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An extra copy of *nim*E^{cyclinB} elevates pre-MPF levels and partially suppresses mutation of *nim*T^{cdc25} in *Aspergillus nidulans*

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Previous work has shown that *nim*A encodes a cell cycle regulated protein kinase that is required along with the p34^{cdc2} histone H1 kinase (MPF) for mitosis in *Aspergillus nidulans*. We have now identified two other gene products required for mitosis in *A.nidulans*. *nim*T encodes a protein similar to the fission yeast *cdc25* tyrosine phosphatase and is required for the conversion of pre-MPF to MPF and *nim*E encodes a B-type cyclin which is a subunit of MPF. A new genetic interaction between *nim*E^{cyclinB} and *nim*T^{cdc25} type genes is reported. Increased copy number of *nim*E^{cyclinB} can suppress mutation of *nim*T^{cdc25} and also lead to increased accumulation of tyrosine phosphorylated p34^{cdc2} (pre-MPF). This biochemical observation suggests an explanation for the genetic complementation. If *nim*E^{cyclinB} recruits p34^{cdc2} for tyrosine phosphorylation to form pre-MPF it follows that increased expression of *nim*E^{cyclinB} would increase the level of pre-MPF. The increased level of pre-MPF generated may then allow the mutant *nim*T^{cdc25} protein to convert enough pre-MPF to MPF and thus permit some mitotic progression. We also demonstrate that correct cell cycle regulation by the p34^{cdc2} protein kinase pathway is essential for correct developmental progression in *A.nidulans*.

Key words: Aspergillus nidulans cell cycle regulation/cdc-25/cyclin B/*nim*E/*nim*T

Introduction

The control of the G_2-M transition of the cell cycle is partly dependent on the regulated activity of a serine/threonine protein kinase known as the M phase or maturation promoting factor (MPF). This kinase comprises at least two subunits: a catalytic subunit generally known as p34^{cdc2}, whose abundance does not change significantly throughout the cell cycle and is encoded by homologs of the *Schizosaccharomyces pombe cdc2* gene; and a regulatory subunit called cyclin which is rapidly destroyed at the exit from mitosis and must accumulate again for MPF activation (for reviews see Lewin, 1990; Nurse, 1990; Maller, 1991). Although several different classes of cyclin proteins have been identified on the basis of sequence similarities and kinetics of appearance and destruction, it is the B-type cyclins that are involved in the G_2-M transition *in vivo* (reviewed by Minshull *et al.*, 1989).

Several groups have investigated the phosphorylation state of p34^{cdc2} and its effect on MPF activity. *In vitro* experiments using *Xenopus* extracts showed that during interphase monomeric cyclin free p34^{cdc2} is unphosphorylated and inactive. It becomes complexed with cyclin and subsequently becomes phosphorylated on particular serine, threonine and tyrosine residues to form an inactive precursor called pre-MPF, which is subsequently activated. This suggests that cyclin acts to target p34^{cdc2} for phosphorylation (Solomon *et al.*, 1990). Mapping of these phosphorylation sites has shown that phosphorylation of tyrosine 15 (Y15) is inhibitory (Gould and Nurse, 1989; Krek and Nigg, 1991). Y15 is located in the ATP-binding domain of p34^{cdc2}, suggesting that the inhibitory tyrosine phosphorylation operates by interfering with kinase activity. It is possible that the cyclin-p34^{cdc2} complex is the preferred substrate for tyrosine phosphorylation due to conformational changes of the p34 subunit induced by binding with cyclin. Studies *in vivo* using sea urchin eggs have also shown a close correlation between cyclin binding to p34^{cdc2} and its tyrosine phosphorylation. These studies showed that only a fraction of the p34^{cdc2} population is recruited by cyclin B to target it for phosphorylation (Meijer *et al.*, 1991). Co-expression studies in baculovirus have demonstrated that tyrosine phosphorylation of the cyclin-p34^{cdc2} complex is dependent on the product of the *S.pombe wee1* gene (p107^{wee1}) (Parker *et al.*, 1991). This protein has sequence similarities to known serine/threonine kinases and *in vitro* can act as a serine/tyrosine kinase. p107^{wee1}, together with a partner encoded by *mik1*, are required either directly or indirectly for the tyrosine phosphorylation of p34^{cdc2} in *S.pombe* (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991).

Pre-MPF is activated by the dephosphorylation of Y15 of p34^{cdc2} which is mediated by the *cdc25* gene product (Dunphy and Newport, 1989; Gould and Nurse, 1989). Genetic experiments have shown that initiation of mitosis is dependent on the product of the *cdc25* gene (Russell and Nurse, 1986). Furthermore, mutant *cdc2* alleles containing a phenylalanine residue at position 15 and therefore lacking the inhibitory phosphorylation, bypass the requirement for *cdc25* function to enter mitosis (Gould and Nurse, 1989). Activation of pre-MPF in *cdc25* mutants of *S.pombe* can be achieved by expression of a human phosphotyrosine phosphatase (Gould *et al.*, 1990). In the absence of obvious sequence similarity to known phosphotyrosine phosphatases or tyrosine kinases, it was hypothesized that *cdc25* leads to mitotic induction by either activating a phosphatase or inhibiting a kinase. Recently, however, evidence has been gained which points to the *cdc25* gene product directly dephosphorylating Y15. Addition of the bacterially produced product of the *Drosophila melanogaster* homolog of *cdc25*

(*string*, Edgar and O'Farrell, 1989) to extracts of G_2 arrested *Xenopus* oocytes led to the dephosphorylation of Y15 of pre-MPF. This reaction is inhibited by vanadate, an inhibitor of phosphotyrosine phophatases. Addition of a peptide comprising the C-terminal 60% of the protein, which contains the region conserved among cloned *cdc25* homologs, also led to dephosphorylation of Y15. However, these proteins initially failed to show any activity on artifical substrates for phosphotyrosine phosphatases (Kumagai and Dunphy, 1991) but have been subsequently shown to act catalytically to dephosphorylate such substrates (Dunphy and Kumagai, 1991). Similar results have been gained using bacterially produced human or *Drosophila* cdc25 protein to dephosphorylate and activate pre-MPF (Strausfeld *et al.*, 1991; Gautier *et al.*, 1991). Further support for the hypothesis of direct dephosphorylation by cdc25 came from the observation that the *S.pombe* cdc25 protein has limited but significant sequence similarities to a phosphatase from vaccinia virus within a region that is critical for activity (Moreno and Nurse, 1991; Strausfeld *et al.*, 1991). This protein is capable of utilizing phosphoserine and phosphotyrosine as substrates (Guan *et al.*, 1991). Taken together, these observations raise the strong possibility that cdc25 directly activates pre-MPF by removing inhibitory phosphates present on pre-MPF to promote mitosis.

Although the tyrosine phosphorylation of the cyclin B-recruited $p34^{cdc2}$ is inhibitory, it is a necessary step in the formation of pre-MPF. Activation of pre-MPF to MPF by tyrosine dephosphorylation requires cdc25 and therefore both cyclin and cdc25 can be considered as positive regulators of the G_2-M transition.

We are using the filamentous fungus *Aspergillus nidulans* as a model system to investigate cell cycle control. A number of genes have been identified by conditional mutations which alter cell cycle progression under restrictive conditions (Morris, 1976). Within the class of mutations that lead to a failure to enter mitosis (the *nim* or never-in-mitosis mutations), temperature-sensitive alleles of five genes (*nim*A, *nim*B, *nim*E, *nim*T and *nim*U) have been shown to result in a G_2 arrest at restrictive temperature (Bergen *et al.*, 1984). Of these, only the *nim*A gene has been studied extensively. It encodes a serine/threonine-like kinase the activity of which, together with *nim*A mRNA levels, undergoes cell cycle regulation and peaks at the G_2-M boundary (Osmani *et al.*, 1987, 1988a, 1991a). Overexpression of *nim*A from an inducible promoter leads to premature initiation of mitosis (Osmani *et al.*, 1988a). It has been postulated that NIMA kinase is required in addition to MPF for entry into mitosis (Osmani *et al.*, 1991b). In this report we show that the *nim*E and *nim*T genes of *A.nidulans* encode homologs of the products of the *S.pombe* *cdc13* (cyclin B) and *cdc25* genes, respectively. Interestingly, we have found that the temperature-sensitive phenotype of *nim*T23 is partially suppressed in strains carrying an extra copy of *nim*E. These strains are capable of vegetative mycelial growth at the restrictive temperature but fail to undergo asexual spore development. The increase in *nim*E copy number is associated with an increase in the amount of tyrosine phosphorylated $p34^{cdc2}$ (pre-MPF) at the *nim*T23 arrest point. Thus, the suppression of the *nim*T23 mutation by $nimE^{cyclinB}$ could be due to additional cyclin increasing the pool of pre-MPF and hence substrate for NIMT, which then increases the relative effectiveness of the mutant *nim*T23 protein.

