190	10			ND[a]					
+30 min	143	54	3					7	51	42
+45 min	128	62	10		ND					
+60 min	63	104	29	4	ND					
+90 min	28	125	36	11				1	5	84

Cytochalasin A (CA) was used to perform block-and-release experiments on germinating conidia of strain A28 as described in MATERIALS AND METHODS. CA was added following 6 hr of growth in YEG at 37° and the block was maintained for 4 hr. Germlings were fixed and stained with Calcofluor and DAPI. Although they were able to successfully complete two rounds of nuclear division, the CA treated germlings failed to form septa. The block was released by transferring germlings to fresh prewarmed YEG. At the indicated intervals, germlings were fixed and stained with Calcofluor and DAPI. Upon release from the block, germlings rapidly proceeded to form septa.

[a] ND = not determined.

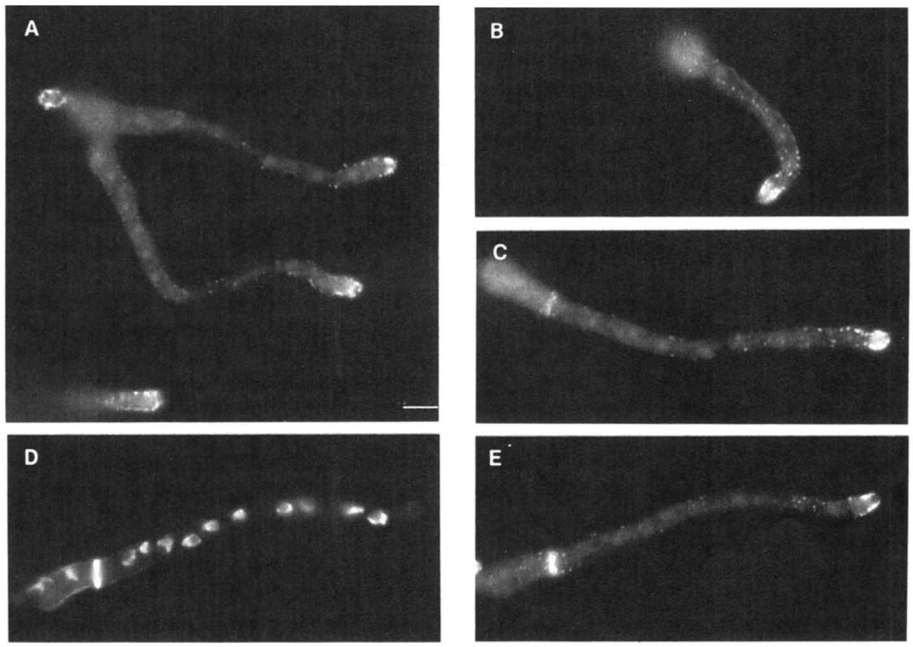

FIGURE 5.—Immunolocalization of actin in germinating conidia. Conidia of strain A28 were germinated on coverslips for 10 hr in YEG at 32°. Germlings were fixed and stained with the anti-actin N.350 monoclonal antibody and a fluorescein-conjugated secondary antibody as described in MATERIALS AND METHODS. A, B, C and E show fluorescein labeling of actin-containing structures and D shows the same germling as in E stained with Calcofluor and Hoechst 33258 to identify septa and nuclei, respectively. Bar, 2 μm.

regions of germlings that grew out in the presence of CA (data not shown).

Because actin-containing structures of *A. nidulans* do not stain with phallotoxin fluorescent conjugates (B. OAKLEY, R. MORRIS, G. MAY and our unpublished data), we used a commercial monoclonal anti-actin antibody (Amersham N.350) to stain germinating conidia. Intense punctate staining was obvious at the germ tube tip with additional cortical spots or plaques located basally (Figure 5A). Actin filaments were occasionally visible and appeared to emanate from and sometimes connect the cortical spots (Figure 5B). Actin was localized at the site of septum formation (Figure 5, C and E). Actin appeared either as an accumulation of spots (Figure 5C) or as dense bands (Figure 5E). Moreover, actin localization at the septum was always coincident with localization to the germ tube tip (Figure 5, C and E), suggesting that *A. nidulans* can simultaneously maintain distinct actin-containing structures within a single cell. Septa were also identified by Calcofluor staining and the distribution of nuclei was visualized by Hoechst 33258 staining. Figures 5D and 5E show the same germling stained with Calcofluor and Hoechst 33258 and with anti-actin N.350 monoclonal antibody. We conclude that actin is necessary and intimately involved in septum formation of *A. nidulans*.

