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nimO, an *Aspergillus* gene related to budding yeast *Dbf4*, is required for DNA synthesis and mitotic checkpoint control

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SUMMARY

The *nimO* predicted protein of *Aspergillus nidulans* is related structurally and functionally to Dbf4p, the regulatory subunit of Cdc7p kinase in budding yeast. nimOp and Dbf4p are most similar in their C-termini, which contain a PEST motif and a novel, short-looped Cys_2-His_2 zinc finger-like motif. DNA labelling and reciprocal shift assays using ts-lethal *nimO*18 mutants showed that *nimO* is required for initiation of DNA synthesis and for efficient progression through S phase. *nimO*18 mutants abrogated a cell cycle checkpoint linking S and M phases by segregating their unreplicated chromatin. This checkpoint defect did not interfere with other checkpoints monitoring spindle assembly and DNA damage (dimer lesions), but did prevent activation of a DNA replication checkpoint. The division of unreplicated chromatin was accelerated in cells lacking a component of the anaphase-promoting complex (*bimE*^{APC1}), consistent with the involvement of *nimO* and APC/C in separate checkpoint pathways. A *nimO* deletion conferred DNA synthesis and checkpoint defects similar to *nimO*18. Inducible *nimO* alleles lacking as many as 244 C-terminal amino acids supported hyphal growth, but not asexual development, when overexpressed in a ts-lethal *nimO*18 strain. However, the truncated alleles could not rescue a *nimO* deletion, indicating that the C terminus is essential and suggesting some type of interaction among *nimO* polypeptides.

Key words: *nimO*, *Dbf4*, *bimE*^{APC1}, *Aspergillus*, DNA replication, Cell cycle, Checkpoint

INTRODUCTION

DNA replication is precisely coordinated with other cell cycle events to occur once per cell cycle (reviewed by Nasmyth, 1996). The cell achieves strict control over S phase by mobilizing a complex of proteins which activate DNA synthesis at discrete chromosomal origins of replication. A six-protein origin recognition complex (ORC) binds to origins constitutively and serves as an assembly site for additional proteins, collectively termed the pre-replicative complex (pre-RC), that associate from late M through G_1/S and are required to initiate DNA synthesis. Among the additional pre-RC factors are the MCM family of proteins, Cdc45p and Cdc6p (see Aparicio et al., 1997).

Initiation of DNA replication occurs first by formation of the pre-RC which renders the origin replication-competent, and second by activation of the pre-RC so that origin sequences can be unwound and replication forks established (Diffley et al., 1994). The mechanism for origin activation apparently involves the modification and redistribution of MCM proteins and Cdc45p from the pre-RC which is mediated by two protein kinases. Clb5/6-Cdc28p kinase facilitates movement of the pre-RC by phosphorylating Mcm4p (Hendrickson et al., 1996). Cdc7-Dbf4p kinase appears to modify multiple MCM proteins (Hardy et al., 1997; Lei et al., 1997; Sato et al., 1997). Once phosphorylated, MCM proteins and Cdc45p may facilitate opening of the origins and aid in the propagation of the replication fork (Aparicio et al., 1997; Tanaka and Nasmyth, 1998).

Cdc7p is an essential enzyme present throughout the cell cycle but active only during G_1/S (Sclafani et al., 1988). Dbf4p is likewise essential (Kitada et al., 1992) but is periodically transcribed, with the peak of mRNA synthesis occurring at G_1/S (Chapman and Johnston, 1989). Activation of Cdc7p kinase occurs through phosphorylation, perhaps by Clb5/6-Cdc28p kinase (Yoon et al., 1993; Ohtoshi et al., 1997); and by association with Dbf4p at G_1/S (Jackson et al., 1993; Dixon and Campbell, 1997; Shellman et al., 1998). Dbf4p also interacts with replication origins, functioning as a molecular escort to deliver Cdc7p kinase to its substrates at the pre-RC (Dowell et al., 1994). Recent work has demonstrated that Cdc7-Dbf4p kinase is needed to fire both early- and late-replicating origins, and is thus required for initiation and for efficient progression of S phase (Bousset and Diffley, 1997; Donaldson et al., 1997).

In the budding and fission yeasts, mutations that arrest the cell cycle just before or at the onset of DNA synthesis lead to a mitotic catastrophe phenotype in which unreplicated chromosomes undergo division of chromatin, or reductional anaphase, before terminal arrest (Masai et al., 1995; Piatti et al., 1995; Toyn et al., 1995; Tavormina et al., 1997). In these mutants, which include *dbf4* and *cdc7*, anaphase progression apparently results from attachment of microtubules to the single kinetochore of an

unreplicated, mono-oriented chromosome. This override of S phase by G_1/S-arresting mutants has led to the idea that after START, activation of the mitotic checkpoint is triggered by the onset of DNA synthesis (Li and Deshaies, 1993) or that, as suggested by *dbf4* and *cdc7* mutations, commitment to mitosis may occur in late G_1 with mitotic restraint enforced by a distinct G_1/M-phase checkpoint that is known to operate in certain genetic backgrounds of yeast (Toyn et al., 1995).

Given the central involvement of *Dbf4* in DNA replication and mitotic checkpoint control, there is considerable interest in determining its precise function and determining if similar G_1/S control mechanisms exist in higher eukaryotes. Potential homologs of *Cdc7* were discovered in fission yeast, *Xenopus*, and human (Masai et al., 1995; Sato et al., 1997), but it is not known if the budding yeast mechanism for controlling Cdc7p activity is conserved. In this study we report a number of close structural and functional similarities between *Dbf4* and the *nimO* gene of *Aspergillus*.

MATERIALS AND METHODS

Plasmid construction

Standard techniques of molecular cloning were used (Ausubel et al., 1994). Fusions of the *nimO* coding region with the *A. nidulans* alcohol dehydrogenase promoter (*alcA::nimO*) were performed in pKK12, which carries the *argB* selectable marker for *Aspergillus* transformation (Kirk and Morris, 1993). A full-length *alcA*::*nimO*$^+$ fusion was made by introducing the 3.6 kb *Afl*II-*Eco*RV *nimO* genomic clone (blunted) into the *Sma*I site of pKK12 to create pSWJ136. *alcA*::*nimO* variants bearing C-terminal gene deletions were constructed after modifying the pKK12 vector to ensure that translation of the truncated alleles would terminate immediately at the end of each insert. A 25 bp adaptor containing a *Not*I site and TAA stop codons in each forward reading frame was cloned into the *Bam*HI site of pKK12 to create pSDW194. One full-length and six C-terminally deleted *nimO* cDNA fragments were blunt-cloned into the *Not*I site of pSDW194, using as the 5′ end an *Afl*II site which lies 29 bp upstream of the *nimO* start codon (Table 1).

The plasmid pSWJ220 used for *nimO* gene deletion was made by replacing all but the first 16 amino acids of the *nimO* coding region with a 2.3 kb *Sma*I-*Pv*uII fragment carrying the *Neurospora crassa pyr4* selectable marker. *pyr4* complements the *pyrG*89 mutation of *A. nidulans* (Waring et al., 1989). The plasmid contains 0.8 kb of 5′ genomic and 2.1 kb of 3′ genomic *nimO* flanking sequences to facilitate homologous integration at *nimO*.

Strains, media and genetic analyses

Standard methods of genetic analysis (Pontecorvo et al., 1953), *Aspergillus* culture (Kafer, 1977), and *Aspergillus* transformation (Ballance et al., 1983) were employed. *A. nidulans* strains used in this study are listed in Table 1. *nim* and *bim* strains were outcrossed a minimum of three times from the original mutants that were generated in FGSC154 and characterized by Morris (1976). All transformants used in this study were shown by Southern blotting to contain a single copy of the plasmid integrated at either the *argB* or *nimO* chromosomal locus. Strains carrying one copy of an *alcA*-driven *nimO* allele integrated at *argB* were from transformation of SWJ396. *alcA*::*nimO* expression was strongly induced in minimal medium (Kafer, 1977) containing 200 mM ethanol or ethanol + fructose (0.04%). Basal expression was obtained in 50 mM glycerol, and repression was achieved using 2% glucose.