Results

Molecular cloning of nimE from a chromosome II-specific cosmid library and suppression of nimT23 by extra dosage of nimE

We are characterizing genes in *A.nidulans* which are required to traverse the G_2 phase of the cell cycle. Temperature-sensitive mutations in two of these genes, *nim*E6 and *nim*T23, have been mapped to chromosome II (Morris, 1976). In order to identify clones containing the wild type *nim*E and *nim*T genes, we obtained a cosmid library of 410 independent chromosome II-specific clones (Brody *et al.*, 1991) from the Fungal Genetics Stock Center. Cosmid DNA from 10 pools of 41 individual clones each were cotransformed into a *nim*E6 *pyr*G89 double mutant using the *A.nidulans pyrG* gene (Oakley *et al.*, 1987) as a selectable marker for transformation. After 16–20 h of growth at the permissive temperature (32°C) the transformants were placed at the restrictive temperature (42°C). One of the cosmid pools was able to complement the temperature sensitivity of *nim*E6. DNA from each cosmid in the pool was prepared separately and tested for *nim*E6 complementation as above. This identified a single clone (W01H08-L) containing the complementing DNA which was localized by subcloning to a 3.9 kb *Eco*RI–*Kpn*I fragment (Figure 1).

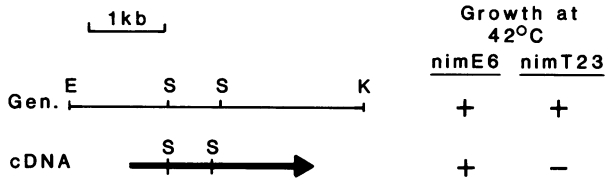

Fig. 1. Complementation of *nim*E6 and *nim*T23 by clones of the wild type *nim*E gene. An *Eco*RI (E)–*Kpn*I (K) genomic fragment of cosmid W01H08-L which was the smallest fragment identified via transformation as capable of full complementation of *nim*E6 and partial complementation of *nim*T23 as judged by growth at 42°C (+) is depicted. Full length cDNA isolated using the *Sal*I (S)–*Kpn*I fragment as a probe showed complementing activity for *nim*E6 only.

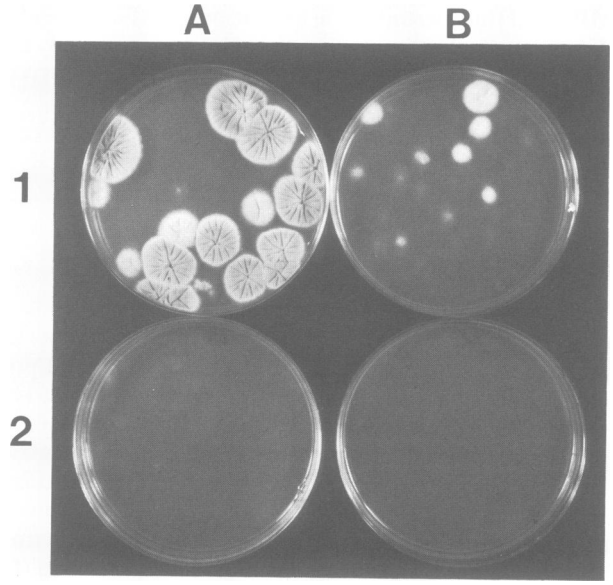

Fig. 2. Phenotype of complementing transformants. A *nim*E6 strain (**A**) and a *nim*T23 strain (**B**) were transformed with either (1) the *Eco*RI–*Kpn*I subclone of the cosmid W01H08-L plus the *A.nidulans* *pyr*G gene (Oakley *et al.*, 1987) or (2) the *pyr*G gene only. Plates were incubated at 42°C for 3 days.

We also attempted to identify a clone that complemented *nim*T23 using the same procedure as described above. Full complementation of *nim*T23 to a wild type phenotype at restrictive temperature was not achieved. However, one cosmid was identified that partially complemented *nim*T23. While these transformants showed significant vegetative growth at 42°C, colonies failed to develop asexual spores (conidia). This cosmid was the same as that selected for complementing *nim*E6 (W01H08-L). Similar complementation of *nim*T23 was also obtained with the 3.9 kb *Eco*RI−*Kpn*I subclone (Figure 1 and 2).

As both the *nim*T23 and *nim*E6 mutations were complemented by the same piece of DNA, we tested the possibility that *nim*T23 and *nim*E6 were allelic. Complementation analysis using heterokaryons and diploids carrying both mutations indicated that *nim*E6 and *nim*T23 were alleles of different loci. Furthermore, sexual crosses showed no linkage between *nim*E and *nim*T, indicating that these genes are well separated on chromosome II (data not shown).

The 3.9 kb *Eco*RI−*Kpn*I fragment of cosmid W01H08-L was subcloned into plasmid pRG3 (Waring *et al.*, 1989) creating plasmid pNIM11. pRG3 carries the *Neurospora crassa pyr4* gene which can be used to complement the *A.nidulans pyr*G89 mutation to select transformants. pNIM11 was used to transform *nim*E6, *pyr*G89 and *nim*T23 *pyr*G89 double mutants. Pyr$^+$ transformants capable of growth at 42°C were subjected to Southern blot analysis to identify strains that contained a single copy of pNIM11 integrated into the genome (data not shown). In order to screen for gene replacement events in these transformants, these strains were then cured of the plasmid by growth on non-selective media containing 5-fluoroorotic acid (5-FOA) which selects against the presence of the *pyr4* marker. The resulting 5-FOA resistant strains were then tested on: (a) selective media at 32°C to test for the loss of pNIM11; (b) non-selective media at 32°C to test viability; and (c) non-selective media at 42°C to test the *nim*E and *nim*T genotype. Representative plates from the scoring of 140 fans from each strain are shown in Figure 3. 42% of the 5-FOA resistant fans derived from the *nim*E6 strain were able to grow at 42°C. In these transformants loss of the plasmid repaired the *nim*E6 lesion indicating that pNIM11 had integrated at the *nim*E locus. Such gene replacement events did not occur in the 5-FOA resistant transformants of *nim*T23. These data demonstrate that pNIM11 contains the wild type *nim*E gene and that one extra copy of *nim*E is able to partially suppress the temperature sensitivity of *nim*T23.

Several cDNA clones were isolated using the *Sal*I−*Kpn*I fragment of W01H08-L as a probe and these were tested as linear pieces of DNA in cotransformation experiments for complementation of *nim*E6 and *nim*T23. Site specific integration of a cDNA at its own locus can result in mutant allele complementation whereas heterologous integration in the absence of promoter sequences does not. Only full