Mutations affecting septum formation: We have generated a large collection of *A. nidulans* ts mutants and screened through this collection for mutants that exhibit defects in septation. We mutagenized strain A28 with 4-nitroquinoline 1-oxide (see MATERIALS

TABLE 3

Summary of the genetic characterization of the new sep mutants

Mutant	$Ts^+:Ts^-$[a]	Dominant/ recessive[b]	Linkage group[c]	Locus/ mutation designation
ts2-78	59:41	R	II	*sepJ1*
ts2-105	50:50	R	I	*sepA3*
ts2-525	49:51	R	III	*sepH1*
ts2-528	48:51	R	II	*sepI1*
ts3-13	62:38[d]	R	ND[e]	*sepE1*
ts8-115	64:36	R	II	*sepG1*

[a] Segregation of temperature-sensitivity from the first outcross of each mutant to AH12.

[b] D = dominant; R = recessive.

[c] Diploids were constructed between each mutant and strain A104. These diploids were used for standard mitotic mapping analysis as described in MATERIALS AND METHODS.

[d] From the cross of ts3-13 to AH12, the *argB2* mutation segregated ($Arg^+:Arg^-$) 69:25 while all other markers segregated 1:1. This suggests that *sepE1 argB2* double mutants may exhibit some inviability. Indeed, of the 38 Ts^- segregants, only 3 were additionally Arg^-.

[e] ND = not determined.

AND METHODS) to yield 30% survival. This survival rate yielded a broad spectrum of conidial color variants as well as colony morphology variants that were easily distinguishable on plates. Replica plating yielded 1,156 ts mutants from approximately 49,000 survivors of mutagenesis. We employed a Calcofluor plate assay (see MATERIALS AND METHODS) to identify those mutants that appeared to exhibit defects in septation. Putative septation mutants were further characterized by germinating them on coverslips at restrictive and permissive temperature followed by staining with Calcofluor and DAPI. Mutants that failed to accumulate multiple nuclei were not considered further. Eleven putative ts septation mutants were chosen for further analysis.

Putative septation mutants were crossed to strain AH12, and six mutants showed monogenic segregation for the ts growth phenotype and cosegregation of the ts growth phenotype with the septation defect (Table 3). All six mutations were recessive in heterozygous diploids. Complementation analysis was performed among all of the *sep* mutants isolated in this study as well as with strains carrying the *sepA*, *B*, *C* and *D* mutations, with one exception. We were unable to perform complementation analysis between the mutant isolate ts2-105 and various *sepA* strains due to the repeated failure to isolate a diploid. Table 3 shows that the six mutants defined at least five new complementation groups designated *sepE*, *G*, *H*, *I* and *J*. One mutant originally designated *sepF* was later excluded from this group since germlings containing this mutation arrested growth predominantly with four highly condensed nuclei. In all but one case, pair-wise crosses between strains carrying *sep* mutations recombined to yield wild-type progeny suggesting that none of these genes are closely linked. The exceptional case

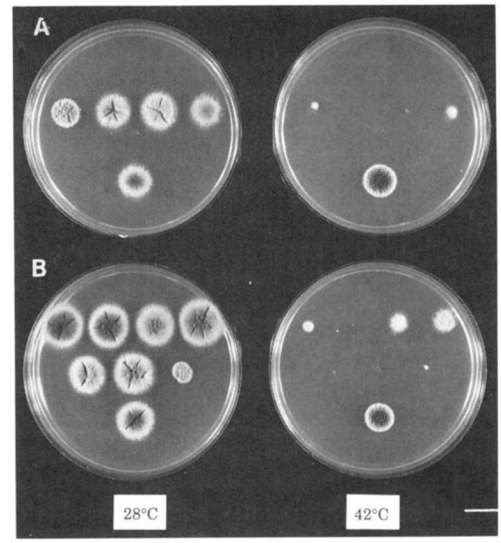

FIGURE 6.—Growth phenotypes of septation mutants of *A. nidulans*. Near isogenic strains were inoculated onto duplicate plates and grown for 48 hr at either 28° or 42°. A, top row (from left to right); ASH20 (*sepA*), ASH15 (*sepB*), AJM19 (*sepC*), and AJM2 (*sepD*), bottom row; A28 (wild type). B, first row (from left to right); ATS55 (*sepE*), ATS32 (*sepF*), ATS22 (*sepG*), and ATS44 (*sepH*), second row (from left to right); ATS19 (*sepI*), ATS18 (*sepJ*), and ATS50 (*sepA3*), third row; A28 (wild type). The differential shading of the colonies is due to the presence of different conidial color markers in the strain backgrounds. Bar, 5 mm.

was the cross between the mutant isolate ts2-105 and ASH34 (*sepA2*) that yielded no wild-type progeny. This indicates that the mutation in ts2-105 is tightly linked to or defines an additional allele of *sepA*. Failure to form diploids and phenotypic analysis suggests that ts2-105 is an additional allele of *sepA*, therefore we have designated it *sepA3*. Parasexual genetic analysis (PONTECORVO and KAFER 1958) with a mitotic mapping strain was used to assign some of the *sep* mutants to linkage groups (Table 3).