Strains in which the only functional *nimO* gene was *alcA::nimO*$^+$ (ΔSWJ648, −652, and −653) were constructed by one-step gene replacement of the *nimO*18 locus. tBAK511 (*nimO*18; *alcA::nimO*$^+$at *argB*) was crossed with SWJ299 (*pyrG*89) to obtain tSWJ638 (*pyrG*89; *nimO*18; *alcA::nimO*$^+$ at *argB*). This strain grows normally on both glucose and ethanol at the permissive temperature of 30°C, but requires ethanol (or glycerol) to induce *alcA::nimO*$^+$ expression and thereby permit growth at restrictive temperature (43°C). Deletion of *nimO*18 in tSWJ638 was by transformation with pSWJ220

Table 1. *Aspergillus nidulans* strains

Strain	Genotype	Source
PCS439	*riboA*1; *yA*2	This study
SWJ299	*pyrG*89; *nicA*2; *wA*2	,,
SWJ601	*argB*2; *pantoB*100; *riboA*1	,,
SWJ238	*nimO*18; *riboA*1; *yA*2	,,
SWJ241	*nimO*18; *nicA*2	,,
SWJ619	*nimO*18; *nicA*2; *wA*2	,,
SWJ396	*nimO*18; *argB*2; *pabaA*1; *pyroA*4	,,
SWJ010	*bimE*7; *pabaA*1; *yA*2	James et al., 1995
SWJ243	*nimO*18; *bimE*7; *pabaA*1; *yA*2	This study
Strains carrying one *alcA* promoter-driven *nimO* allele integrated at the *argB* locus* (transformants of SWJ396):		
tBAK511‡	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSWJ136 (*argB*$^+$ *alcA::nimO*$^+$)	This study
tSWJ620§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW213 (*argB*$^+$ *alcA::nimO*$^+$)	,,
tSDW567§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW207 (*argB*$^+$ *alcA::nimO*Δaa538-647)	,,
tSDW556§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW206 (*argB*$^+$ *alcA::nimO*Δaa437-647)	,,
tSWJ628§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW215 (*argB*$^+$ *alcA::nimO*Δaa403-647)	,,
tSDW644§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW214 (*argB*$^+$ *alcA::nimO*Δaa328-647)	,,
tSDW643§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW205 (*argB*$^+$ *alcA::nimO*Δaa260-647)	,,
tSDW535§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW204 (*argB*$^+$ *alcA::nimO*Δaa151-647)	,,
tSDW575	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW194 (*argB*$^+$ *alcA* control plasmid)	,,
Strains carrying one *alcA* promoter-driven *nimO* allele integrated at the *nimO* locus*:		
tSWJ622§	*nimO*18; *argB*2; *pabaA*1; *pyroA*4 +pSDW213 (*argB*$^+$ *alcA*::*nimO*$^+$)	This study
Strains used for creating and analyzing a deletion of the *nimO*18 allele*:		
tSWJ638‡¶	*nimO*18; *argB*2; *pyrG*89; *nicA*2; *wA*2 +pSWJ136 (*argB*$^+$ *alcA::nimO*$^+$)	This study
ΔSWJ648/ 652/653‡	*nimO*18::*pyr4*$^+$; *argB*2; *pyrG*89; *nicA*2; *wA*2 +pSWJ136 (*argB*$^+$ *alcA::nimO*$^+$) +pSWJ220 (*nimO*18::*pyr4*$^+$) (these three strains are transformants of tSWJ638 with pSWJ220 which carry a deletion of the *nimO*18 allele and an *alcA* promoter-driven allele of *nimO*$^+$ integrated at the *argB* locus)	,,
tSWJ664§‖	*nimO*18; *argB*2; *riboA*1; *yA*2 +pSDW207 (*argB*$^+$ *alcA*::*nimO*Δaa538-647)	This study

*Transforming plasmid and plasmid genotype are indicated after host strain genotype.

‡The *alcA::nimO* construct in these strains is derived from a genomic clone of *nimO* (see Materials and Methods).

§The *alcA::nimO* construct in these strains is derived from a cDNA clone of *nimO* (see Materials and Methods).

¶Produced from a cross of tBAK511 × SWJ299.

‖Produced from a cross of tSDW567 × PCS439.

linearized with *Sma*I and *Kpn*I. Eight out of 127 pyr^+ transformants selected on medium containing ethanol + fructose behaved as predicted for deletion of an essential gene: each was incapable of growth on glucose and became completely dependent on ethanol. The expected patterns for gene replacement at the *nimO*18 locus were observed by Southern blotting of the 8 ethanol-dependent strains and they contained *alcA::nimO*$^+$ as their only copy of *nimO*; three are shown (Fig. 1).

For DNA labelling and microscopic studies, cells were grown in complex medium (0.5% yeast extract, 2% glucose, trace elements, and supplements). When added to cultures, drugs were as follows: hydroxyurea (Acros Chemical Company) was added to a final concentration of 100 mM from a 2 M aqueous stock, nocodazole (Sigma Chemical Co.) to a final concentration of 5 μg/ml from a 2.5 mg/ml stock in DMSO, and 4-nitroquinoline-1-oxide (Acros Chemical Company) to a final concentration of 1 μg/ml from a 5 mg/ml stock in acetone. Equivalent volumes of water, DMSO, or acetone were added as appropriate to control cultures.

Gene isolation and analysis

The *nimO* gene was isolated by complementing the ts-lethality of *nimO*18 with pools and sub-pools of Linkage Group VII-specific *A. nidulans* cosmid DNAs (Brody et al., 1991) until one fully complementing cosmid, W10C05, was identified. A 2.1 kb *Hin*dIII-*Xba*I cosmid subclone with complementing activity was used to screen an *A. nidulans* lambda gt10 cDNA library (generously provided by S. Osmani). Five independent phage isolates were obtained and judged to be identical or overlapping by restriction mapping. A 3.145 kb *nimO* cDNA plus 581 bp of 5′ genomic DNA was sequenced manually (Sequenase Version 2.0 DNA Sequencing Kit, U.S. Biochemical Corporation), and analyzed using DNA Strider 1.0 for Macintosh and the UWGCG software.

The *nimO*18 lesion was localized to the region containing amino acids 1-403 by screening six C-terminally truncated *alcA::nimO* cDNA fragments for ability to fully complement *nimO*18 on all carbon sources (see Table 1). This phenotype differed from most transformants, which instead exhibited glucose-repressible, ethanol-inducible rescue. Constitutive complementation by gene conversion or by plasmid integration at the *nimO* locus to restore a wild-type *nimO* gene (*nimO*$^+$ + *alcA::nimO*18) occurred with constructs bearing 403 or more N-terminal amino acids (not shown). The entire coding region plus 192 bp of 3′ flanking sequence was amplified from *nimO*18 mutant DNA using three primer sets: (1) amino acids 1-214: forward 5′-TGT GTG TAT TGT TAC CTT-3′; reverse 5′-AGC ACC

