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S-Phase, G2, and Nuclear Division Mutants of *Aspergillus nidulans*

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Twenty-two temperature-sensitive cell cycle mutants of the fungus *Aspergillus nidulans*, which block in interphase at restrictive temperature, were analyzed by the reciprocal shift method of Jarvik and Botstein (Proc. Natl. Acad. Sci. U.S.A. 70:2046–2050, 1973) and Hereford and Hartwell (J. Mol. Biol. 84:445–461, 1974) to determine whether these mutations were blocked at the G1, S, or G2 phase of the cell cycle. We found five mutants to be blocked in S and nine to be blocked in G2. Two of the G2 mutants were atypical in that they were not able to accomplish the G2 to M transition at restrictive temperature but nevertheless could initiate subsequent cycles of DNA replication. None was blocked in G1. There were nine strains that could not be classified. The block imposed by restrictive temperature was irreversible in three of these strains, and the six other strains were unclassifiable due to their aberrant terminal nuclear phenotypes.

A large number of temperature-sensitive (*ts*) mitotic mutants and strains with mutations in tubulin have been identified and characterized in the fungus *Aspergillus nidulans* (6, 7, 9, 11–13, 16, 17). The *ts* mitotic mutants fall into four phenotypically different classes designated by the gene symbols *nim*, *bim*, *sep*, and *nud*, to indicate mutations that block interphase (*nim*), mitosis (*bim*), septation (*sep*), and nuclear movement (*nud*) (6). From complementation analysis it is known that each class contains several different genes. The interphase mutants, the largest group, consist of at least 23 complementation groups (6; Upshall and Mortimore, Genetics, in press). In this paper, we continue the phenotypic classification of the interphase mutants by using the reciprocal shift method of Jarvik and Botstein (5) and Hereford and Hartwell (4) to determine whether these mutations block in G1, S, or G2.

Twenty-two interphase mutants of *A. nidulans* were analyzed by the reciprocal shift method to test whether the *ts* mutational block in each of the mutants preceded, was coincident with, or followed an S-phase block imposed by the DNA synthesis inhibitor hydroxyurea (HU). The particular experimental design used in this study is similar to that used by Hartwell (3) to characterize interphase mutants of *Saccharomyces cerevisiae* relative to DNA synthesis. One requirement of the reciprocal shift method is a reliable assay for measuring the completion of the pathway of interest. Hartwell (2) used cell division as his endpoint for completion of the cell cycle. In *A. nidulans* cell division does not necessarily occur at each cell cycle and is not a useful marker (15); therefore, we have used nuclear division to indicate completion of the cell cycle. More specifically, since the asexual spores (conidia) of *A. nidulans* are uninucleate and before germination are arrested in G1 (1), we have used the appearance of binucleate germlings to indicate completion of the first cell cycle after germination.

Of the 22 *ts* mutants analyzed, 5 strains were found to block in S phase and 9 strains to block in G2. Two of the nine strains blocked in G2 were atypical (see below). None was blocked in G1. The block imposed by restrictive temperature was irreversible in three strains, and six strains were not able to be classified because they had aberrant nuclear morphologies at the end of one or both reciprocal experiments.

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MATERIALS AND METHODS

Strains. Two groups of strains of *A. nidulans* were used. The first group consisted of the *ts nim* (never in mitosis) mutants isolated by Morris (6). The *nim* strains were originally identified by the absence of mitotic figures when cultures were shifted from permissive to restrictive temperature. The second group consisted of mutants that are *ts* at 42°C but that upon growth at 37°C generate aneuploid colonies (Upshall and Mortimore, in press; Mortimore and Upshall, in preparation). These mutations have been designated *hfaB3* and *hfaF1* (high frequency of aneuploids) and *sod*^{V1}*B1* (stability of disomy). In addition to the generation of aneuploid colonies, these strains also have low mitotic indices at 42°C and fulfill the criteria established for *nim* strains by Morris (6, 7). In all experiments, FGSC 187 (*pabaA1*, *yA2*; Fungal Genetic Stock Center, Arcata, Calif.) was used as the non-*ts* control.