Phenotypic characterization of *sep* mutants: Previous studies did not extensively characterize the phenotypes of the *sepA–D* mutants (MORRIS 1976; TRINCI and MORRIS 1979) and therefore we have included these mutants in this study. All phenotypic analyses were performed on outcrossed segregants that were either prototrophic or contained a single auxotrophic mutation that could be easily supplemented. The growth phenotypes of the *sep* mutants at permissive and restrictive temperatures are shown in Figure 6. At the permissive temperature, each allele of *sepA* that we have tested exhibits a colonial morphology, whereby their radial growth rate is reduced. Otherwise, the remainder of the *sep* mutants are indistinguishable from the parent strain A28 at permissive temperature. At the restrictive temperature, the majority of the *sep* mutants produce a tight growth arrested phenotype. The *sepD*, *sepG* and *sepH* mutants grew to some extent, but failed to conidiate, at 42°.

We further characterized the *sep* mutants in three

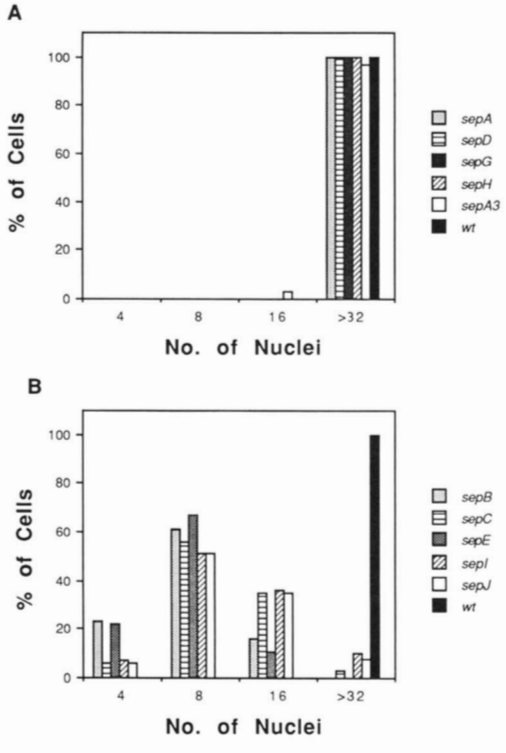

FIGURE 7.—Nuclear counts in septation mutants. Conidia from near isogenic strains containing various *sep* mutations were germinated on cover slips for 16 hr in YEG at 42°, fixed and stained with Hoechst 33258 and Calcofluor. The strains used were as follows; ASH5 (*sepA*), ASH13 (*sepB*), AJM19 (*sepC*), AJM4 (*sepD*), ATS55 (*sepE*), ATS22 (*sepG*), ATS44 (*sepH*), ATS19 (*sepI*), ATS18 (*sepJ*), ATS50 (*sepA3*) and A28 (wild type). By this analysis, *sep* mutants sort into two classes. One class (A) form as many nuclei as wild-type strains. A second class arrest nuclear division around the eight nuclei stage (B), suggesting a block to further progression of nuclear division.

ways. First, we performed nuclear counts following germination at restrictive temperature (Figure 7). The mutations sort into two groups. The *sepA*, *D*, *G* and *H* mutations block septum formation and strains containing these mutations produce as many nuclei as a wild-type strain when grown at the restrictive temperature (Figure 7A). A second group, consisting of the *sepB*, *C*, *E*, *I* and *J* mutations, possess hyphae that are devoid of septa and in addition contain reduced numbers of nuclei (Figure 7B). The majority of these mutants arrest nuclear division around the time of septum formation (8 nuclei stage). This result suggests that these mutations may block septum formation by inhibiting the progression of the third nuclear division cycle.