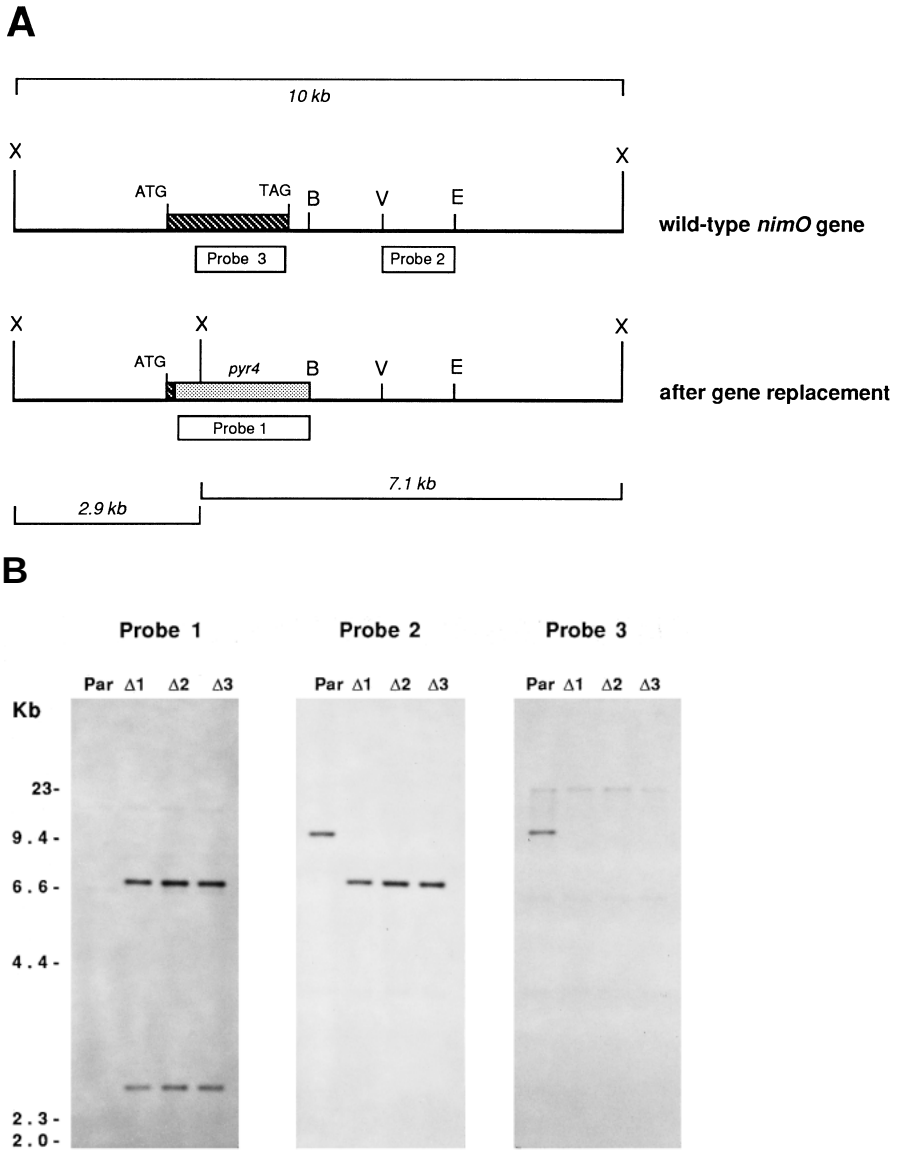

Fig. 1. Deletion of the *nimO* gene. The *nimO*18 allele was deleted from a ts-lethal *nimO*18 strain (tSWJ638). tSWJ638 carries one copy of *alcA*::*nimO*$^+$ inserted at the *argB* locus and the *pyrG*89 mutation as a selectable marker. (A) Schematic diagram of the *nimO*18 gene replacement and strategy for southern blotting of transformants. X, *Xho*I; B, *Bam*HI; E, *Eco*RI; V, *Eco*RV; (B) Southern blots of DNAs digested with *Xho*I were analyzed using three different probes: Probe 1 is a 2.3 kb *Eco*RI fragment containing the *pyr4* gene of *N. crassa*; Probe 2 is a 1.3 kb *Eco*RV-*Eco*RI fragment corresponding to the 3′ flanking region; and Probe 3 is a 1.8 kb *Bam*HI-*Xba*I fragment internal to the *nimO* coding region. The high molecular mass band detected by Probe 3 at ~23 kb corresponds to the *alcA*::*nimO*$^+$ allele that is integrated at the *argB* locus. Par, parental strain; Δ, deleted strain.

ATC CCC AGC AGA CTC-3′; (2) amino acids 157-468: forward 5′-T GTT CGC AGC AAA TGC TG-3′; reverse 5′-CCA TGA TAG AAC GGG CCT TTG-3′; and (3) amino acids 441-647 plus 192 bp 3′ DNA: forward 5′-GAG ACT CCG GAT GCT CCT-3′; reverse 5′-CAA ATG CAT ATC AGC GAA-3′. The PCR-amplified products were cloned into pGEM-T® (Promega Corporation), sequenced, and compared to the wild-type sequence.

DNA labelling and microscopic studies

DNA synthesis in the ts-lethal *nimO*18 mutant was measured by [^{3}H]adenine labelling. Freshly harvested conidia of strain SWJ238 at a density of 4×10^6 per ml were grown in complex media at 30°C and 43°C with vigorous shaking (275 rpm) in the presence of 1.33 μCi per ml [2,8-^{3}H]adenine (NEN Life Science Products). Duplicate 0.5 ml samples were taken every 30 minutes beginning at 2 hours until 10.5 hours and processed as described by Bergen and Morris (1983) to determine specific incorporation into DNA. The counts per minute (cpm) for the duplicate samples were averaged. For all time points, the duplicate cpm values were between 0.1-20% of each other.

Measurements of nuclear morphology and number on cells stained with the DNA-specific dye 2,4-diamidino-2-phenylindole (DAPI) were performed as described previously (James et al., 1995) using a Nikon Optiphot photomicroscope with epifluorescence optics.

RESULTS

nimO is required for DNA replication and for activation of a mitotic checkpoint

The recessive, temperature sensitive lethal *nimO*18 mutation (Morris, 1976) was first described as a cell cycle mutant with abnormal nuclear morphologies (Bergen et al., 1984). More recent application of flow cytometric techniques revealed a general defect in DNA synthesis (not shown; see James et al., 1995). To precisely characterize this defect, the synthesis of [2,8-^{3}H]adenine-labelled DNA was measured at the restrictive temperature (Fig. 2A). During germination of *nimO*18 conidia for 10.5 hours, approximately four rounds of DNA synthesis occurred at the permissive temperature (30°C), while essentially no DNA was synthesized at the restrictive temperature (43°C). *nimO* is therefore necessary for DNA synthesis, but the experiment does not show whether it is needed for initiation, elongation, or both. This question was resolved by measuring DNA synthesis in a reciprocal shift assay. *nimO*18 conidia were germinated at permissive temperature (30°C) in the presence of the DNA synthesis inhibitor hydroxyurea (HU). HU arrests cells in early S phase, just after the initiation of DNA synthesis. Following pre-arrest in HU, the cells were washed into HU-free medium, and shifted to the restrictive temperature (43°C). If *nimO* is needed exclusively for initiation of S phase, then shifting to the restrictive temperature (after initiation has already occurred) should permit DNA synthesis to resume normally. If, however, *nimO* is required for elongation of DNA, then DNA synthesis should not resume following the shift. Fig. 2B demonstrates that although *nimO*18 mutant cells were able to resume DNA synthesis at the permissive and restrictive temperatures upon removal of HU, DNA synthesis after the shift to restrictive temperature was much diminished. Since *nimO*18 mutant cells were capable of DNA synthesis after the shift, albeit more slowly, *nimO* must be necessary for the initiation of S phase. However, because the rate of DNA synthesis was slowed substantially under these conditions, *nimO* function must also be required after initiation for the efficient progression of S phase.

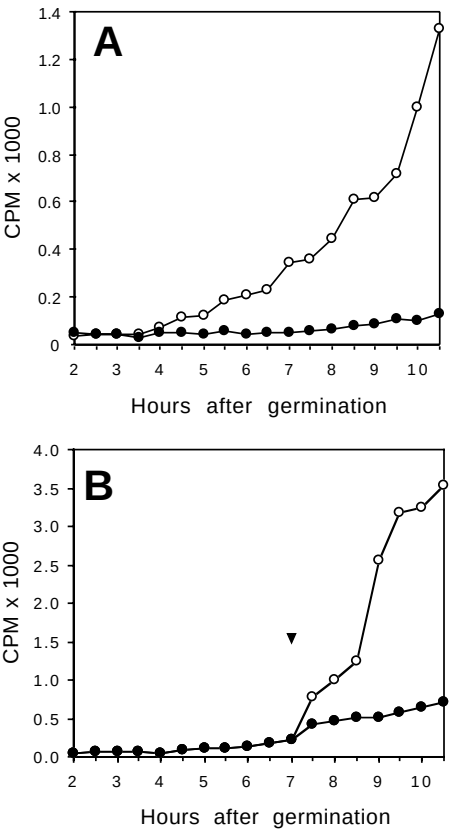

Fig. 2. *nimO* is required for DNA synthesis and for efficient progression through S phase. (A) Conidia of SWJ238 (*nimO*18) were germinated at permissive (30°C) and restrictive (43°C) temperatures, and the synthesis of DNA was monitored by incorporation of [2,8-^{3}H]adenine into DNA. ○, incorporation at 30°C; ●, incorporation at 43°C. (B) Conidia of SWJ238 were pre-arrested with 100 mM hydroxyurea (HU) at 30°C for 7 hours, then hydroxyurea was removed, cells were washed once with HU-free medium, and the culture was split and incubated at 30°C or 43°C in the absence of HU. ○, incorporation of [2,8-^{3}H]adenine into DNA, 30°C + HU → 30°C – HU; ●, 30°C + HU → 43°C – HU. Arrowhead designates removal of HU. Each of the experiments was repeated a minimum of three times.