Experimental methods. Asexual spores were germinated in YG medium (0.5% yeast extract, 2% glucose, plus 0.2% Tween 80 to help synchronize germination). In a typical experiment, 12 ml of medium in a 25-ml flask was inoculated with 4×10^6 spores per ml and incubated in a constant-temperature water bath with vigorous agitation. Permissive and restrictive temperatures were 32 and 44°C, respectively. Samples were fixed in an equal volume of 2% glutaraldehyde for 5 to 10 min, washed, and suspended in water. Nuclei were stained with the DNA-specific fluorescent dyes DAPI (2,4-diamidino-2-phenylindole) (1) or mithramycin (8). These dyes and HU were purchased from the Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Asexual spores were germinated by incubation in complete medium either at permissive temperature in the presence of an inhibitory concentration of HU or at restrictive temperature in the absence of HU for a period of time sufficient to allow most of the spores to germinate and accumulate at the respective HU and *ts* block points. The spores germinated at permissive temperature in HU were then shifted to restrictive temperature and removed from HU. Reciprocally, the spores germinated at restrictive temperature in the absence of HU were shifted to permissive temperature and HU was added to the medium. By analyz-

TABLE 1. Predicted results for reciprocal block experiments on cell cycle mutants

Cell phase	Completion of mitosis during the second incubation[a]	
	Expt 1	Expt 2
G1	−	+
S	−	−
G2	+	−

[a] In experiment 1, spores were germinated at restrictive temperature. The germlings were then shifted to permissive temperature in the presence of the DNA synthesis inhibitor HU. These cells can complete the cell cycle and become binucleate only if S phase was completed at restrictive temperature, that is, if the cell cycle block were after the end of S. For experiment 2, spores were germinated at permissive temperature but in the presence of HU and then washed free of the drug while being shifted to restrictive temperature. Completion of the cell cycle in this experiment could only happen if the genetic block was before S phase. Failure to complete the cell cycle in either experiment shows that the genetic block and the drug-induced block are interrelated; they both inhibit S phase.

ing the effects of these reciprocal shifts on the ability of germlings to undergo nuclear division (and become binucleate), we were able to determine the relationship between the block points of the mutations in relation to S phase in 14 of the 22 mutants analyzed. Note that the experimental design makes the implicit assumption that the mutants block at specific points in the cell cycle under restrictive conditions. This assumption turned out to be unwarranted in the case of two of the *ts* mutants (see below).

There are theoretically four possible results to this experiment (Table 1). If the first set of conditions, restrictive temperature followed by HU, and only this set of conditions blocks completion of the cycle, then the *ts* block must be exerted before S phase, i.e., in G1. If both the first and second set of conditions, restrictive temperature followed by HU and HU followed by restrictive temperature, block completion of the cell cycle, then the *ts* block is located in S phase. If the second set of conditions, HU followed by restrictive temperature, and only this set of conditions blocks completion of the cycle, then the *ts* block must be after S phase, i.e., in G2. Finally, if neither set of conditions blocks completion of the cell cycle, the *ts* mutation must be independent of S phase, i.e., on some other pathway, blockage of which inhibits nuclear division.

An important requirement of the reciprocal shift method is that both methods of blocking the cell cycle be rapid in onset, highly effective, and readily reversible. We have shown previously that HU inhibits DNA synthesis in *A. nidulans*, that the HU block is effective, and that it is rapidly induced and rapidly reversible within minutes after removal of HU from the medium (1). We have also shown that inhibition of DNA synthesis in *A. nidulans* by HU prevents the next nuclear division just as it does in a variety of other systems (see, for example, reference 3), and in this paper we again show, for each of the *ts* interphase mutants, that HU prevents nuclear division. Fewer than 3% of the germlings of any mutant underwent nuclear division during 7 h in the presence of HU (Table 2), whereas in the absence of HU, all strains completed one or more nuclear divisions. We also show for each of the mutants that the HU block is reversible. When HU was removed from the medium, each of the HU-blocked strains recovered and completed one or more nuclear divisions during the course of 3 h in the absence of the drug (Table 2). Similarly, we demonstrated for each mutant that restrictive temperature (44°C) causes a block before nuclear division and that in most cases the *ts* block is reversible, since when germlings were shifted from restrictive temperature to permissive temperature (32°C) for 3 h cells of most strains became multinucleate (Table 2). Three strains (*nimL*, *nimM*, and *nimN*) that failed to undergo nuclear division when shifted from 44 to 32°C are apparently irreversible *ts* lethals and were not amenable to analysis by the reciprocal shift method. Therefore, the position of the *ts* block in these strains could not be determined.