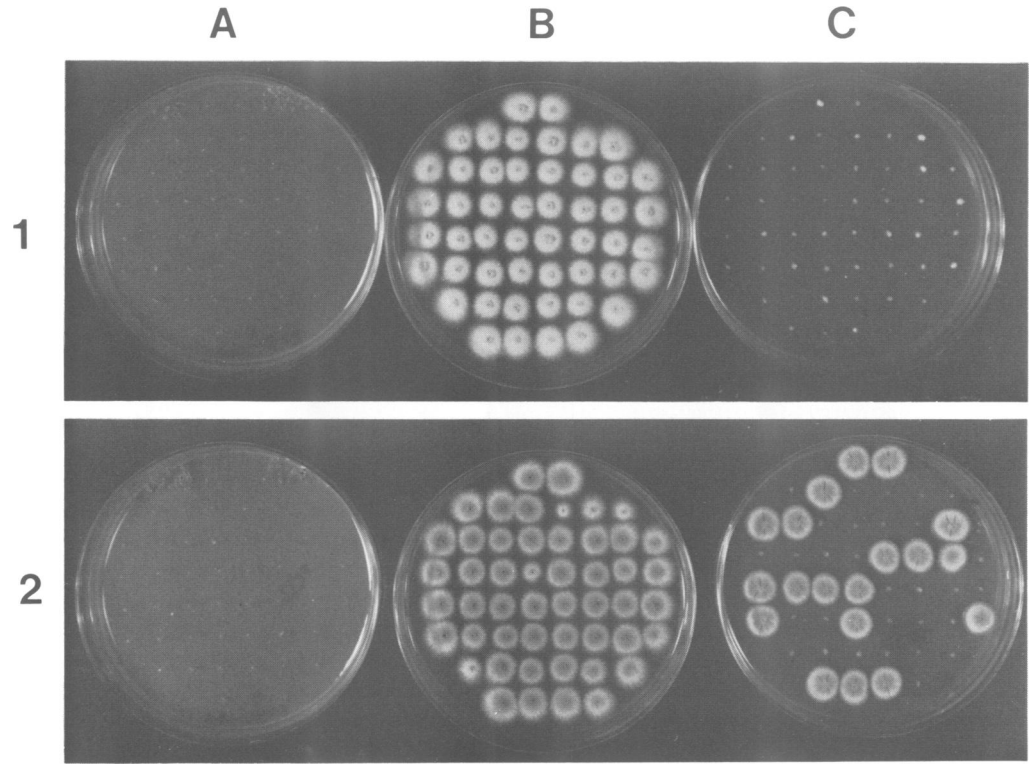

Fig. 3. Phenotypes of 5-flouroorotic acid (5-FOA) resistant pNIM11 transformants of *nim*E6 and *nim*T23. *nim*T23 *pyr*G89 (1) and *nim*E6 *pyr*G89 (2) strains were transformed with pNIM11, which contains the *Eco*RI−*Kpn*I fragment of cosmid W01H08-L cloned into pRG3 (Waring *et al.*, 1989), a vector which carries the *N.crassa pyr*4 gene as a selectable marker for transformations. Pyr$^+$ transformants were selected on media lacking uridine and uracil (UU). Transformants containing a single copy of pNIM11 were plated on media containing UU and 5-FOA to select for loss of the *pyr4* marker. 5-FOA resistant derivatives were plated on the following media and incubated for 2 days at the indicated temperature: (**A**) selective media (−UU), 32°C; (**B**) non-selective media (+UU), 32°C; (C) non-selective media (+UU), 42°C. 5-FOA resistant strains derived from pNIM11 transformants of *nim*E6 which had lost pNIM11, as indicated by the lack of growth in (A), were capable of growth at 42°C indicating the repair of the temperature sensitive *nim*E6 lesion. Although the 5-FOA resistant strains derived from *nim*T23 were viable as they grew in (B), all were temperature sensitive indicating no gene replacement events at *nim*T. These plates are representative of 140 5-FOA resistant colonies selected from each strain.

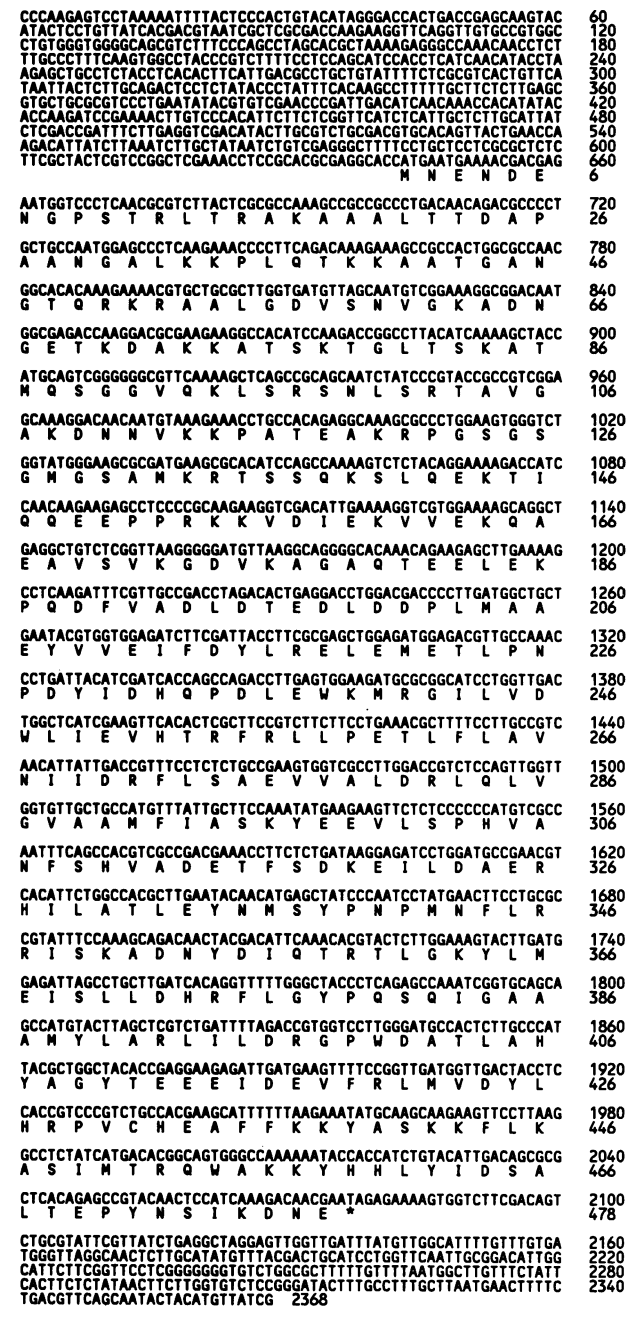

Fig. 4. Nucleotide and derived amino acid sequence of the *nim*E cDNA. The sequence data of *nim*E have been deposited in the EMBL Nucleotide Sequence Database under the accession number X64602.

complementation of *nim*E6 and no complementation of *nim*T23 was observed (Figure 1). These data confirm that we had cloned *nim*E and demonstrate that expression of the extra copy of *nim*E is required for the partial suppression of *nim*T23.

nimE encodes a B-type cyclin

Sequence analysis of *nim*E cDNAs revealed an open reading frame encoding a predicted 54 kDa protein of 478 amino acids (Figure 4). Database searches indicated the predicted NIME protein to have significant sequence similarity to the cyclin family of proteins. The aspartic acid residue in the destruction box sequence (RAALGDVSN, amino acids 52–60) and the sequence FLRRISK (amino acids 344–350) are motifs indicative of a B-type cyclin (Minshull *et al.*, 1989, 1990; Glotzer *et al.*, 1991). NIME shows 35–39% identity (55–59% similarity) to cyclin B sequences in the GenEMBL database, but 51% identity (67% similarity) to the product of the *S.pombe cdc13* gene (Figure 5).

Molecular cloning of a S.pombe cdc25 homolog from A.nidulans by PCR

The product of the *cdc25* gene of *S.pombe* is a key regulator of the G_2–M transition. Homologs of *cdc25* have been identified in *Drosophila* (Edgar and O'Farrell, 1989), humans (Sadhu *et al.*, 1990) and budding yeast (Russell *et al.*, 1989). We wished to clone the *A.nidulans cdc25* homolog as a first step towards investigating its interaction with other cell cycle regulators in *A.nidulans*. Alignment of the *cdc25* homologs show that the C-terminal portions of the proteins are related (Sadhu *et al.*, 1990). These alignments revealed regions that are conserved and can be used to design degenerate PCR primers for amplifying *cdc25*-related sequences, an approach successfully used in the cloning of the human homolog (Sadhu *et al.*, 1990).

PCR amplification of *A.nidulans* cDNA was used to identify a PCR product that contained an open reading frame which predicted a protein with significant sequence similarity to that of *S.pombe cdc25*. The PCR product was used as a probe to identify cDNA clones, one of which approximated the size of the mRNA estimated by Northern blotting at 2.9 kb (data not shown). The sequence of the cDNA contained an open reading frame that predicts a 62 kDa protein of 556 amino acids (Figure 6), which is related to all the cdc25 homologs, but is most closely related to the *S.pombe cdc25* gene product (Figure 7A). In addition to the C-terminal similarities common to all cdc25 homologs, the *S.pombe* and *A.nidulans* CDC25 proteins share 60% identity in a 40 amino acid region near the N-terminus which is absent from the other homologs (Figure 7B). Southern analysis using the *A.nidulans cdc25* cDNA to probe wild type DNA resulted in a single site of hybridization, indicating that no genes of related sequence are present in the *A.nidulans* genome (data not shown).