*nimO*18 mutants exhibited a defect in checkpoint control, arresting in mitosis with a chromosome mitotic index (CMI) of greater than 50% (Fig. 3A). Furthermore, the majority of mitotic cells segregated their chromatin into two distinct and well-separated masses that often were unequal in size (Figs 3C, 4), suggesting that in the absence of DNA replication chromosome segregation could occur via microtubule capture of the single kinetochore. To further define the checkpoint defect and abnormal division of chromatin, nuclear division was assayed in two ways. In the first experiment, cells were incubated at restrictive temperature (43°C) in the presence of HU. In the second experiment, cells were pre-arrested in S phase by incubation with HU, and then shifted to the restrictive temperature with and without HU. By treating cells with HU at permissive temperature, cells could initiate DNA synthesis

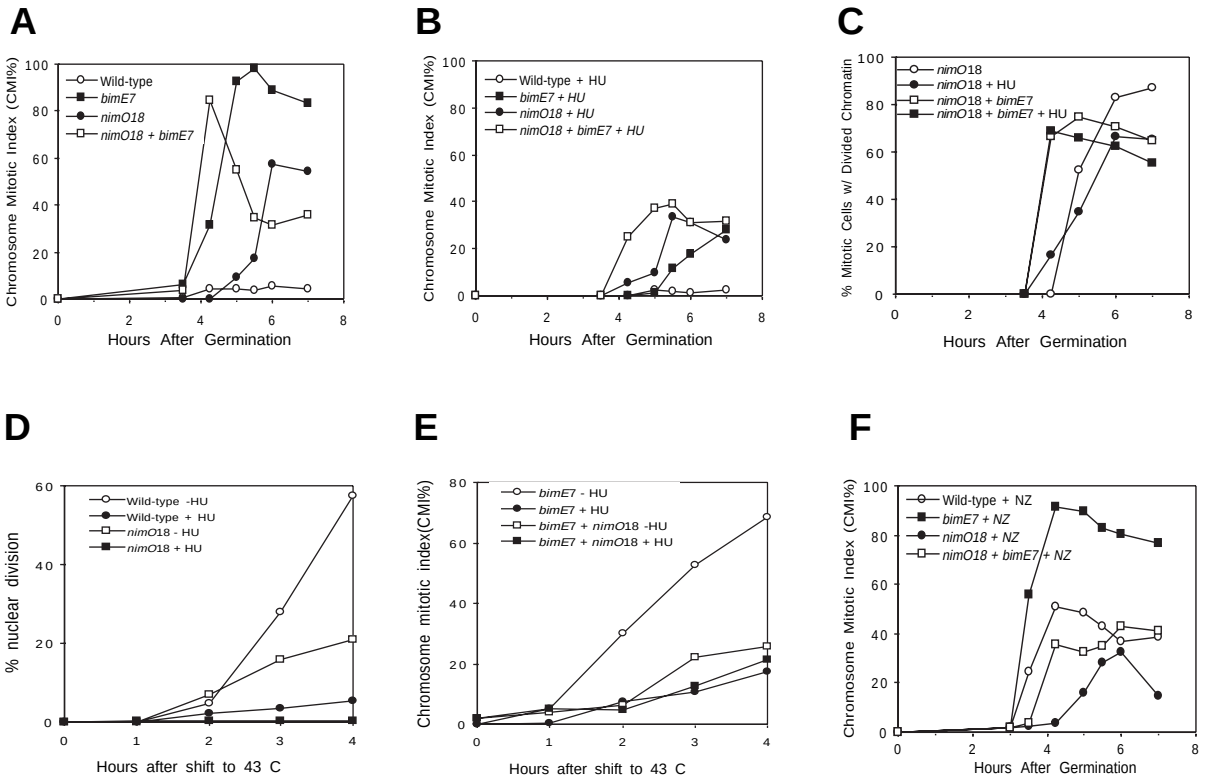

Fig. 3. *nimO*18 mutants reveal a mitotic checkpoint defect that permits the division of unreplicated chromatin in the absence of $bimE^{APC1}$, a component of the anaphase-promoting complex. (A) Chromosome mitotic index (CMI%) of single and double mutants with *nimO*18 and *bimE*7. Conidia were germinated at restrictive temperature (43°C) in complex medium. Samples were taken at the times indicated and fixed and stained with DAPI to determine nuclear morphology and CMI. All measurements are based on observations of at least 200 cells per time point for this and the following experiments. (B) Chromosome mitotic index (CMI%) of single and double mutant cells germinated at 43°C in the presence of 100 mM hydroxyurea (HU), and treated and measured as in A. (C) Division of chromatin in mitotic cells carrying *nimO*18 and/or *bimE*7. The graph shows the percentages of mitotic cells from A and B in which the chromatin was clearly segregated into two equal or unequal masses. (D) Division of chromatin by cells pre-arrested in S phase by treatment with HU. Wild-type and *nimO*18 cells were pre-arrested with HU at 30°C for 7 hours, and then shifted to 43°C in the presence or absence of HU. Following the pre-arrest, hydroxyurea was removed, cells were washed once with HU-free medium, and then the culture was split and returned to the restrictive temperature in the presence and absence of HU. (E) Chromosome mitotic index (CMI%) of *bimE*7 or *bimE*7 + *nimO*18 mutant cells pre-arrested with HU at 30°C, followed by a shift to 43°C. The experiment was performed as described in D, except that the CMI was measured. (F) Chromosome mitotic index (CMI%) of single and double mutant strains germinated in the presence of 5 μg/ml nocodazole in order to trap cells in mitosis.

and thus activate the S phase arrest checkpoint before imposing the restrictive temperature for *nimO*18.

When *nimO*18 cells were incubated at restrictive temperature in the presence of HU, a substantial proportion arrested in mitosis (~40%, Fig. 3B). The majority of mitotic cells segregated their chromatin (Fig. 3C), showing that mitotic induction occurred without DNA replication. In the second experiment, a small proportion of the cells pre-arrested in HU and then shifted to 43°C without HU were able to continue the cell cycle (~20%, Fig. 3D). These observations parallel the reciprocal shift assay shown in Fig. 2B, and indicate that since some, but relatively few, cells can complete the cell cycle after the shift, *nimO* function is required not only for initiation but also during S phase. When incubation was continued in the presence of HU at restrictive temperature, the chromatin in *nimO*18 cells remained uncondensed and did not divide (Fig. 3D), indicating that a DNA replication checkpoint was activated by pre-arrest in HU before the shift to restrictive temperature.

If the premature division of chromatin in *nimO*18 reflects the operation of a normal mitotic apparatus, then it should depend on the formation of a functional spindle. Abnormal mitotic progression leading to lethal anaphase arrest could result, for example, from a defect in the spindle assembly checkpoint. To test this, *nimO*18 cells were incubated at restrictive temperature with the anti-microtubule drug nocodazole which inhibits spindle formation and traps cells in mitosis. Under these conditions, *nimO*18 cells accumulated a CMI approaching 40% (Fig. 3F) but did not segregate DNA, indicating that the division of unreplicated chromatin requires a functional spindle, and suggesting that the spindle assembly checkpoint was activated in the absence of DNA replication.

The anaphase-promoting complex or cyclosome (APC/C) in budding yeast is required for anaphase segregation of chromosomes, by dissolving the glue that holds together pairs of sister chromatids; for the completion of mitosis, involving the degradation of mitotic cyclins; and as part of a checkpoint operating during G_1 to restrain the onset of S and M phases (reviewed by Townsley and Ruderman, 1998). If the aberrant chromosome segregation in *nimO*18 occurs by reductional

anaphase of unreplicated, mono-oriented chromosomes, then the APC/C should be irrelevant to this checkpoint defect. The relationship between G_1/S control and APC/C was examined in cells lacking both *nimO* and one component of the *Aspergillus* APC/C, *bimE*^{APC1}. The temperature sensitive *bimE7*^{APC1} mutation causes a stringent pre-anaphase mitotic arrest and partially inactivates an S phase checkpoint by allowing chromosome condensation and spindle assembly (but not segregation) in the presence of hydroxyurea (James et al., 1995; Ye et al., 1996). When germinated at restrictive temperature in the absence or presence of HU, *nimO*18; *bimE*7 cells achieved a high CMI (Fig. 3A,B), and this double mutant (but not *bim*E7 alone) segregated its unreplicated DNA efficiently (Figs 3C, 4). As predicted, then, the APC/C was not required to segregate unreplicated chromatin. Interestingly, the rate, but not the degree, of mitotic induction of double mutants was accelerated relative to each single mutant.

nimO encodes a protein with similarity to a G_1/S regulator of *S. cerevisiae*

nimO was cloned by complementation and a 3145 bp cDNA (GenBank accession number AF014812) was sequenced. The bona fide *nimO* gene was isolated because two cDNA clones fully complemented *nimO*18 ts-lethality at high frequency (*n*=85 *nimO*$^+$ transformants). Homologous integration or gene conversion by the plasmid-borne cDNA can often complement or repair the mutant allele whereas heterologous insertion of a promoterless cDNA does not. As further proof, a transformant of *nimO*18 containing a single homologously integrated *alcA::nimO*$^+$ plasmid (tSWJ622) was crossed with *pantoB*100 (SWJ601). *pantoB* is very tightly linked to *nimO* (<0.1 cM). Phenotypically, tSWJ622 rescued the ts-lethality of *nimO*18 when grown on ethanol but not glucose. In the cross, all progeny exhibiting ethanol-dependent rescue of *nimO*18 failed to recombine with *pantoB*100 (*n*=65), indicating tight linkage of the integrated plasmid to the *nimO* locus.