In the first of two reciprocal shift experiments (restrictive temperature followed by HU), spores from each of the *ts* mutants were germinated at restrictive temperature (44°C) for 7 h, at which time HU was added. After 10 min of incubation, the germlings were shifted to permissive temperature and then, after 2 to 3 h, fixed, stained, and scored for nuclear division. Under these conditions, seven mutants (*nimA1*, *nimA5*, *nimA7*, *nimB2*, *nimE6*, *nimT23*, and *nimU24*) exhibited substantial nuclear division (Fig. 1); two other mutants (*hfaB3* and *hfaF1*) exhibited some nuclear division; and five mutants (*sod*^{V1}*B1*, *nimC3*, *nimG10*, *nimK14*, and *nimQ20*) failed to undergo nuclear division (Fig. 2; Table 2). Five other mutants (*nimH*, *nimI*, *nimO*, *nimP*, and *nimS*) had grossly abnormal nuclear morphologies at restrictive temperature which made it impossible to classify them with any degree of confidence (Fig. 3). Strain *nimD* was not tightly blocked in interphase at restrictive temperature. These six strains (*nimD*, *nimH*, *nimI*, *nimO*, *nimP*, and *nimS*) therefore have not been classified.

For the five mutants that failed to undergo nuclear division

TABLE 2. Cell cycle phase at which *A. nidulans ts* mutants block[a]

Genotype	Frequency of cells completing one nuclear division						Cell phase
	Expt 1[b]			Expt 2[c]			
	A	B	C	D	E	F	
sod^{V1}*B1*	0	1	0.01	0.01	1	0.01	S
hfaB3	0	1	0.29	0	1	0.01	G2
hfaF1	0	1	0.20	0.01	1	0.08	G2
nimA1	0.03	1	0.79	0	1	0.05	G2
nimA5	0.04	1	0.71	0	1	0.05	G2
nimA7	0.07	1	0.77	0	1	0.05	G2
nimB2	0	1	0.59	0	1	0.01	G2
nimC3	0	1	0.05	0	1	0.02	S
nimE6	0.01	1	0.87	0.03	1	0.06	G2
nimG10	0	1	0	0.03	1	0.11	S
nimK14	0	1	0.07	0	1	0.06	S
nimQ20	0	1	0.06	0.03	1	0.05	S
nimT23	0.01	1	0.92	0	1	0.03	G2
nimU24	0.01	1	0.62	0.01	1	0.02	G2
Wild type	1	1	1	0	1	1	No block

[a] We tested 22 *ts* mutants of *A. nidulans* to determine at which cell cycle phases these mutants block. This was accomplished by the double reciprocal block method as described in Table 1 and the legends to Fig. 1 and 2. Of the 22 strains tested, 3 were found to be *ts* lethal (*nimL*, *nimM*, and *nimN*), since these strains failed to recover when transferred from restrictive to permissive temperatures. Due to unusual nuclear phenotypes when germinated at restrictive temperature, six strains (*nimD*, *nimH*, *nimI*, *nimO*, *nimP*, and *nimS*) cannot be categorized confidently. The strains presented in this table fall into the patterns predicted in Table 1 for strains that block in either S phase or G2. No strains were found that fit the predicted pattern of mutants that block in G1.

[b] A, Strains were kept at 44°C; B, strains were shifted from 44 to 32°C for 3 h; C, spores were germinated at 44°C for 7 h, incubated with HU for 10 min, and then shifted to 32°C with HU for 2 to 3 h.

[c] D, Strains were kept at 32°C in the presence of HU for 7 h; E, strains were incubated at 32°C with HU and then were incubated for 3 h at 32°C in the absence of HU; F, strains were incubated at 32°C with HU and then shifted to 44°C without HU.

in the first of the two reciprocal shift experiments (restrictive temperature followed by HU), theory predicts either that the *ts* mutation precedes DNA synthesis (S phase) or that the *ts* mutational block is in DNA synthesis per se. The second of the two experiments (HU followed by restrictive temperature) differentiates between these possibilities. The five mutants that failed to undergo nuclear division in the first experiment also failed to undergo nuclear division in the second experiment, demonstrating that the *ts* mutation caused a block in S phase in each of these mutants (Fig. 2; Table 2).