Identification of nimT as the cdc25 homolog of A.nidulans

Phenotypic analysis of strains carrying the *nim*T23 mutation indicated that the *nim*T gene product is required for the transition through G_2 and for the tyrosine dephosphorylation of $p34^{cdc2}$ in the same manner that *cdc25* is required for G_2 progression in *S.pombe* (Osmani *et al.*, 1991b). We therefore wished to ascertain if the *cdc25* homolog cloned using PCR corresponded to the *nim*T gene. The *cdc25* cDNA was able to fully complement the temperature sensitivity of *nim*T23 when introduced via cotransformation (data not shown). Four of these complementing transformants were outcrossed to a wild type strain and a total of 144 progeny from these crosses were tested for temperature sensitivity. No temperature-sensitive segregants were recovered (data not shown) indicating that the complementation was due to gene replacement events that repaired the temperature-sensitive lesion of *nim*T23. Hence, we conclude that the *cdc25* homolog isolated using PCR is the wild type *nim*T gene.

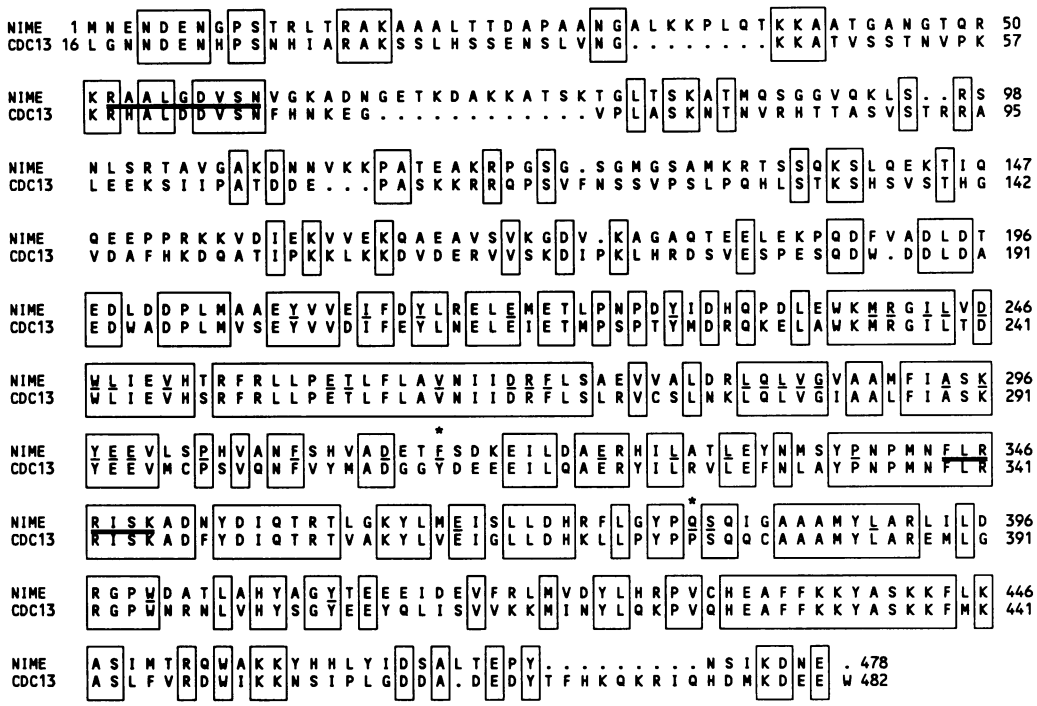

Fig. 5. Alignment of the NIME and cdc13 (*S.pombe*) amino acid sequences. The derived amino acid sequences of NIME and cdc13 (CDC13) (Booher and Beach, 1988) were aligned using the UWGCG program BESTFIT (Devereux *et al.*, 1984). Gaps, as indicated by a period, have been inserted to optimize the alignment. The sequences show 51% identity and identical residues are boxed. Double lines separate the destruction box sequence (Glotzer *et al.*, 1991) and the B-type cyclin specific sequences of FLRR-SK (Minshull *et al.*, 1989). Single lines separate residues at positions that are highly conserved between A- and B-type cyclins (Minshull *et al.*, 1989). Asterisks mark positions where the predicted NIME sequence differs from this consensus, although other cyclin sequences deviating from consensus at these two positions can be found on the GenBank database (release 68.0)

Extra dosage of nimE in a nimT23 strain allows vegetative growth, but does not allow normal development of spore forming cells

We have observed that extra dosage of *nim*E allowed significant mycelial growth of *nim*T23 strains at 42°C, but did not allow sporulation (Figure 2). In order to determine the stage of differentiation at which these strains were blocked, we grew a wild type strain and a *nim*T23 strain containing one extra copy of *nim*E at 42°C under conditions that would allow conidiation to occur. After 20–24 h, both strains developed aerial hyphae, called vesicles, which are the first visible indication of differentiation (for review see Timberlake, 1990). These cultures were incubated for a total of 40 h to allow completion of the differentiation process. Samples were then fixed and observed by phase contrast microscopy. Whereas the wild type strain had developed normally (Figure 8), the strain bearing *nim*T23 plus one extra copy of *nim*E showed a number of morphological defects. Only one-third of the vesicles had developed cells even resembling primary sterigmata, the next cell type delineated from the vesicles, but these were extended and less numerous than on wild type conidophores (Figure 9, A and D). Furthermore, the vesicles of this strain showed considerable branching (Figure 9, B–E), a phenomenon not observed for the wild type strain. Few vesicles showed further differentiation of conidiophore structures (Table I).

How does increased expression of nimE^{cyclinB} suppress mutation of nimT^{cdc25}?

To investigate the potential mechanism by which extra *nim*E^{cyclinB} suppresses mutation of *nim*T^{cdc25} we measured the effect of *nim*E^{cyclinB} dosage on the biochemical phenotype caused by *nim*T23. We first measured the level of phosphotyrosine on p34^{cdc2} in cells containing the *nim*T23 mutation alone or with an extra copy of *nim*E^{cyclinB}. The strains were shifted from the permissive temperature to the restrictive temperature (42°C) and p34^{cdc2} was affinity purified using p13–Sepharose from protein extracted before the temperature shift and after 3 h at 42°C. As expected the level of phosphotyrosine on p34^{cdc2} increased at the G_2 arrest point of *nim*T23 in the absence of extra cyclin (Figure 10A, lane 1 versus lane 2). In the strain containing extra *nim*E^{cyclinB} the phosphotyrosine detected on p34^{cdc2} increased to a higher level than that present in the strain not containing the extra *nim*E^{cyclinB} when the *nim*T23 mutation was imposed (Figure 10A, lane 2 versus lane 4). In addition, the level of tryosine phosphorylated p34^{cdc2} was also increased in the *nim*T23 strain containing extra *nim*E^{cyclinB} even when grown at the permissive temperature (Figure 10A, lane 1 compared with lane 3). This observation indicates that extra cyclin leads to an increased level of tyrosine phosphorylated p34^{cdc2} accumulating before and after the *nim*T23 mutation is imposed.