The cDNA encodes a putative ORF of 647 amino acids (72.8 kDa), assuming the first in-frame methionine is used. An in-frame stop codon occurs 27 bp upstream of the putative initiation codon, implying that the coding region does not extend beyond the 5′ end of the clone. The cDNA contains a 5′ untranslated region of 236 nucleotides, and a long 3′ untranslated region of 964 nucleotides.

A GenEMBL search revealed homology only with the budding yeast G_1/S regulator Dbf4p, with 28.6% identity and 48.6% similarity (TFASTA, init1 = 119; Fig. 5). No other protein exhibited a degree of similarity close to Dbf4p. nimOp and Dbf4p are similar in length, in the distribution and abundance of charged residues (~19.5% basic and ~12.8% acidic), and they share strong PEST motifs (aa 600-617 in *Dbf4* and aa 558-573 in *nimO*). The greatest identity lies in a novel Cys_2-His_2 zinc finger-like motif (aa 597-616 in *nimO* and aa 642-661 in *Dbf4*). The zinc fingers are identical in length and alignment of the Cys and His residues, and occur close to the C-termini.

The *nimO*18 lesion was localized to the N-terminal 403 amino acids (see Materials and Methods). When the mutant region corresponding to amino acids 1-468 was sequenced and compared with the wild-type sequence, a single nucleotide change was found in each of three independently generated PCR clones. This change, a T to A tranversion in the coding strand, resulted in a Val → Glu substitution at residue 329 (GTA to GAA codon change; Fig. 5). This mutation occurs in a relatively conserved region between *nimO* and *Dbf4* (39.5% identity and 56.7% similarity over 41 amino acids). No additional mutations were discovered in the remaining 3′ region corresponding to amino acids 441-647.

nimO is essential for cell viability and DNA synthesis

The *nimO*18 allele was deleted from tSWJ638 (*pyrG*89; *nimO*18; *alcA::nimO*$^+$) to produce strains containing *alcA::nimO*$^+$ as their only copy of *nimO* (see Fig. 1). These strains grew normally on ethanol + fructose but died on glucose medium, thus establishing that *nimO* is an essential gene. In addition, they exhibited poor growth and conidiation on glycerol medium, suggesting that a relatively high induction of *alcA*::*nimO*$^+$ was required for normal growth (not shown).

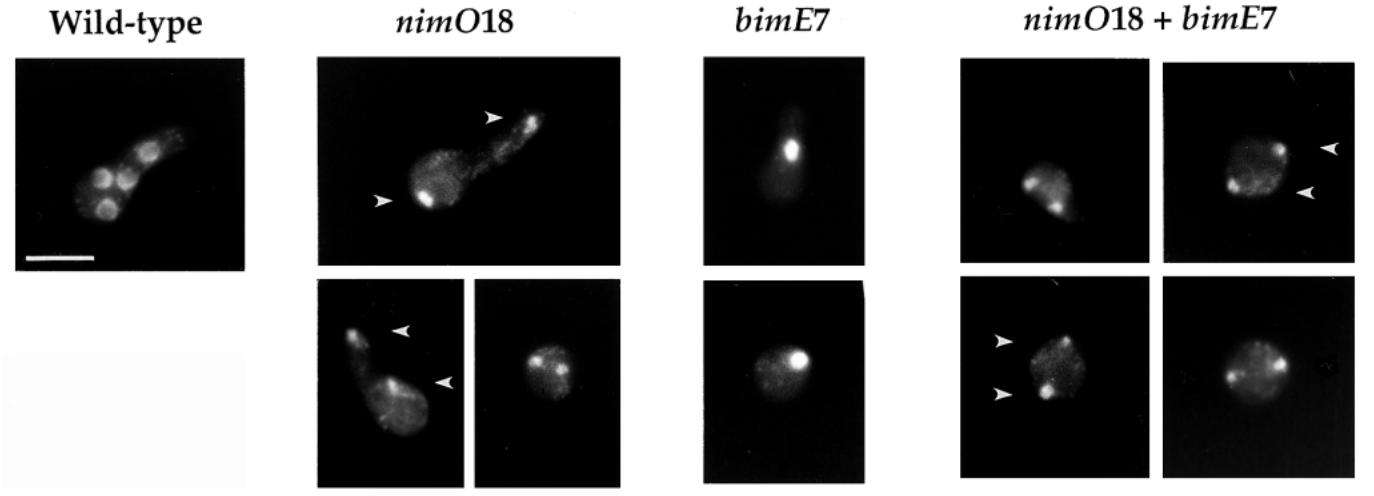

Fig. 4. Nuclear morphology and division of chromatin in single and double mutants with *nimO*18 and *bimE*7. Micrographs show DAPI-stained cells germinated in complex medium for between 5 and 7 hours at 43°C. Arrows in selected photomicrographs indicate segregated DNA masses within a single cell. Bar, ~5 μm.

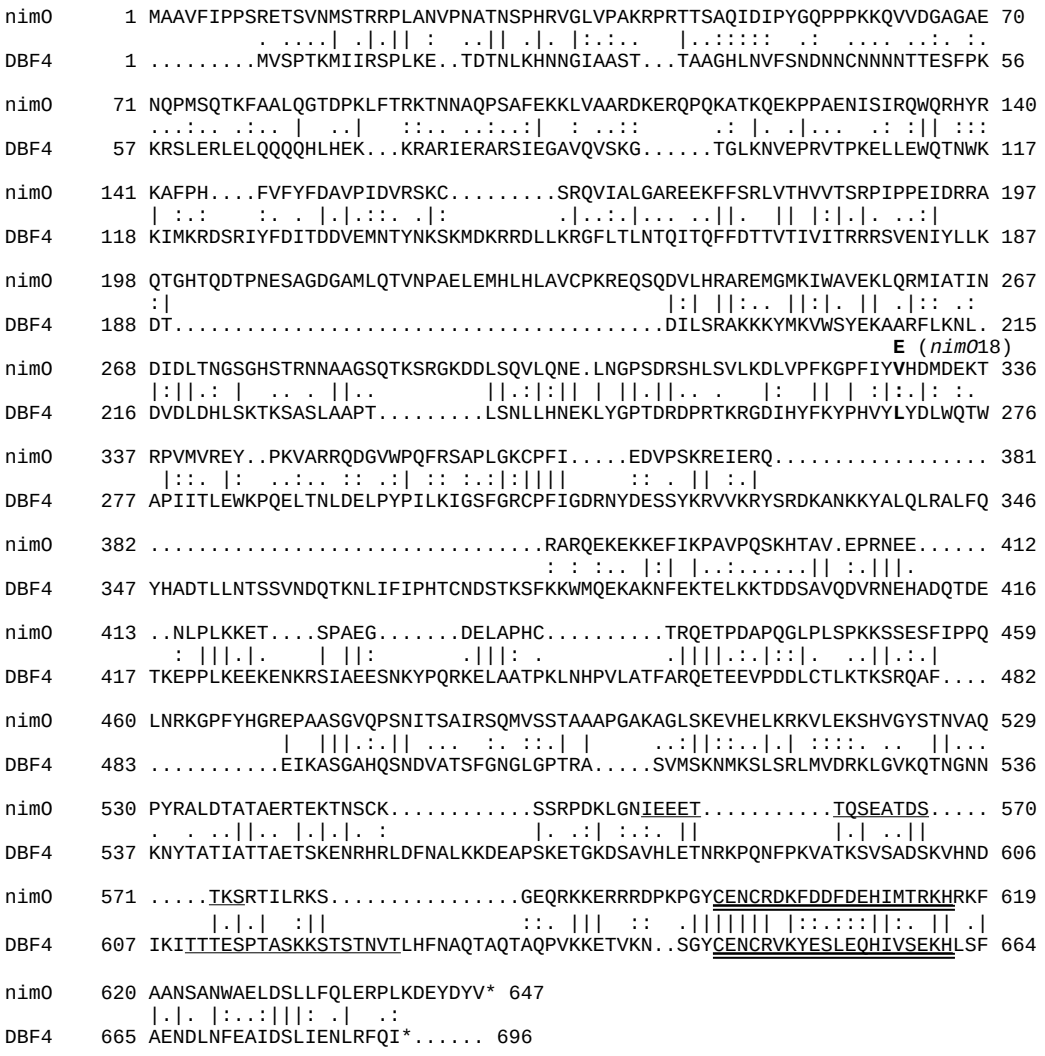

Fig. 5. Comparison of the amino acid sequences of *nimO* and *Dbf4* proteins. The nimOp sequence (upper) and the Dbf4p sequence (lower) were aligned using the GAP program of the UWGCG package with the gap weight and length weight set at 3.0 and 0.1, respectively. Identities are indicated by a '|', and conservative substitutions by a ':'. Double underline, zinc finger region; single underline, potential PEST motifs. Bolded residues show the *nimO*18 mutation resulting from a Val → Glu substitution at residue 329.