According to theory (Table 1), the seven mutants that exhibited substantial nuclear division (59 to 92%) in the first experiment (restrictive temperature followed by HU), but no appreciable nuclear division in the second experiment (HU followed by restrictive temperature) must be blocked after S phase, i.e., in G2. The same general conclusion can be drawn for *hfaB3* and *hfaF1*, except that the low frequency (20 to 30%) of nuclear division by these strains in experiment 1 (Table 2) suggests that they are in some way different from the initial group of seven mutants. These two strains also differed from the other mutants in that their nuclei continued to enlarge when they were kept under restrictive conditions, whereas the nuclei of the other strains did not enlarge (Fig. 4).

DISCUSSION

We have used the reciprocal shift method of Jarvik and Botstein (5) and Hereford and Hartwell (4) to classify *ts* interphase mutants of *A. nidulans*. Of the 22 mutants analyzed, 8 were nonclassifiable for technical reasons, including incomplete penetrance, irreversibility of the *ts* block, and aberrant nuclear morphology. Of the remaining 14 mutants, 5 were blocked in S and 9 were blocked in G2. Surprisingly, none of the interphase mutants was found to block in G1, even though G1 mutations have been identified in other organisms (2, 10, 14).

Of the nine strains that appeared to be blocked in G2, seven behaved in accordance with theoretical expectations. Most (60 to 90%) of the germlings of *nimA1*, *nimA5*, *nimA7*, *nimB2*, *nimE6*, *nimT23*, and *nimU24* were able to complete nuclear division when shifted from restrictive temperature to HU and were unable to complete nuclear division when presented with the reciprocal shift. According to the logic of