Previous studies demonstrated that the *sepA* mutant could form septa at appropriate positions throughout the fungal mycelium within 100 min following a shift from the restrictive to the permissive temperature (TRINCI and MORRIS 1979). To see which of our mutants behaved in a similar manner, strains were germinated overnight at the restrictive temperature and then shifted to the permissive temperature. At intervals of 30, 60 and 90 min, coverslips were removed, fixed and stained with Hoechst 33258 and Calcofluor. Figure 8 shows that *sepA* mutant cells were capable of forming septa following a shift down. In addition, a fraction of the cells containing the *sepD*, *G* and *H* mutations were also capable of forming septa following a shift to permissive growth conditions. In contrast, the remaining *sep* mutants failed to form septa following a shift down [we have observed that *sepB* mutant cells can form septa 8 hr following a shift down (data not shown)]. Thus, all strains that produced abundant numbers of nuclei were able to form septa following a shift to permissive temperature. Conversely, all strains that showed reduced numbers of nuclei failed to form septa following a shift to permissive temperature.

Finally, we examined the nuclear and hyphal morphology of strains carrying various *sep* mutations following a 16-hr incubation at restrictive temperature. The *sepA* mutation has obvious effects on hyphal morphology (Figure 9, A and F). This mutant produces abnormally wide hyphae with excessive dichotomous branching, suggesting that it possesses defects in cell wall growth and/or the maintenance of hyphal tip polarity. Nuclear morphology in this mutant, as well as in the *sepH* mutant, appeared similar to wild type (Figure 9, B, F and G). In contrast, the *sepB* and *sepJ* mutations, as well as other mutations that resulted in reduced numbers of nuclei during germination at the restrictive temperature (*sepC*, *E* and *I*), produced nuclei exhibiting highly aberrant morphologies (Figure 9, C, D and E). These nuclei generally appeared multi-lobed, elongated and fragmented. Genetic analysis of the *sepB* and *sepJ* mutants has demonstrated that the aberrant nuclear morphology cosegregates with temperature-sensitive growth and with the absence of septa. Taken together, our results suggest that this group of mutants fail to form septa because of an inability to successfully complete specific events during the third mitotic division.

Based on these phenotypic analyses, we have classified the nine *sep* mutants into three distinct phenotypic classes that are summarized in Figure 10. Class I mutants are represented by mutations in the *sepB*, *C*, *E*, *I* and *J* genes. These mutations appear to block septum formation by arresting at mitosis during the third round of nuclear division in germlings. Consequently, when germinated at the restrictive temperature, the number of nuclei in these germlings ranges from 4–16 with a median number of 8. Presumably, the aberrant nuclear morphologies arise as a result of the block to nuclear division. Class II and III mutations block septation and appear to have no effect on nuclear division as judged by light microscopy. Mutations in the *sepA* gene also cause altered hyphal morphology. Mutations in the remaining group of

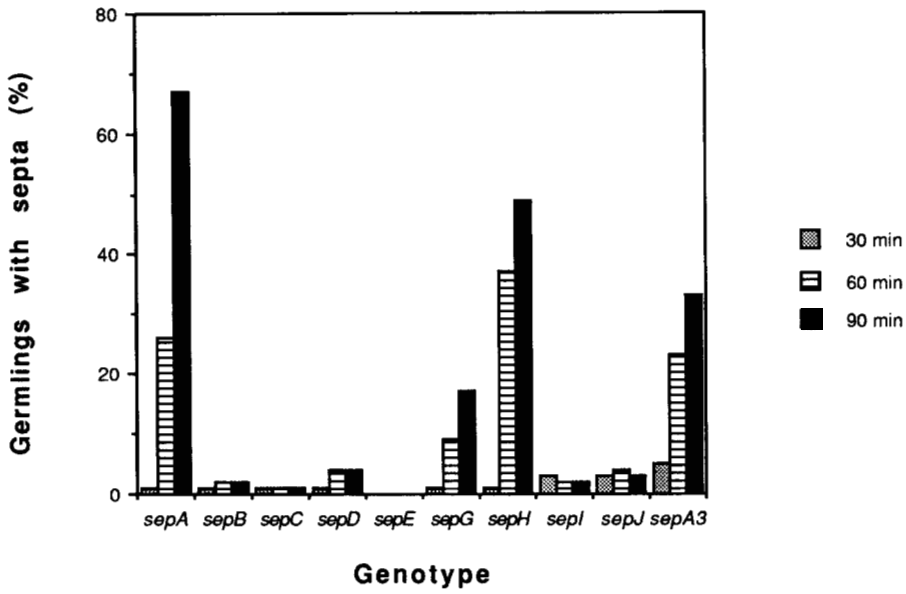

FIGURE 8.—Temperature shift analysis of septation mutants. Conidia from near isogenic strains containing various *sep* mutations were germinated on cover slips for 16 hr in YEG at 42° and then shifted to permissive temperature as described in the MATERIALS AND METHODS. Germlings were fixed and stained with Calcofluor and DAPI or Hoechst 33258. See Figure 7 for description of the strains used in these experiments. Septation mutants sort into two classes. *sepA, D, G* and *H* mutants are capable of forming septa during a 90-min interval following a shift to permissive growth conditions. *sepB, C, E, I* and *J* mutants are incapable of forming septa under these conditions.

genes show no effect on hyphal morphology or mitosis but nevertheless block septation.