An ethanol-requiring, glucose-inviable *nimO* deletion strain (ΔSWJ653) was examined for cell cycle defects by analyzing nuclear morphology and DNA synthesis. When conidia of ΔSWJ653 were germinated at 32°C on complex media containing glucose, most cells arrested with a very short germ tube and a single uncondensed nucleus. After a delay of ~10 hours the CMI began to rise, eventually reaching ~22% after 15 hours (Fig. 6A). No cells were observed to complete mitosis and produce interphase daughter nuclei, but among those arrested in mitosis, ~10% divided their chromatin at later time points (12-15 hrs, not shown). Thus the deletion, to a lesser degree than *nimO*18, can override the mitotic checkpoint and arrest in mitosis with segregated chromatin. Together with the discovery that the deletion strain could not synthesize DNA (Fig. 6B), these experiments indicate that the *nimO*18 ts-lethal phenotype is probably caused by complete, or nearly complete, loss of *nimO* function.

Functional analysis of the *nimO* C terminus

A series of six *alcA*-regulated *nimO* C-terminal truncations was tested for their ability to complement the ts-lethality of *nimO*18 (Fig. 7). Three *alcA::nimO* plasmids carrying truncations of 109, 210, and 244 amino acids restored hyphal growth to *nimO*18 in an ethanol-inducible, glucose-repressible manner (Figs 7, 8). These transformants barely grew on glycerol, which permits basal expression from *alcA*, but grew well when high-level expression was induced by ethanol. However, wild-type growth was not restored because their ability to form conidia was greatly reduced or eliminated (Figs 7, 8). Assuming similar levels of protein expression and stability for each truncated allele, these observations would suggest that the level or quality of *nimO* function was adequate for hyphal growth, but was not sufficient to promote asexual development.

To establish whether the C terminus of *nimO* is essential, we analyzed the progeny of a cross between a *nimO* deletion (ΔSWJ653) and the shortest C-terminal truncation of 109 amino acids (tSWJ664). ΔSWJ653 is deleted at the *nimO* chromosomal locus but grows on media containing ethanol because it harbors one copy of *alcA*::*nimO*$^+$ at the *argB* locus. tSWJ664 carries *nimO*18 and one copy of *alcA*::*nimO*Δ538-647 at *argB*. If the C terminus of *nimO* is essential, then recombinant progeny carrying the deletion + *alcA*::*nimO*Δ538-647 should not be recovered. Among the progeny from this cross, only those carrying *alcA*::*nimO*$^+$ or *alcA*::*nimO*Δ538-647 in combination with the deletion would be completely ethanol-dependent. Among 50 ethanol-dependent, glucose-

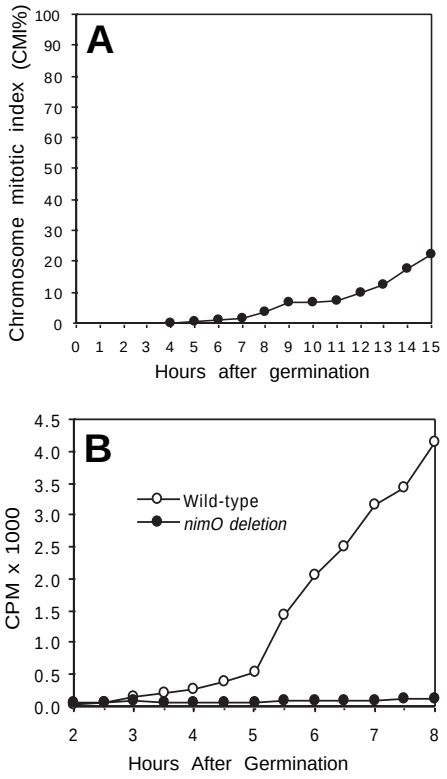

Fig. 6. Cell cycle arrest phenotype of a *nimO* deletion. (A) Chromosome mitotic index of the deletion strain. Conidia were germinated for 15 hours at the restrictive temperature (43°C). Beginning at 4 hours, samples were taken hourly and fixed and stained with DAPI to determine number and morphology of nuclei. All measurements were based on observations of at least 200 cells per time point; (B) DNA synthesis defect in the deletion strain. The synthesis of DNA in germinating conidia was monitored by incorporation of [2,8-^{3}H]adenine into DNA, as detailed in the Materials and Methods and Fig. 3. ○, incorporation by strain PCS439 (wild-type); ●, incorporation by ΔSWJ653 (*nimO* deletion).

inviable progeny examined by Southern blotting, only the wild-type *alcA*::*nimO*$^+$ allele was found in combination with the deletion (not shown). Thus, the deletion was not rescued by the truncated allele, indicating that the C terminus of *nimO* is indispensable for gene function. The ability of this same 109 amino acid truncation to rescue growth when strongly induced in a *nimO*18 background therefore suggests some form of interaction at the restrictive temperature between the *nimO*18 temperature-sensitive polypeptides and the truncated polypeptides.

DISCUSSION

nimOp is structurally related to budding yeast Dbf4p

Unique structural similarities occur between *Aspergillus nimO* and *S. cerevisiae Dbf4*. The genes are similar in size and share only limited overall identity (28.6%). However, the C terminus of each contains a novel, short-looped Cys_2-His_2 zinc finger, and a PEST motif which may control protein stability (Rogers et al., 1986). The zinc fingers, which are identical in length and critical residues, differ from the consensus in having 9 rather than 12 amino acids in the central loop, and in being solitary rather than occurring in tandem repeats (Berg, 1990). Examples of functional solitary or short-looped Cys_2-His_2 zinc fingers are known (Scotland et al., 1993; Pedone et al., 1996), but not motifs that are both solitary and short-looped like nimOp/Dbf4p.

Synthesis of *Dbf4* is cell cycle regulated, with the peak of transcription occurring at G_1/S (Chapman and Johnston, 1989). G_1/S-specific transcription of many budding and fission yeast genes, including *Dbf4*, is controlled through an 8 bp promoter motif, the *Mlu*I cell cycle box or MCB, which contains an invariant CGCG core (Merrill et al., 1992). The recently discovered *Aspergillus* MCB equivalent (A/TCGCGT/ANA/C) occurs twice in the *nimO* 5′ flanking region. Furthermore, a transcription factor called stuAp binds specifically to these motifs in *nimO* and in other G_1/S- or S phase-specific *Aspergillus* genes (Dutton et al., 1997), suggesting that *nimO* expression is likely to be G_1/S-regulated.

nimO is required for DNA replication and mitotic checkpoint control

As the regulatory subunit of Cdc7p, Dbf4p activates this kinase at G_1/S and facilitates its binding to origins of replication, where it triggers DNA synthesis through phosphorylation of substrates in the pre-RC (see Lei et al., 1997; Sato et al., 1997). Close functional similarities exist between *nimO* and *Dbf4*. First, *nimO* and *Cdc7*/*Dbf4* are required for the initiation of DNA synthesis. Second, *nimO* and *Cdc7/Dbf4* are needed for S phase progression following initiation. This dual role to trigger S and mediate efficient replication throughout S contrasts with earlier studies pointing to a singular role in initiation (Johnston and Thomas, 1982). However, recent studies in budding yeast have demonstrated that when Cdc7p is inactivated at mid S-phase, early origins fire but late ones do not. As a consequence, S phase progression is slowed, presumably because DNA synthesis can proceed only from the early-activated subset of origins (Bousset and Diffley, 1997; Donaldson et al., 1997). A role for *nimO* in S phase progression is clearly indicated by reciprocal shift assays, where inactivation of *nimO* following early S phase (HU) arrest allows DNA synthesis and nuclear division to resume, but at a much-reduced rate relative to controls.