As extra *nim*E^{cyclinB} allows the vegetative growth of strains containing the *nim*T23 mutation at the restrictive temperature, we assayed the level of tyrosine phosphorylated p34^{cdc2} under these growth conditions. For comparison, a wild type strain had to be employed because in the absence of extra *nim*E^{cyclinB} the *nim*T23 strain would not grow significantly at the restrictive temperature (Figures 2 and 3). Under conditions where extra cyclin allows a strain containing the *nim*T23 mutation to undergo vegetative growth, there

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GAATTCAACCTGCTGCAATTGAGAAACATTGGACGTCTAGTTTACCGTGACTGCATCCAT   60
TCATACTCGCCGTCGTAGCGTATGCTCTGCTAGATAGCGTCGCCGTTTCGTGCACTCGGA  120
TCAGAGGGTTAGAGGGCTTGAGGGAAACAGTCATTTGCTGCCATGGAACATTCCTCGCCT  180
                                    M  E  H  S  S  P              6

TTGGCTGCCATGCAGCCTCCGTCTGTAATGCTGGGGCATTGTTTCCGTTCAGATGCACCG  240
L  A  A  M  Q  P  P  S  V  M  L  G  H  C  F  R  S  D  A  P     26

ACGTCTTATCATGGATTTAGTCCTCTACCAGGGCTAGGCCCCGGCGGGTTTAACTTCAAG  300
T  S  Y  H  G  F  S  P  L  P  G  L  G  P  G  G  F  N  F  K     46

GACTTGTCCATGAAGAGGTCTAACGGAGACTACTTCGGTACGAAGGTCGTCCGGGGCTCA  360
D  L  S  M  K  R  S  N  G  D  Y  F  G  T  K  V  V  R  G  S     66

TCTCCAACAGCGAGCCTTGCTGCAGATCTGTCTCAGAATTTTCACATTGATCAGAGCCCC  420
S  P  T  A  S  L  A  A  D  L  S  Q  N  F  H  I  D  Q  S  P     86

CAGGTTGCTACGCCACGACGGTCTTTGTTCTCTGCTTGCCTACTGGGAAATGGAAACAGA  480
Q  V  A  T  P  R  R  S  L  F  S  A  C  L  L  G  N  G  N  R    106

CGTGGGGTCGATGATGCGATGACCACTCCGCCCCTTCCATCCTCATCACCTGCACCCGCG  540
R  G  V  D  D  A  M  T  T  P  P  L  P  S  S  S  P  A  P  A    126

ATGGATATCATGGACATGTCCCCACTGCCTCACAAACCTCCATTTATCAGTACGCCTGAA  600
M  D  I  M  D  M  S  P  L  P  H  K  P  P  F  I  S  T  P  E    146

ATCGAGCTGGACTCGCCAACTCTGGAGAGCTCCCCAATGGACACGACTATGATGTCAACT  660
I  E  L  D  S  P  T  L  E  S  S  P  M  D  T  T  M  M  S  T    166

GATGGCCTTGTTCCGGATTCACCAACGGTTCTACCGAAGGATGGTAAACAAGAGAGAAGA  720
D  G  L  V  P  D  S  P  T  V  L  P  K  D  G  K  Q  E  R  R    186

CGTCCGACTTTTCTGCGGCCTAGCTTGGCGAGAAGCAAAGCGCAGTCCTTCCAGGTCGGA  780
R  P  T  F  L  R  P  S  L  A  R  S  K  A  Q  S  F  Q  V  G    206

ATGACCAGACCTGCCCCGGAAAGCCAAGGACCGCCCTTCAAGTTTCAGACCAATGGAATC  840
M  T  R  P  A  P  E  S  Q  G  P  P  F  K  F  Q  T  N  G  I    226

AACAAGACATCATCAGGCGTAGCTGCATCATTGGAGGACATGTTTGGCGAATCCCCACAG  900
N  K  T  S  S  G  V  A  A  S  L  E  D  M  F  G  E  S  P  Q    246

CGTGAACGGCCCATGATGCGCATCAATTCAACCAGTGGCCTCAACTCGCGTTTAAGACCA  960
R  E  R  P  M  M  R  I  N  S  T  S  G  L  N  S  R  L  R  P    266

CCATTAGGAAGCGGGAGCCATGTCCGTGGCAATGGTTCACCATCGGCGGCTTCAGTTCGG 1020
P  L  G  S  G  S  H  V  R  G  N  G  S  P  S  A  A  S  V  R    286

AAAAGTGCGCACCCGAATATGCGGCCCCGCAAGCAATGCAGAAGATCATTGAGTATGTAC 1080
K  S  A  H  P  N  M  R  P  R  K  Q  C  R  R  S  L  S  M  Y    306

GAACATCCCGAGGATGTAATTGCCGACAGCGAGGTTTCTTATACATCAAATGCACCACTC 1140
E  H  P  E  D  V  I  A  D  S  E  V  S  Y  T  S  N  A  P  L    326

CAGTCCATTAGCGACTTCGAAGAAACACAGGCTCTGCAGCTACCTCACTTCATTCCCGAG 1200
Q  S  I  S  D  F  E  E  T  Q  A  L  Q  L  P  H  F  I  P  E    346

GAGCAAGCAGACAATTTAGGTCGCATCGACAAGGCCACCCTCGTGGATATCAAAGAGGGA 1260
E  Q  A  D  N  L  G  R  I  D  K  A  T  L  V  D  I  K  E  G    366

AAATATGATAATATGTTTGACAATATCATGATCATTGATTGCCGGTTTGAATATGAATAC 1320
K  Y  D  N  M  F  D  N  I  M  I  I  D  C  R  F  E  Y  E  Y    386

GATGGCGGGCACATCGTCGGAGCTGTCAATTACAATGACAAGGAGAATCTGGCCGCCGAA 1380
D  G  G  H  I  V  G  A  V  N  Y  N  D  K  E  N  L  A  A  E    406

CTTTTGCGGACCCGAAGCCACGGACTGCTATAGTCTTCCACTGCGAGTATTCGGTACAC  1440
L  F  A  D  P  K  P  R  T  A  I  V  F  H  C  E  Y  S  V  H    426

AGGGCTCCTCTTATGGCCAAGTATATCCGGCATCGGGATCGTGCTTATAATGTGGATCAT 1500
R  A  P  L  M  A  K  Y  I  R  H  R  D  R  A  Y  N  V  D  H    446

TACCCCCAACTATCTTATCCTGATATGTACATTCTAGAAGGGGGCTACAGTGGTTTCTTT 1560
Y  P  Q  L  S  Y  P  D  M  Y  I  L  E  G  G  Y  S  G  F  F    466

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A  E  H  R  S  L  C  Y  P  Q  N  Y  V  E  M  S  A  K  E  H    486

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R  N  N  P  G  D  R  K  L  L  A  S  P  F  N  D  S  P  G  S    546

CGCTTTCCTGGCCGTCGCATGCTTTCATACTAATTTCAGTATGGAAATTCAACAAAGCAT 1860
R  F  P  G  R  R  M  L  S  Y  *                               556

CTTCATTTGCGGTTCCTCCACCCATGCCCTCTGCATGGTTTAGCGACCTATGATTCTTGG 1920
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AACAGTCCAAGTTCACCCTCACTCGCTTTCAGATTTTCTACCTATCTTCCCTACGATATT 2100
TACCCATACCTTATTCACCACCACATTAAAACATCCCATATCGCGTCAGTCTCACCACAA 2160
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AACATAAACACATTAGTTTCCTTTGTTAGAGAATGGAATAATTCCGGAATTC  2632
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Fig. 6. Nucleotide and derived amino acid sequence of the *nim*T cDNA. The sequence data of *nim*T have been deposited in the EMBL Nucleotide Sequence Database under the accession number X64601.

is a large increase in the level of tyrosine phosphorylation of p34^{cdc2} which is precipitated by p13−Sepharose (Figure 10B, compare lane 1 with lane 4). In addition, there is an increase in the actual amount of p34^{cdc2} that is precipitated by p13−Sepharose (Figure 10C, compare lane 1 with lane 4). These data indicate that the presence of extra cyclin allows growth and nuclear division under conditions when high levels of the p34^{cdc2} protein is phosphorylated on tyrosine.

As the *nim*T23^{cdc25} mutation is reversible we assayed the level of H1 kinase activity associated with the p13 precipitated p34^{cdc2} from cells containing normal levels or extra cyclin B (Figure 11). Cells were shifted to the restrictive temperature for 3 h to allow accumulation of the elevated levels of tyrosine phosphorylated p34^{cdc2}. After the addition of benomyl, the cells were returned to permissive temperature to allow entry into, but not through, mitosis. The presence of additional cyclin B allows the accumulation of a larger pool of inactive tyrosine phosphorylated p34^{cdc2} which is able to become active as an H1 kinase when the *nim*T^{cdc25} function is returned, as the presence of additional cyclin B yields significantly more H1 kinase activity (Figure 11, compare lane 2 with 3 and 4). This indicates that extra cyclin B causes the accumulation of high levels of potential p34^{cdc2} H1 kinase activity that is able to become active if *nim*T^{cdc25} is allowed to function.