DISCUSSION

The process of cytokinesis results in the partitioning of cells as a consequence of growth. Additionally, cytokinesis allows for the asymmetric segregation of developmental and/or metabolic information (HORVITZ and HERSKOWITZ 1992). Although the cells of *A. nidulans* are multinucleated, cytokinesis in this organism shares many features with other organisms. First, cytokinesis in *A. nidulans* is temporally coordinated with the nuclear division cycle. Formation of the first septum in germlings requires the completion of three rounds of nuclear division. Thereafter, each mitosis is closely followed by a round of septum formation (CLUTTERBUCK 1970). Second, cell division occurs at a specific site. In *A. nidulans* the first septum forms at the basal end of the germ tube (Figure 1) and in growing mycelia, cellular compartments separated by septa are uniform in size (FIDDY and TRINCI 1976). Finally, we have shown that septum formation in *A. nidulans* is an actin-dependent process. As a multicellular organism *A. nidulans* forms a wide variety of cell types (CHAMPE and SIMON 1992) and thus the opportunity exists to examine the coordination of cytokinesis with a variety of morphological and developmental events. To this end, we report the preliminary phenotypic characterization of the *sep* mutants. At the restrictive temperature, these ts mutants form multinucleated cells that fail to make septa.

Temporal relationship between the nuclear division cycle and septation: The uninucleate G_1-arrested conidia of *A. nidulans* undergo their first nuclear division as the spore swells and breaks dormancy. The second nuclear division is generally concomitant with germ tube emergence, such that most cells with four nuclei have established a polarized growth axis and possess a germ tube. We have found that the first septum is located at the basal end of the germ tube and is not made until the third nuclear division has been completed. The reasons *A. nidulans* delays septation until this point are not known. We have observed that this is the first mitosis to occur once the cells possess a germ tube into which nuclei have migrated. Forming septa at any point prior to this could enhance the probability of producing anucleate compartments. Moreover, subsequent to this point, each cellular compartment in a hyphal element behaves in an essentially autonomous manner. Mitosis is synchronous only within compartments (ROSENBERGER and KESSEL 1967; FIDDY and TRINCI 1976). Additionally, intercalary compartments appear to reorient their growth axes independently and ultimately enter the asexual developmental pathway at different times (FIDDY and TRINCI 1976; S. HARRIS and J. HAMER, unpublished data). Thus, the formation of the first septum is a critical juncture in the vegetative stage of the life cycle of *A. nidulans* as it marks the transition from a unicellular state to multicellular growth. We speculate that germinating conidia possess a regulatory mechanism to ensure that the first septum is not made until sufficient numbers of well spaced nuclei are present. Such a mechanism must also govern the position of the first septum in order to enhance the likelihood that there will be nuclei on either side of it.

We have considered three possible mechanisms by which the formation of the first septum could be regulated. A gene product that is essential for septum formation may not be synthesized until late in the process of conidial germination. Alternatively, assembly of the first septum may be delayed until the germling has attained a critical size or volume. Finally,