Third, mutations in *nimO* and *Dbf4* allow nuclear division of unreplicated chromosomes due to the loss of a mitotic checkpoint. Furthermore, abrogation of this mitotic checkpoint by ts-lethal *nimO*18 mutants influences downstream checkpoints in a manner that closely parallels ts-*dbf4* mutants (Toyn et al., 1995; Tavormina et al., 1997): mitosis is not restrained by treatment with HU, indicating that initiation of DNA synthesis is required to activate a DNA replication checkpoint in both species; and the segregation of unreplicated chromatin was restrained by the UV-mimetic 4-nitroquinoline-1-oxide (4-NQO, not shown) and the anti-microtubule inhibitor nocodazole, indicating that a DNA damage checkpoint and a spindle assembly checkpoint are intact and do not require DNA replication for their activation. Interestingly, γ-irradiation of ts-*dbf4* mutant cells did not restrain mitosis in budding yeast, suggesting that: (a) initiation of DNA synthesis is needed to activate the checkpoint which responds to double strand breaks; and (b) separate checkpoints exist for the detection of dimer lesions (damage by 4-NQO) and double strand breaks

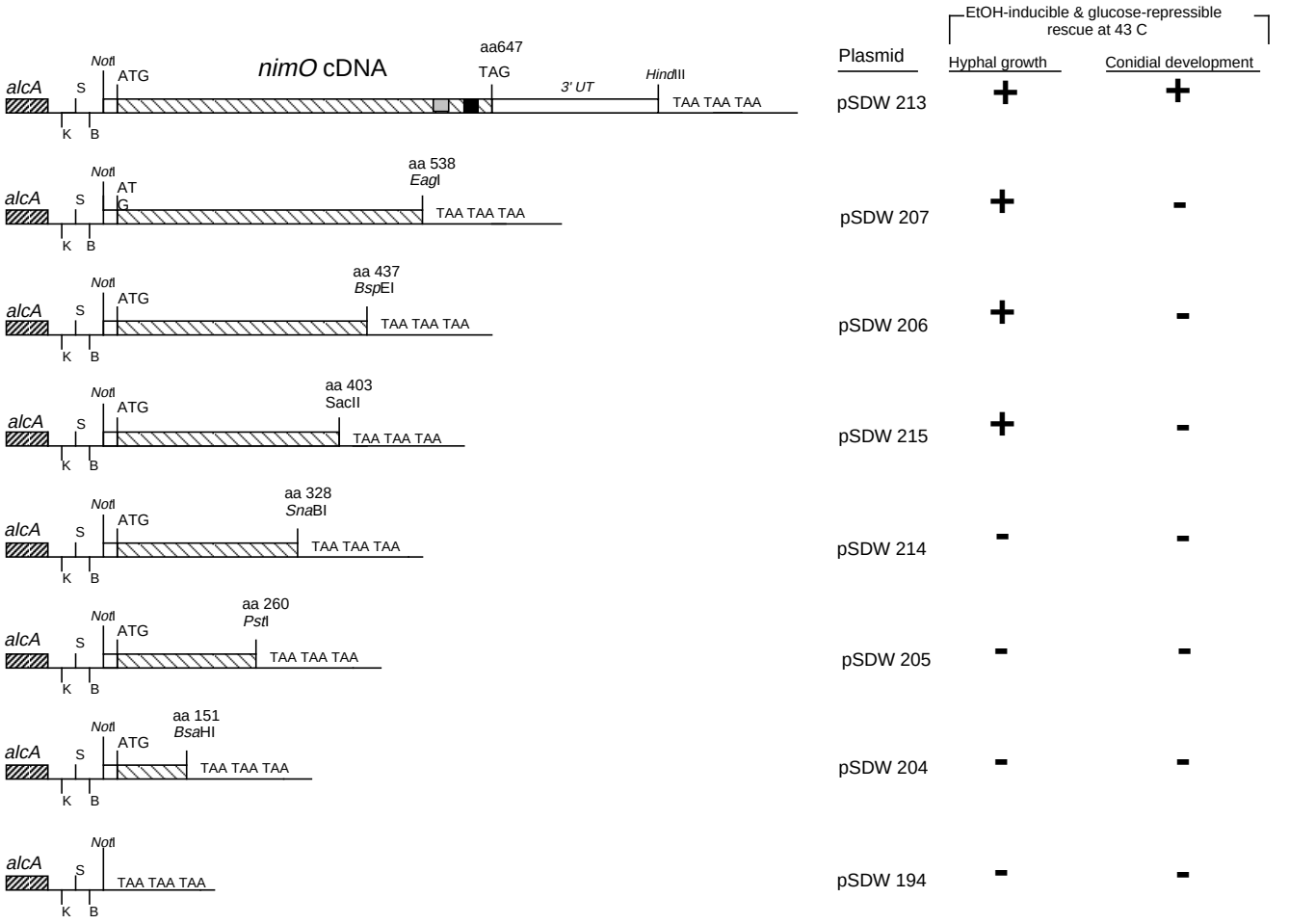

Fig. 7. Ethanol-dependent rescue of *nimO*18 ts-lethality by *alcA*::*nimO* C-terminal truncations. One full-length and six partial-length *nimO* cDNA fragments bearing C-terminal deletions were tested on ethanol, glycerol, and glucose for their ability to complement *nimO*18 ts-lethality at 43°C. *nimO* coding region is indicated by the shaded area (▨). Zinc finger is indicated by the black box (■). PEST motif is indicated by the stippled box (□). +, complementation; –, no complementation. K, *Kpn*I; S, *Sma*I; B, *Bam*HI.

(Tavormina et al., 1997). *nimO*18 has not yet been tested with γ-irradiation.

The anaphase-promoting complex or cyclosome (APC/C) controls the metaphase to anaphase transition and exit from mitosis by activating a proteolytic system that ubiquitinates and degrades mitotic proteins (reviewed by Townsley and Ruderman, 1998). For example, separation of sister chromatids at anaphase occurs by destruction of proteins such as Pds1p, which appears to regulate dissolution of the glue holding sister chromatids together (Yamamoto et al., 1996). In budding and fission yeasts a number of mutants that inhibit DNA synthesis are known abrogate a mitotic checkpoint and enter anaphase in the absence of DNA replication because of a 'reductional anaphase' in which spindle microtubules capture an unreplicated chromosome at its single kinetochore. Since APC/C triggers anaphase by dissolving the glue that tethers sister chromatids, unreplicated chromosomes would appear to bypass this APC/C function (Piatti et al., 1995). Thus, if the aberrant chromosome segregation in *nimO*18 represents a reductional anaphase stemming from the inability to synthesize DNA, then this unequal segregation of chromatin should also occur in cells lacking APC/C. This idea was tested in strains containing *nimO*18 and a ts-lethal APC/C mutant, *bimE*7^{APC1}. *bimE*^{APC1} is absolutely required for chromosome segregation, as revealed by the tight pre-anaphase mitotic arrest of ts-*bimE*7 (James et al., 1995). Its involvement in the *Aspergillus* APC/C was demonstrated biochemically (Lies et al., 1998). *nimO*18; *bimE*7 double mutants bypassed the mitotic checkpoint and divided chromatin efficiently, indicating that, as predicted, APC/C is not required for chromatin segregation when DNA synthesis is inhibited. This phenomenon appears generally true for *Aspergillus* G_1/S mutants. For example, reductional anaphase occurs in the *nimQ*20 mutant. *nimQ* is homologous to the budding yeast pre-RC component, MCM2, and is required for DNA synthesis (Ye et al., 1997).

In addition to regulating anaphase, APC/C controls exit from mitosis in part by destroying the G_2/M regulator cyclin B to inactivate mitotic CDKs (Zachariae et al., 1998). Similarly, APC/C is part of a G_1 checkpoint that restrains the onset of S and M phases by preventing the premature accumulation of B-type cyclins (Irniger and Nasmyth, 1997; Kominami et al., 1998). Interestingly, both *nimO*18; *bimE*7 and *nimQ*20; *bimE*7 double mutants undergo mitotic induction at an accelerated rate