These data demonstrate that the *nim*T23 strain is able to grow in the presence of extra cyclin even though the level of tyrosine phosphorylated p34^{cdc2} is high. As tyrosine phosphorylation of p34^{cdc2} inhibits its function we would have predicted that high levels of tyrosine phosphorylated p34^{cdc2} would arrest cell cycle progression in G_2. Therefore, to investigate how effective the extra dosage of *nim*E^{cyclinB} was at suppressing the cell cycle defect caused by the *nim*T23 mutation, we monitored the number of nuclei per germling in three different strains grown at the restrictive temperature for a period of 12 h (Table II). During germination of spores to germlings the nuclei of *A.nidulans* are in a common cytoplasm as no cell division occurs after mitosis. The number of nuclei per germling is thus a direct measure of the number of nuclear divisions which have occurred for that cell and is therefore a measure of how rapidly the nuclear division cycle is progressing. The wild type strain contained significantly more nuclei per cell when compared to the *nim*T23 or the *nim*T23 plus *nim*E^{cyclinB} strains. However, it is clear that the number of nuclear divisions that can occur in the *nim*T23 strain with an extra *nim*E^{cyclinB} was greater than in the strain that contained the *nim*T23 mutation alone. These data demonstrate that although increased dosage of *nim*E^{cyclinB} allows cells containing the *nim*T23 mutation to grow at the restrictive temperature, the nuclear division cycle in such strains is very much slowed down leading to fewer nuclei per cell than normal.

We also investigated the effect of increased dosage of *nim*E^{cyclinB} in a wild type strain to see if this had any effects on the tyrosine phosphorylation state of p34^{cdc2}. Increased copy number of *nim*E^{cyclinB} led to an increase in the steady state level of tyrosine phosphorylated p34^{cdc2} when compared with a wild type strain (Figure 10B, compare lane 3, normal level of *nim*E^{cyclinB}, with lane 2, extra copies of *nim*E^{cyclinB}). This indicates that increased expression of *nim*E^{cyclinB} leads to an increase in the pool size of tyrosine phosphorylated p34^{cdc2} (pre-MPF). We also noted that in the two samples grown without extra *nim*E^{cyclinB}, the α-PSTAIR antisera detected a doublet (Figure 10C, lanes 1 and 3). However, in the presence of extra *nim*E^{cyclinB} primarily the lower band was detected (Figure 10C, lanes 2 and 4). This difference could be due to tyrosine phosphorylation state.

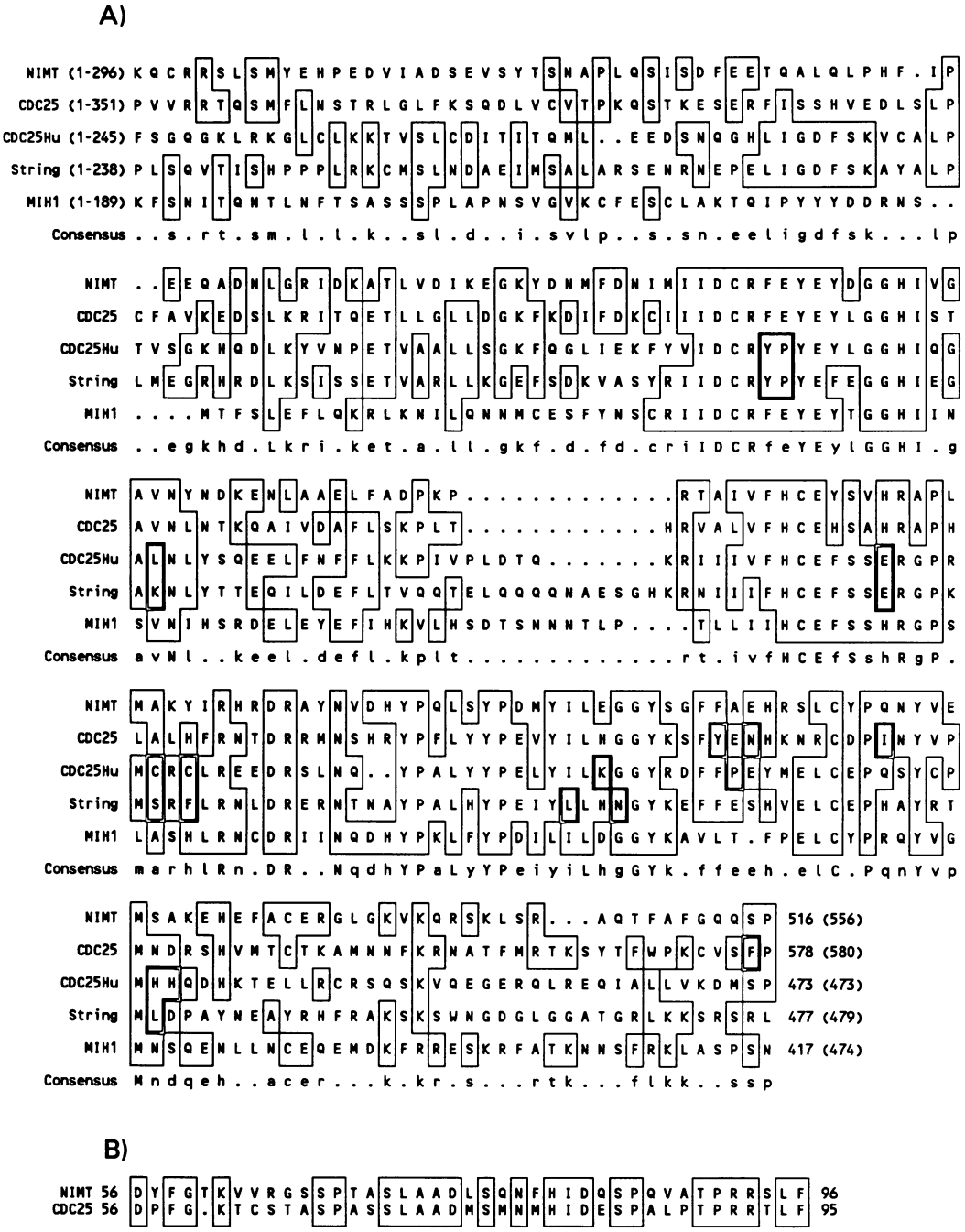

Fig. 7. Alignment of NIMT with CDC25 homologs. (**A**) The derived amino acid sequence of NIMT from amino acids 297−516 were aligned to the multiple alignment of cdc25 homologs as generated by Sadhu *et al.* (1990), using the UWGCG program LINEUP (Devereux *et al.*, 1984). The numbers in parentheses at the end of the sequences indicate the number of amino acids in the full length proteins. Identical residues are boxed, with variant residues within boxes of identity separated by double lines. The consensus generated by the LINEUP program shows invariant residues in uppercase and variant but conserved residues in lowercase. Periods in the consensus sequence indicate no significant conservation of sequence at that position. Gaps, also indicated by a period, have been introduced to optimize the alignment. Within this region, NIMT shows 38.0% sequence identity to *S.pombe* cdc25 (cdc25, Russell and Nurse, 1986), 34.6% identity of human cdc25 (CDC25Hu, Sadhu *et al.*, 1990), 32.6% identity to *Drosophila melanogaster* string (Edgar and O'Farrell, 1989) and 34.6% identity to the *Saccharomyces cerevisiae* MIH1 amino acid sequence (Russell *et al.*, 1989). (**B**) BESTFIT alignment of NIMT and cdc25 revealed that amino acids 56−96 of NIMT and 56−95 of cdc25 show 60% identity (boxed residues). Similar sequences are not present in the other proteins used in the multiple sequence alignment (A). A single gap in cdc25, indicated by a period, has been introduced to optimize the alignment.