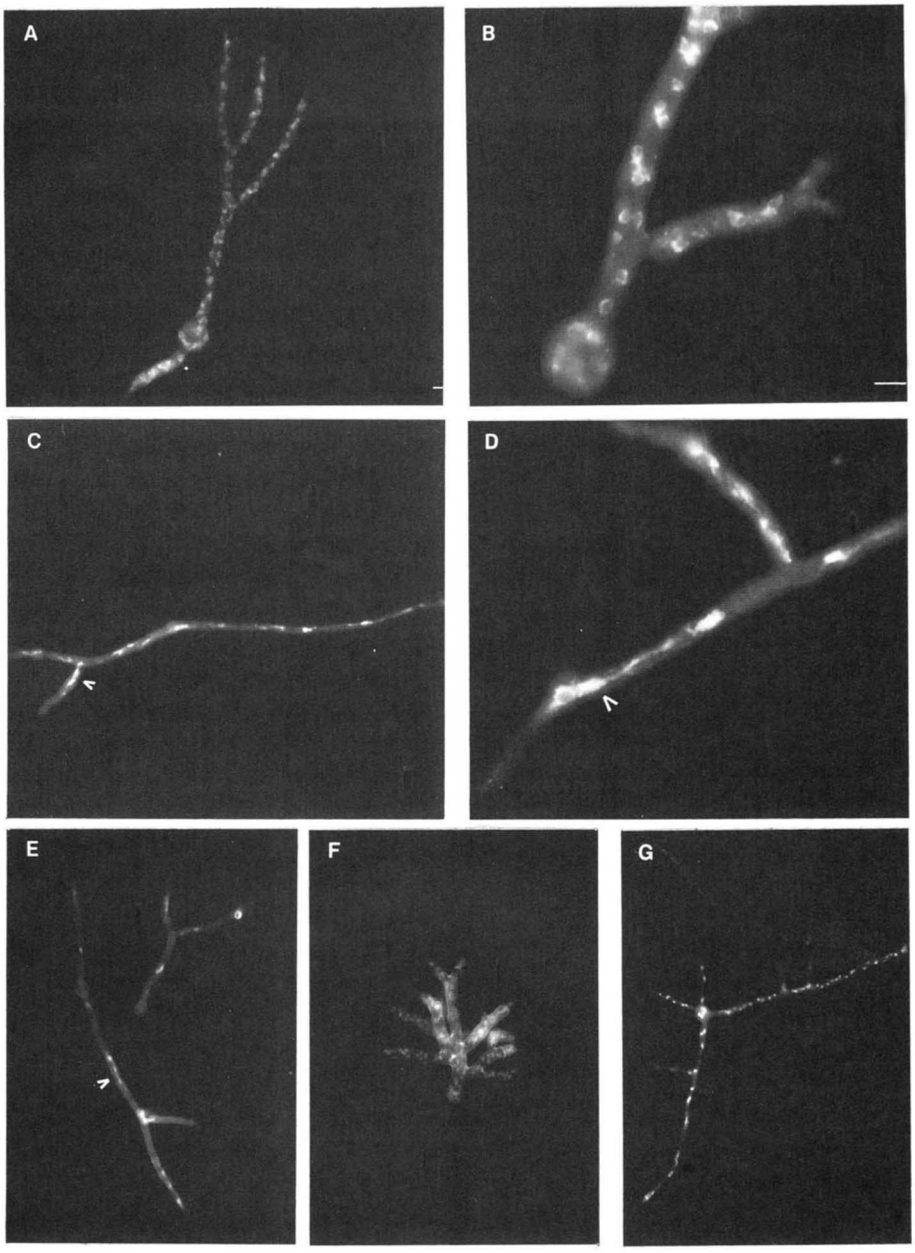

FIGURE 9.—Cell and nuclear morphology of septation mutants of *A. nidulans*. Conidia from near isogenic strains containing various *sep* mutations were germinated on coverslips for 16 hr in YEG at 42°. Germlings were fixed and stained with Calcofluor and Hoechst 33258. A and B, ASH34 (*sepA*); C and D, ASH15 (*sepB*); E, ATS18 (*sepJ*); F, ATS50 (*sepA3*) and G, ATS44 (*sepH*). The arrowheads indicate nuclei exhibiting aberrant morphologies. Bars, 2 μm.

formation of the first septum may be dependent upon completion of an event(s) intimately associated with the third nuclear division. The first possibility seems unlikely, since physiological studies of fungal spore germination have shown that levels of RNA and protein synthesis required for general growth and metabolism have been reached by the time of germ tube emergence (for review see LOVETT 1976). We were able to distinguish between the latter two possibilities by performing a series of block-and-release experiments using the DNA synthesis inhibitor hydroxyurea. Cellular growth, as indicated by measurements of RNA synthesis (BERGEN and MORRIS 1983) and cell size, continues unabated in the presence of HU. However, when germlings containing four nuclei were blocked during the third nuclear division by incubation in the presence of HU, they failed to form septa. Furthermore, release from the HU-imposed block resulted in the resumption of nuclear division and rapid formation of septa in almost all germlings examined. Thus, although attainment of a critical cell size or volume may be necessary for formation of the first septum, it is clearly not sufficient, as germlings must complete the third round of nuclear division before they can make a septum. These results are consistent with models for cell division in other organisms that require signaling between the mitotic apparatus and the cell cortex to initiate cytokinesis (SATTERWHITE and POLLARD 1992).