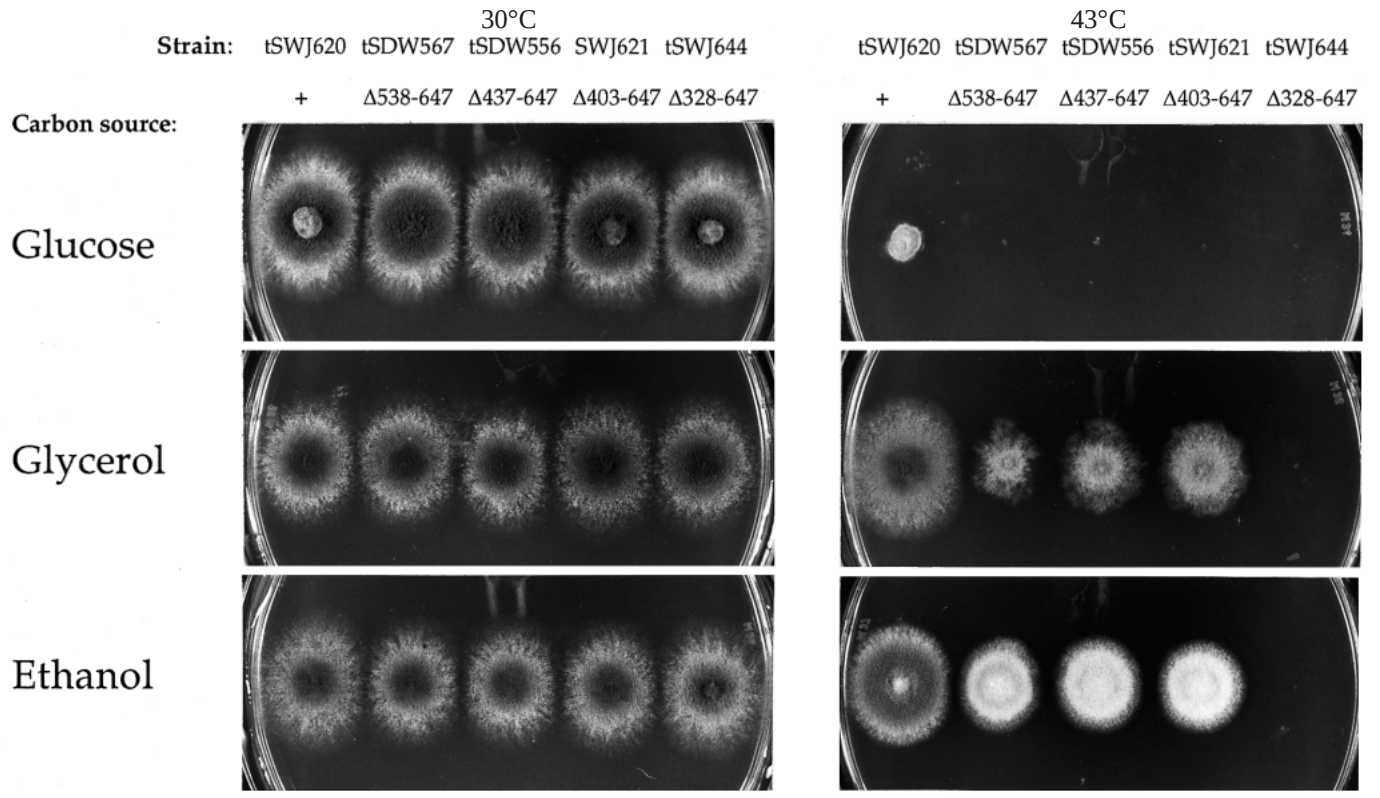

Fig. 8. Growth phenotypes of SWJ396 carrying single copies of *alcA::nimO* C-terminal truncation alleles. Transformants carrying the wild-type cDNA fusion (+) and four truncated alleles (Δ) are shown. Deleted amino acid residues are specified following the Δ symbol. Note the differences in rate of growth on glycerol.

compared to the *nimO*18/*Q*20 and *bimE*7 single mutants. We suggest that this additive acceleration reflects combined defects in the mitotic and G_1 checkpoints. By this model, *bimE* (APC/C) function during G_1 to restrain the onset of S is compromised, shortening G_1 phase so that the arrest point of *nimO*18/*Q*20 (G_1/S) is reached sooner than in *nimO*18/*Q*20 single mutants. This model is generally supported by studies in budding yeast (see Townsley and Ruderman, 1998) and more specifically by biochemical analyses of *nimQ*20; *bimE*7 which revealed that at G_1/S in *Aspergillus*, a critical transition occurs in which the APC/C G_1 checkpoint declines concomitant with activation of a mitotic checkpoint controlled through inhibitory phosphorylation on tyrosine-15 of $p34^{cdc2}$ (Ye et al., 1997). This involvement of $p34^{cdc2}$ contrasts with budding yeast, which does not depend on tyrosine phosphorylation of $p34^{cdc28}$ for the checkpoint linking S and M (Amon et al., 1992; Sorger and Murray, 1992).

nimO is essential for cell viability

nimO is essential, because strains containing *alcA::nimO*$^+$ as their only copy of *nimO* die under repressing conditions (complex media with 2% glucose), and depend completely on ethanol for growth. The cell cycle arrest phenotypes of the *nimO* deletion and *nimO*18 are essentially similar, suggesting that *nimO*18 behaves as a null mutation at restrictive temperature. Under repressing conditions the deletion completely inhibited DNA replication and displayed a mitotic checkpoint defect by arresting with divided chromatin. However, in *nimO*18 these defects were more pronounced, suggesting that: (1) the ts allele is a gain of function mutation conferring a unique influence on checkpoint control; or (2) the deletion is slightly leaky. Leaky synthesis of nimOp on repressing media might diminish the checkpoint defect and still prohibit DNA synthesis. Moderate leakiness does occur in minimal medium containing 2% glucose, where germinating conidia of the deletion produced long, branched germ tubes and executed 3-5 cell cycles before arresting growth (not shown). Also, *nimO*18 ts-lethality was weakly rescued on glucose minimal medium by *alcA*::*nimO*$^+$ (see Fig. 8). Thus, while complex media repressed *alcA::nimO*$^+$ more efficiently than minimal media, this repression apparently is not absolute.

nimO C-terminal mutants reveal a potential interaction between *nimO* polypeptides

Since the greatest similarity between nimOp and Dbf4p occurs in their C-termini, this region in *nimO* was investigated by using *alcA*-driven C-terminal truncations to test for complementation of *nimO*18 and a *nimO* deletion. *nimO* alleles lacking as many as 244 C-terminal amino acids rescued *nimO*18 ts-lethality, but could not complement the *nimO* deletion. Thus, the C terminus of *nimO* is essential for gene function except in the case where full-length *nimO*18 ts polypeptides are present together with the truncated polypeptides. Similar results were obtained for *Dbf4*, where a 40 amino acid C-terminal truncation complemented ts-*dbf4* mutations but not a deletion (Kitada et al., 1992). These genetic interactions among *nimO* and *Dbf4* alleles may be explained by their participation in a multimeric complex. For example, Cdc7p/Dbf4p kinase was recently shown to form oligomeric complexes in vivo, apparently via direct association between Cdc7 polypeptides (Shellman et al., 1998).

Strains expressing the largest truncation of 244 amino acids grew faster than those harboring shorter truncations, as can be seen on media containing glycerol (Fig. 8). Assuming similar levels of protein expression and stability for each truncated allele, these observations would suggest an inhibitory function for the C terminus of *nimO*. Interestingly, Jackson et al. (1993) reported evidence of a negative role for the *Dbf4* C terminus. Removal of the C-terminal 127 amino acids from Dbf4p which eliminates both the PEST and zinc finger motifs was observed to enhance the interaction with Cdc7p in a two-hybrid assay.

Although truncated *nimO* alleles restored hyphal growth to *nimO*18 strains when their expression was strongly induced, wild-type growth was not achieved because asexual development was inhibited. In this process aerial hyphae differentiate into conidiophores and form terminal ranks of primary and secondary sterigmata from which chains of uninucleate conidiospores are produced by budding (reviewed by Timberlake and Marshall, 1988). We cannot rule out the possibility that developmental arrest resulted from the combined effects of C-terminal truncation plus a pattern of *alcA*-driven expression that is altered from the endogenous *nimO* expression pattern. However, dosage effects represent the simplest and most likely explanation, because: (1) growth vigor of the strains improved with increased expression (glycerol versus ethanol), suggesting that more expression is better yet still insufficient for conidiation; and (2) the arrest of asexual development was non-specific: some conidiophores aborted before development of primary and secondary sterigmata, while others developed small numbers of primary only or primary plus secondary sterigmata, and rarely a few conidia (not shown). Therefore, a role for *nimO* as a concerted regulator of development seems unlikely. Instead, we suggest that conidiation may require accelerated rates of nuclear division which cannot be supported by the mutant *nimO* proteins.

Accumulating evidence implicates budding yeast Dbf4p as a linchpin in the regulation of DNA replication. Although possible homologs of *Cdc7* have been found in other species (Masai et al., 1995; Sato et al., 1997), it is not known if they are activated by association with a Dbf4p-like molecule. This report identifies the first potential homolog of *Dbf4*, and presents evidence to suggest that regulation of Cdc7p kinase activity may occur by a conserved mechanism.

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