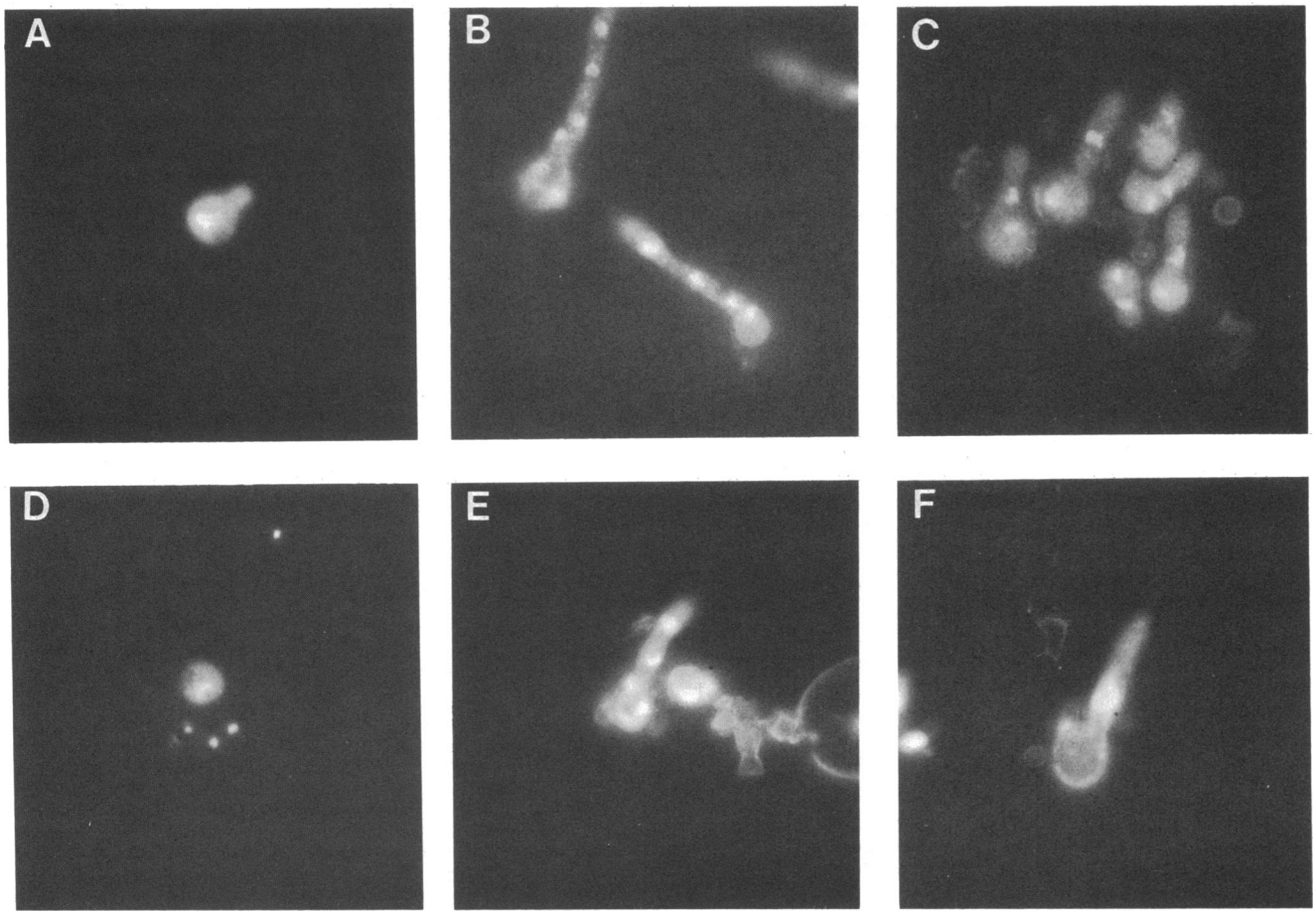

FIG. 1. DAPI fluorescence of double reciprocal block experiments of *nimT*. The results of the two reciprocal block experiments for *nimT* are presented by representative micrographs from the six samples. The samples were fixed and stained as described in the text. In the first experiment, spores were germinated for 7 h at 44°C (A) and then either shifted to permissive temperature (32°C) for 3 h (B) or equilibrated with 90 mM HU and then shifted to permissive temperature for 3 h (C). In the second experiment, spores were germinated at 32°C in the presence of 15 mM HU for 7 h (D), at which time the HU was washed out, and the culture was split in two and either returned to permissive (E) or shifted to restrictive (F) temperature for 3 h. The presence of binucleate cells in (C) and the lack of binucleate cells in (F) show that *nimT* blocks in G2 (compare results with Table 1). Magnification, ×2,050.