Septation is an actin-dependent process: Actin-containing structures in filamentous fungi generally stain poorly with fluorochrome-conjugated phalloidins, as high background fluorescence and nonspecific staining are observed (BARJA *et al.* 1991; ROB-

Class I	• *sepB, C, E, I* and *J* • no septa are formed following a shift down • aberrant nuclear morphology
Class II	• *sepA* • septa form following a shift down • associated morphological defects
Class III	• *sepD, G* and *H* • septa form following a shift down • no nuclear or morphological defects

FIGURE 10.—Phenotypic classification of *A. nidulans sep* mutants. See text for details.

ERSON 1992; THOMPSON-COFFE and ZICKLER 1993). Although the sole *A. nidulans* actin gene exhibits some sequence divergence when compared with other non-muscle actin isotypes, it does contain consensus sequences at the two regions believed to be involved in binding phalloidin (VANDEKERCKHOVE *et al.* 1985; FIDEL *et al.* 1988; MEAGHER 1991). Since *A. nidulans* actin does not stain with phalloidin, it is possible that these specific residues are obscured through interaction with an actin-associated protein.

We have used the monoclonal anti-actin antibody N.350 to stain actin-containing structures in *A. nidulans*. This antibody has been used to localize actin in filamentous fungi by both immunofluorescence and immuno-electron microscopy (BOURETT and HOWARD 1992; ROBERSON 1992; THOMPSON-COFFE and ZICKLER 1993). We observed actin at three locations in germinating conidia. Actin was prominently localized at the tip of the extending germ tube and at cortical spots distributed basally. These spots likely correspond to structures termed filasomes, which have been shown to contain actin by immuno-electron microscopy (BOURETT and HOWARD 1992; ROBERSON 1992). In addition, we observed actin localization at the septum, ranging from an accumulation of spots to a dense band. This localization at the septum appears to be transient, as the majority of septa did not stain with the N.350 antibody. Conversely, we occasionally observed actin localization at the presumptive septum (based on its location within the germ tube) in the absence of a Calcofluor-stained structure. Thus, the appearance of actin at the presumptive septum may serve as an early marker for septation relative to the appearance of Calcofluor staining. These results are consistent with reports of the transient appearance of actin at the site of septum formation in other fungi (KILMARTIN and ADAMS 1984; MARKS and HYAMS 1985; RUNEBERG *et al.* 1986). In contrast to yeast cells, actin staining in *A. nidulans* appeared simultaneously at the site of septum formation and at the apical regions. Further study of how actin and its interactions with various actin-associated proteins mediate events involved in septation and tip growth in *A. nidulans* should reveal how the tip cell can simultaneously maintain actin-containing structures at both its basal and apical regions.

We further assessed the role of actin in septum assembly by performing block-and-release experiments using the microfilament depolymerizing agent CA. The observation that germlings made one or more septa within the first cell cycle upon release from the CA block suggests that early events in the process of septum assembly had already occurred. Moreover, since septa were made in the appropriate spatial arrangement within regions of the germling that had been exposed to CA, the sites for septum assembly must have already been determined. This suggests that filamentous actin may not be required for the selection of the appropriate site for septum assembly and for the initial steps in the process. It remains possible, however, that the actin spots associated with filasomes (BOURETT and HOWARD 1992) may play a role in determining the site for septum assembly. Filamentous actin does appear to be necessary for events that occur later in the process of septum assembly. This may include functions such as the delivery of the precursors required for localized cell wall biosynthesis.

Phenotypic analysis of septation mutants: It has been known for some time that cytokinesis requires the transmission of signals between the mitotic apparatus and the cortex (reviewed in SATTERWHITE and POLLARD 1992). Similar types of processes are likely to be involved in the spatial and temporal regulation of septum formation in *A. nidulans*. Presumably, this is a complex process that may involve many as yet unidentified gene products. Although biochemical and cytological approaches have been successfully employed to identify some of the proteins that mediate the formation of contractile rings, these approaches are limited by the abundance and stability of the particular protein. Thus, we employed a genetic approach to examine the mechanisms underlying the regulation of septum formation in *A. nidulans*.

Cytokinesis mutants in *A. nidulans* are easily detected as cells with multiple nuclei, long germ tubes and no septa. Four *sep* mutants were identified in a previous search for ts cell cycle mutants of *A. nidulans* (MORRIS 1976). We generated a second collection of ts mutants and identified five additional *sep* mutants using a simple Calcofluor plate screen. Using a variety of approaches we have sorted the nine *sep* mutants into three distinct phenotypic classes. The finding that all but one of the *sep* mutants (*sepA*, see RESULTS, and

MORRIS 1976) are represented by single alleles suggests that many more *sep* genes await identification. Their identification may reveal the presence of additional phenotypic classes of *sep* mutants.