Discussion

Identification of cyclin B and cdc25 homologs in A.nidulans

We have described the identification of two genes that are required for G_2 progression in *A.nidulans*, *nim*E and *nim*T, as encoding homologs of cyclin B and of fission yeast cdc25, respectively. NIME and NIMT are most similar in sequence to the products of the *S.pombe cdc13*$^+$ and *cdc25*$^+$ genes. In the case of NIME and cdc13, this sequence similarity does not extend beyond the region which is conserved between

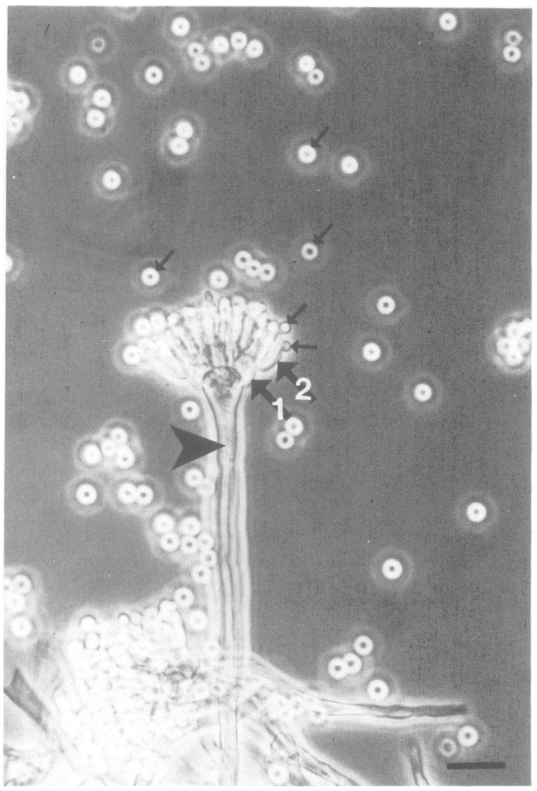

Fig. 8. Conidiophore development in a wild type strain. Conidia were inoculated into medium solidified with 0.7% agar and poured onto a plate solidified with 2% agar. After 40 h of growth at 42°C, aerial hyphae were harvested, fixed and viewed by phase contrast microscopy. A mature conidiophore is shown with the following cell types: conidia, either just forming or free in the fixative (small arrows); primary sterigmata (1); secondary sterigmata (2); vesicle (large arrowhead). Bar = 10 μm. The fixing and mounting of conidiophores causes many mature conidia to be sheared from the conidiophore.

other cyclin B homologs (Figure 5; reviewed by Minshull *et al.*, 1989). Alignment of the NIMT and cdc25 sequences showed, as with alignment to other cdc25 homologs, that there is a significant similarity between the C-terminal halves of the proteins (Figure 7a). Experiments using the C-terminal 60% of the *Drosophila* homolog of cdc25 (String) have shown this peptide to be active and correlates roughly with this region of sequence similarity between the previously identified cdc25-like proteins (Kumagai and Dunphy, 1991). In addition to this conserved domain, NIMT and *S.pombe* cdc25 share a 40 amino acid region of 60% identity near the N-terminus that is not present in the other cdc25 homologs (Figure 7b). Although no role for this region of the protein is known, such conservation of sequence suggests that it may play some role in NIMT/cdc25 function. With the inclusion of two SP and one TP motifs in this region, one obvious hypothesis would be of regulation by phosphorylation. Although this has not yet been tested, the *S.pombe* cdc25 protein has been shown to be phosphorylated on serine and threonine residues *in vivo* (Moreno *et al.*, 1990).

Suppression of the nimT23 mutation in strains carrying an extra copy of nimE

Strains carrying the temperature-sensitive *nim*T23 allele arrest in the G_2 phase of the cell cycle when incubated at the restrictive temperature (Bergen *et al.*, 1984), express low levels of histone H1 kinase activity and high levels of tyrosine phosphorylated $p34^{cdc2}$ (Osmani *et al.*, 1991b). During experiments designed to clone *nim*T by complementation, it was discovered that the temperature sensitivity of *nim*T23 was suppressed in strains that contained an additional copy of wild type *nim*E. These strains showed significant vegetative mycelial growth but failed to undergo normal development to produce asexual spores (conidia) (Figures 2 and 9).

Some evidence has accumulated to suggest that cyclin B may function in part to recruit $p34^{cdc2}$ into an inactive species called pre-MPF consisting of $p34^{cdc2}$ coupled to cyclin. In order to maintain pre-MPF in its inactive form, the $p34^{cdc2}$ in the complex is phosphorylated on a tyrosine residue located in the putative ATP binding site. Only part of the $p34^{cdc2}$ pool is thought to be recruited into pre-MPF in each cell cycle (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991). Pre-MPF is then activated in G_2 by tyrosine dephosphorylation mediated by $cdc25^+$ to yield active MPF. Our experiments show that increased copy number of cyclin B results in an increased pool of pre-MPF and also leads to suppression of a mutation in $nimT^{cdc25}$. By introducing extra $nimE^{cyclinB}$ into a wild type cell we were able to observe an increase in the pool size of steady state levels of pre-MPF (Figure 10B). This effect was more clearly observed at the arrest point of $nimT^{cdc25}$ when an extra copy of $nimE^{cyclinB}$ was present in the strain. In a strain containing one extra copy of $nimE^{cyclinB}$ and the *nim*T23 mutation, compared with a strain with just the *nim*T23 mutation, we routinely detected elevated levels of tyrosine phosphorylated $p34^{cdc2}$ at any time after the shift to the restrictive temperature (for e.g. Figure 10A). Additionally, in the *nim*T23 strain grown at the restrictive temperature which was suppressed by extra $nimE^{cyclinB}$, we detected high levels of pre-MPF and the cells were able to grow vegetatively.

Increased copy number of $nimE^{cyclinB}$ leads to an increase in the pool size of pre-MPF and suppresses mutation of $nimT^{cdc25}$. These two observations suggest a possible mechanism by which the suppression of *nim*T23 by extra $nimE^{cyclinB}$ may take place. The increased pre-MPF pool generated by extra $nimE^{cyclinB}$ will effectively increase the substrate pool (pre-MPF) for NIMT-mediated dephosphorylation. Therefore during incubation of the *nim*T23 strain carrying an extra copy of *nim*E at the restrictive temperature, enough pre-MPF is eventually dephosphorylated to enable some nuclei to advance into mitosis. Consistent with this model is the observation that the cell cycle in the *nim*T23 strain suppressed by extra $nimE^{cyclinB}$ is very much slowed down, most likely due to a prolonged G_2 period. In addition, the *nim*T23 mutation probably does not cause total loss of function at the restrictive temperature as after prolonged incubation some nuclear division is apparent (Table II). As Southern analysis failed to reveal other cdc25-related sequences of *A.nidulans*, these data are evidence for slight leakiness of the temperature-sensitive *nim*T23 mutation and thus it is likely that the partially functional protein leads to some mitotic induction when an increased substrate pool of pre-MPF is available.

Based solely on the suppression of mutation of $nimT^{cdc25}$ by extra dosage of $nimE^{cyclinB}$ a second explanation is possible. High copy number suppression of a temperature-sensitive mutation is indicative of a physical interaction