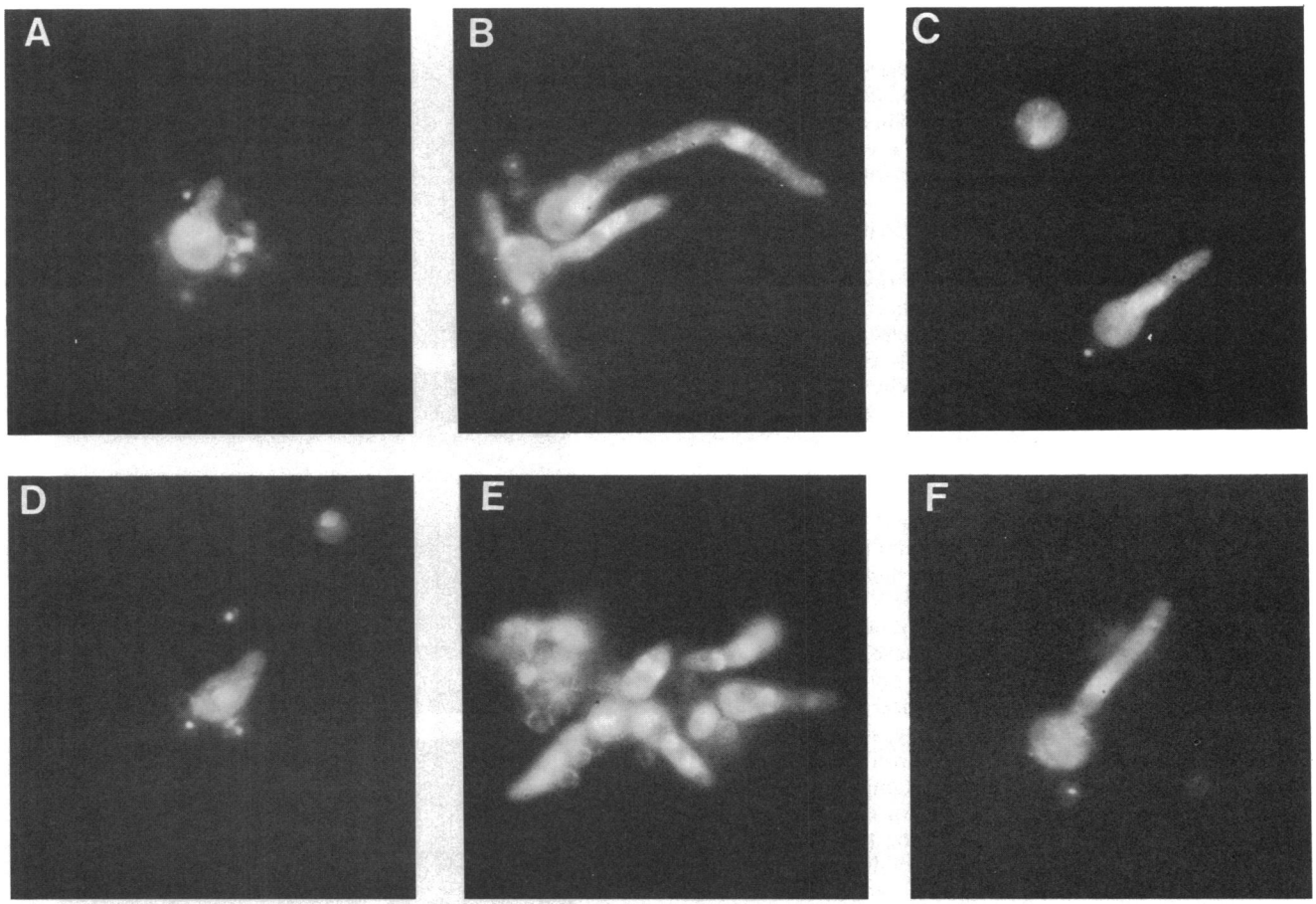

FIG. 2. DAPI fluorescence of double reciprocal block experiments of *nimC*. The six panels here represent the results of reciprocal block experiments with *nimC* and are comparable to the six frames of Fig. 1. Spores were germinated for 7 h at 44°C (A) and then shifted to 32°C without (B) or with (C) 90 mM HU. In the second experiment, spores were germinated at 32°C with 15 mM HU (D), the HU was removed, and the culture was placed either at 32°C (E) or up to 44°C (F). The lack of binucleate cells in (C) and (F) indicates that *nimC* blocks in S phase (Table 1). Magnification, ×2,050.

the reciprocal shift experiment, these strains are blocked in G2 at restrictive temperature. Three of these mutations are allelic (*nimA1*, *nimA5*, and *nimA7*), and, as might be expected, all three exhibited the same phenotype.

Two strains (*hfaB3* and *hfaF1*) behaved differently from the other strains blocked in G2 in that only 20 to 30% of the germlings completed nuclear division in the first experiment (restrictive temperature followed by HU) (Table 2). In theory (Table 1), most (if not all) of the nuclei of a G2 mutant should divide during the first reciprocal block experiment. What then accounts for the low frequency of nuclear division in the *hfa* mutants?

At least two explanations are possible based on technical factors. The *hfa* germlings might be more sensitive than wild-type germlings to sequential treatment with restrictive temperature and HU, or spore germination might be slower in *hfa* strains than in wild-type strains. The first of these explanations cannot be evaluated directly, although it is clear that *hfaB3* and *hfaF1* were no more sensitive to irreversible inactivation by restrictive temperature alone or by HU alone than any other strain. Therefore, we consider this explanation unlikely. The second possibility, slow germination, could lead to a larger fraction of germlings in S phase, at the time that HU was added, consequently reducing the number of germlings in G2 and increasing the number in G1 or S. If this was true then more germlings would be trapped by the HU block and fewer nuclear divisions would occur. The *hfa* strains, however, were not perceptibly slower to germinate than the wild type or any of the other strains; therefore, this explanation also seems unlikely.