Class I mutants (*sepB, C, E, I* and *J*) fail to form septa at the restrictive temperature. Unlike the remaining *sep* mutants, these mutants arrest with a median number of eight nuclei suggesting that a block to further progression of the nuclear division cycle has been imposed. This phenotype is strikingly different from other nuclear division mutants of *A. nidulans* (*nim* and *bim* mutants) that arrest with a single nucleus (OSMANI *et al.* 1987; DOONAN and MORRIS 1989; O'DONNELL *et al.* 1991; MORRIS and ENOS 1992; MAY *et al.* 1992). In class I mutants, nuclei are frequently misshapen, and appear elongated and multilobed by both DAPI and Hoechst staining. This nuclear morphology is somewhat reminiscent of that in mutants which produce an increased frequency of aneuploid nuclei (UPSHALL and MORTIMORE 1984). Although class I mutants could represent leaky mitotic mutations, several observations are inconsistent with this hypothesis. First, the ts growth phenotype of the mutants suggests that the mutations are not leaky (Figure 6). Second, leaky mitotic mutants would be expected to arrest nuclear division in a random manner. Class I mutants arrest with a similar nuclear morphology and a median nuclear number of eight. All of these phenotypes are consistent with the idea that class I genes encode products that are required to ensure the continued progression of the nuclear division cycle past the third mitotic division. Our physiological experiments demonstrate that passage through this particular mitotic division is required to trigger septum formation.

The observation that class I mutants fail to form septa within 90 min [the approximate time required for a single cell cycle in *A. nidulans* (BERGEN and MORRIS 1983)] following a shift to the permissive temperature suggests that these mutants are blocked at an early step in the commitment to septum formation. We speculate that the class I mutants may reveal an early regulatory step or checkpoint in the signaling pathway between the nucleus and the presumptive septation site at the cortex. In this scenario a failure to initiate septation triggers a mitotic arrest in the multinucleate germling. The dependency relationship between septum formation and the third mitotic division suggests that this particular phenotypic class of mutants could exist.

Interestingly, this class of septation mutant would be difficult to detect in a uninucleate organism where this particular arrest phenotype would resemble a typical nuclear division defect. Some yeast genes, however, have been implicated in coordinating mitosis and cytokinesis. The *cdc14* gene of *S. pombe* has been proposed to play a role in the coordination between mitosis and cytokinesis (FANKHAUSER and SIMANIS 1993). However, null and ts alleles of this gene have no effect on mitosis, and the mitotic defect is only observed following overexpression of the *cdc14* gene product for 18 hr. Another *S. pombe* septation gene, *cdc16*, appears to encode a negative regulator of septation because *cdc16* mutants display a multi-sep phenotype (MINET *et al.* 1979). Recently, the *cdc16* gene product has been shown to be required for maintenance of $p34^{cdc2}$ kinase activity in mitosis (FANKHAUSER *et al.* 1993). Loss of this regulatory activity may accelerate the mitotic cycle and lead to premature septation. We are currently investigating the exact nature of the mitotic block in the *A. nidulans* class I mutants.

At the nonpermissive temperature, germlings of class II (*sepA*) and III (*sepD, G* and *H*) mutants arrest growth with large numbers of nuclei and fail to form septa. Members of both mutant classes are capable of forming septa within 90 min following a shift from restrictive to permissive temperature. These results suggest that class II and class III gene products act later in the process of septum assembly. Interestingly, the single class II mutant produces hyphal cells that are approximately twice the width of wild-type cells and display apparent defects in polarity. Given the pleiotropic nature of the class II mutant phenotype, we suspect that the product of this gene is involved in processes required for localized cell wall biosynthesis.

At the restrictive temperature, class III mutants arrest as small branching colonies that fail to produce spore-bearing structures (conidiophores). These mutants display a typical hyphal morphology, and furthermore, the appearance and distribution of nuclei are normal. These genes could play more specific roles in septation such as forming specific parts of the septum (septal pore or Woronin bodies) or encoding enzymes necessary for the synthesis of specific septal wall components. Although these mutants grow slowly at 42°, they fail to conidiate, suggesting that septation is necessary for the initiation of asexual development. Various methods are available for inducing the asexual morphogenesis pathway (TIMBERLAKE 1990) and future work will address the specific requirements for *sep* genes during conidiation.

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