A third and more plausible explanation for the low level of nuclear division by the *hfa* strains in the first reciprocal shift experiment is that at restrictive temperature the *hfa* mutations prevent nuclear division but do not cause cells to be terminally blocked in G2, i.e., the mutation does not block the initiation of the next cell cycle. The evidence that this may be the case is as follows. The *hfa* mutants differ from the other interphase mutants in nuclear morphology. When maintained at restrictive temperature for several generations the nuclei of these strains become extremely large (Fig. 4) and clearly contain much more DNA (by DAPI staining) than either wild-type strains or the other *ts* interphase mutants maintained under the same conditions (also see Upshall and Mortimer, in press). Thus, *hfaB3* and *hfaF1*, although unable to undergo nuclear division at restrictive temperature, appear to be able to start new rounds of DNA replication. Since G2 occupies about 30% of the *Aspergillus* cell cycle at 44°C (1), if initiation of new cell cycles is not blocked at restrictive temperature in these strains, one might expect about 30% of the germlings to be in G2 at any given

time in asynchronous cultures. Thus, when *hfa* germlings were shifted to HU, the 30% of the germlings in G2 would be able to complete nuclear division, whereas the 70% that were earlier in the cell cycle would be blocked by HU and be unable to undergo nuclear division. This agrees with our measurement of 20 to 30% nuclear division by the *hfa* mutants in the restrictive temperature-to-HU shift experiment.

DNA synthesis and nuclear division are usually mutually dependent events. Throughout nature, inhibition of DNA synthesis prevents subsequent nuclear division, and inhibition of nuclear division usually prevents subsequent nuclear DNA synthesis. More particularly, cells blocked in DNA synthesis fail to divide (1), and mutants blocked in nuclear division fail to make DNA (3, 10). Why then are the *hfa* mutants different from other cell cycle mutants in being able to continue DNA replication in the absence of nuclear division? The answer, in part, relates to the way that these mutants were identified. Most cell cycle mutants in other organisms have been identified by the fact that under restrictive conditions cells become blocked at a particular point in the cell cycle with a well-defined terminal phenotype. In contrast, the *hfa* mutants were identified by their ability to generate aneuploids. Their selection did not presuppose any cell cycle block point, and therefore these mutants need not necessarily be blocked at any specific point in the cycle. It should also be noted that the dependence of DNA synthesis on prior nuclear division is not nearly so absolute as the dependence of nuclear division on prior DNA synthesis. There are several well-documented examples of DNA synthesis without prior nuclear division, for example, polytene chromosomes. Thus, it is not surprising that mutants exist that are unable to undergo nuclear division but still can replicate DNA. Nevertheless, since the usual consequence of a block in nuclear division is failure to enter the subsequent cell cycle, the *hfaB3* and *hfaF1* mutations, which uncouple nuclear division from initiation of the next cell cycle, are of substantial interest.

Other G2 mutants also underwent less nuclear division in the first reciprocal shift experiment than the theoretically expected 100%. The same possibilities that explain the low percentage of nuclear division in *hfaB3* and *hfaF1* may also account for the deviation from ideal in these strains. These cells might be unusually sensitive to high temperature or HU or both, be slow to germinate, or be blocked in G2 but able to initiate new cell cycles with some small but finite probability.

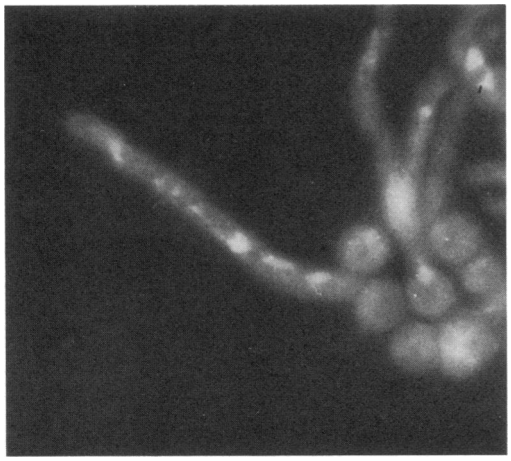

FIG. 3. Fragmented nuclei of *nimO* when germinated at restrictive temperature. Spores of *nimO* were germinated at 44°C for 9 h and fixed and stained as described in the text. Due to the fragmentation of the nuclei, we could not confidently score the reciprocal block experiments of this and similar strains. Magnification, ×2,050.

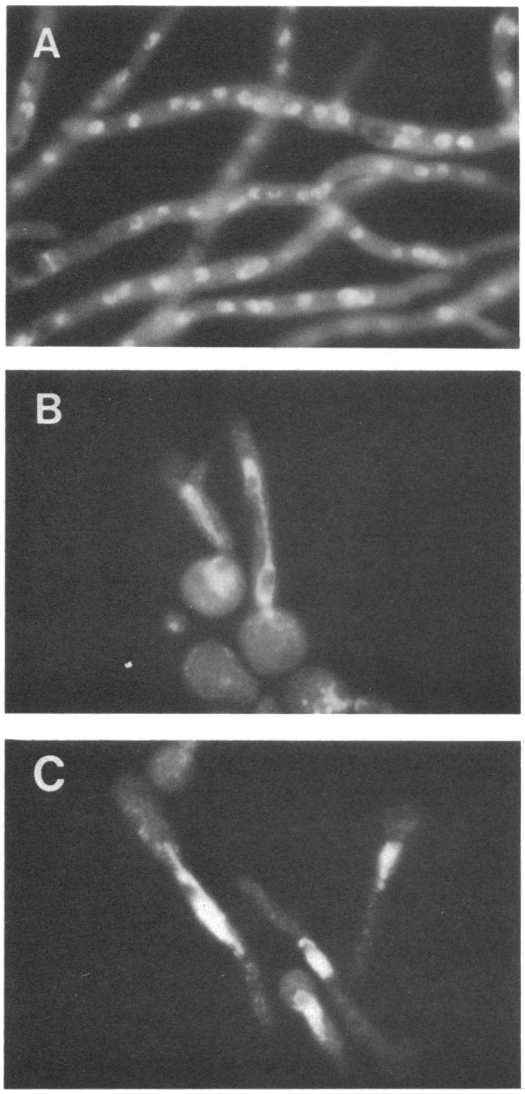

FIG. 4. Terminal phenotype of *hfa* mutants. Spores of FGSC 187 (non-*ts* parental strain), *hfaB3*, and *hfaF1* were germinated at 44°C for 9 h, fixed, and stained as described in the text. When compared with FGSC 187 (A), *hfaB3* (B) and *hfaF1* (C) contain a large, unusually shaped nucleus. The amount of fluorescence leads one to believe that further rounds of DNA synthesis can occur in the absence of karyokinesis in these strains. These mutations may define gene products that are required for mitosis. Magnification, ×2,050.

In five strains the *ts* lesion was interdependent with the HU block, indicating that these mutations inhibited S phase. The precise location of the S-phase lesions, whether in deoxyribonucleotide synthesis, initiation of S phase, elongation of DNA by DNA polymerase, ligation of DNA fragments, etc., cannot be determined from these experiments, which do not differentiate among the various components of DNA synthesis.

Three of the strains examined (*hfaB3*, *hfaF1*, and *sod*^{V1}*B1*) were originally isolated as mutants which generate aneuploid colonies when grown at 37°C (Upshall and Mortimore, in

press). We have shown that *sod*^{VI}*B1* blocks in S and that the *hfa* mutants prevent the G2 to M transition. The *sod*^{VI}*B1* mutation both selects and stabilizes cells with disomic nuclei. Since only cells with two homologs for chromosome VI are stabilized and the mutation itself is on chromosome VI, it is believed that the selection and stabilization is a consequence of gene dosage (Mortimore and Upshall, in preparation). In contrast, the *hfaB3* and *hfaF1* mutations generate an array of different aneuploid segregants when grown at sub-restrictive temperature (37°C). The mechanism(s) by which a mutation effective in G2 would influence chromosome segregation is discussed elsewhere (Upshall and Mortimore, in press), but lesions affecting the maturation or function of the kinetochore, spindle pole body or mitotic spindle maturation, or function or the hypothetical mitotic motor could both generate aneuploids at sub-restrictive temperature (37°C) and prevent mitosis at a higher, fully restrictive temperature.

One surprising result of this study was the lack of mutations that block in G1. Such G1 mutations have been identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (2, 10, 14), and the cell cycle of *A. nidulans* has a measurable G1 (1). Isolation of mutations that block in G1 in *A. nidulans* may require a different selection procedure than that previously used.

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