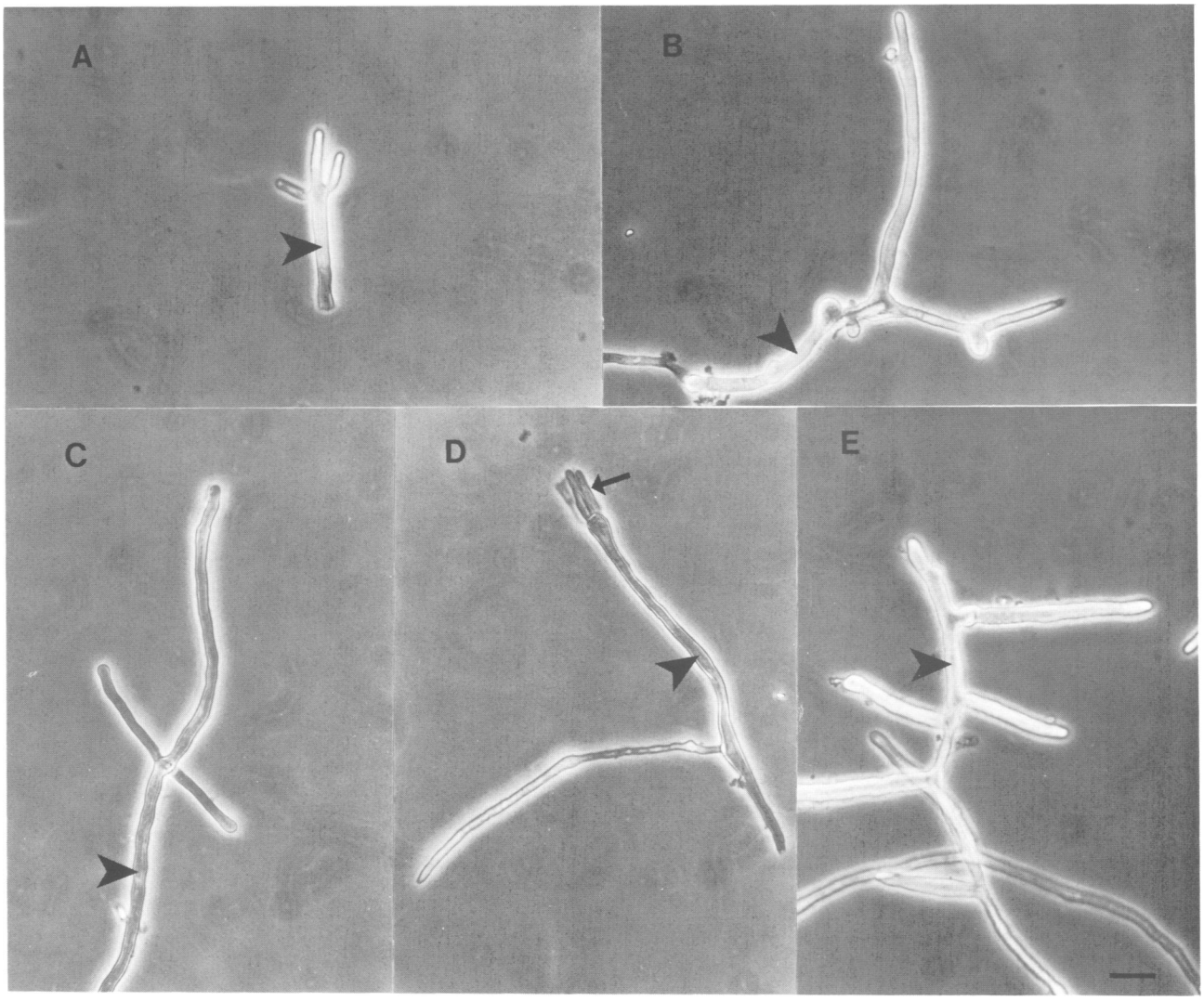

Fig. 9. Defective conidiophore development in the *nim*T23 strain carrying an additional copy of *nim*E^{cyclinB}. Representative samples of aerial hyphae harvested from the *nim*T23 strain carrying an additional *nim*E gene are shown (**A**–**E**). Vesicles are indicated by large arrowheads. The small arrow in (D) indicates abnormal primary sterigmata. Bar = 10 μm. These aerial hyphae are all atypical and appear to be unable to form identifiable primary sterigmata. In addition, the vesicles formed are very much branched, something not seen in the wild type strain.

between the two proteins involved. For instance, elevated p34^{cdc2} can suppress temperature-sensitive mutation of *cdc13* in fission yeast (Booher and Beach, 1987) due to their documented physical interaction. By this scenario the increased copy number of *nim*E^{cyclinB} would promote interaction between the *nim*E^{cyclinB} and *nim*T^{cdc25} proteins and so make the mutant *nim*T^{cdc25} more effective at the restrictive temperature. This would predict that normally *nim*E^{cyclinB} and *nim*T^{cdc25} proteins physically interact in the cell and indeed it has recently been reported that human cyclin B and cdc25 type proteins do physically interact with the cyclin B causing activation of the cdc25 phosphatase activity (Galaktionov and Beach, 1991). Of course, the suppression of *nim*T23 by extra *nim*E^{cyclinB} could be the result of a combination of the two suggested interactions, i.e. (i) increased pre-MPF substrate pool size and (ii) increased effectiveness of the mutant *nim*T^{cdc25} caused by the extra copy of *nim*E^{cyclinB} promoting physical interaction between the two proteins.

Although we favor the above hypothesis, we cannot rule out the possibility that other phosphotyrosine phosphatases unrelated to NIMT may also be dephosphorylating Y15 of p34^{cdc2} under conditions when the level of pre-MPF is artificially high. This possibility does not detract from the hypothesis that the suppression is due to increased pool size of pre-MPF generated by the extra copy of *nim*E^{cyclinB} as demonstrated here.

Cell cycle regulation and development of A.nidulans

The suppression of *nim*T23 by additional NIME allows significant vegetative growth, however, these strains fail to undergo normal asexual spore development. The process of spore formation in *A.nidulans* involves the differentiation of the vegetative mycelia into an aerial structure called a conidiophore that contains several distinct, specialized cell types. First, aerial hyphae, called vesicles, are generated which grow from the mycelial mat growing in the agar of the growth media. The vesicle essentially serves as an aerial extension on which the actual spore forming cells develop (Figure 8). This phase of development was unaffected in the *nim*T23 + *nim*E^{cyclinB} strain (Figure 9 and Table I) and aerial vesicles were produced in this strain on a time scale

Table I. Development of conidiophore structures in a *nim*T23 strain carrying an additional copy of *nim*E^{cyclinB}

Conidiophore development	Wild type	*nim*T23 + *nim*E^{cyclinB}
Vesicle only	0.8%	66.7%
Vesicle + primary sterigmata	1.3%	33.0%[a]
Vesicle + primary sterigmata + secondary sterigmata	97.9%	0.3%

Strains were grown as described in Figure 8, fixed and viewed by phase contrast microscopy. Total count for wild type was 613 and for *nim*T23 + *nim*E, 573. Emergence of aerial vesicles occurred for both strains at 20–24 h. [a]This includes morphologically abnormal cells resembling primary sterigmata (see text).

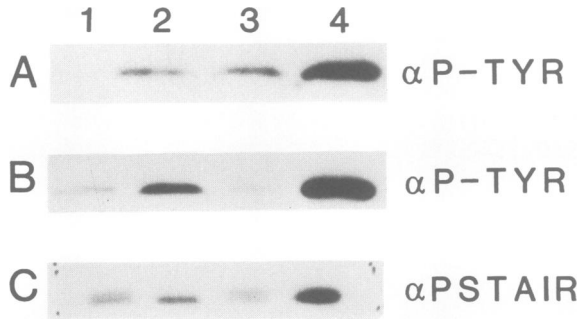

Fig. 10. Effects of extra *nim*E^{cyclinB} and *nim*T23 on tyrosine phosphorylation of pre-MPF. (**A**) Pre-MPF was precipitated using p13–Sepharose from a *nim*T23 strain (SO53) and a *nim*T23 + *nim*E^{cyclinB} strain (TR9) grown at 32°C (lanes 1 and 3) or shifted to 42°C for 3 h (lanes 2 and 4). The p13 purified protein was Western blotted using anti-phosphotyrosine antibodies using [^{125}I]protein A to visualize interactive protein. (**B** and **C**) Proteins were affinity purified and processed as in A. A wild type strain R153 (lane 1) and a *nim*T23 + *nim*E^{cyclinB} strain (TR9, lane 4) were grown at 42°C. A wild type strain (R153) was also grown at 37°C (lane 3) along with a wild type strain transformed with extra *nim*E^{cyclinB} (GR5 + *nim*E^{cyclinB}, lane 2). A portion of the protein affinity purified on p13–Sepharose (7/8) was probed with anti-phosphotyrosine (B) and the remainder (C) probed with anti-PSTAIRE.

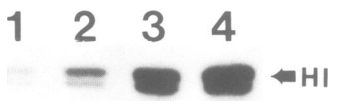

Fig. 11. Additional cyclin B leads to elevated levels of p34^{cdc2} H1 kinase activity. Cells were shifted to 42°C for 3 h and then returned to 26°C in the presence of 5 μg/ml benomyl for 45 min. p34^{cdc2} was affinity purified using p13–Sepharose and its H1 kinase activity determined. An autoradiogram of phosphorylated histone H1 is shown here. A wild type strain (lane 1), a *nim*T23^{cdc25} strain (lane 2) and a *nim*T23^{cdc25} strain containing additional copies of cyclin B (lanes 3 and 4).

similar to the wild type strain. The next phase of development involves the growth and differentiation of the tip of the vesicle to produce a smaller cell type called primary sterigmata. It was this stage of development that was defective in the *nim*T23 + *nim*E^{cyclinB} strain. Wild type primary sterigmata are arrowed '1' in Figure 8. The primary sterigmata further differentiate to produce another cell type called the secondary sterigmata (arrowed as '2' in Figure 8). It is the secondary sterigmata that actually produce the asexual spore by a process of budding coupled to an asymmetric mitosis to generate a string of spores from a

Table II. Effect of extra *nim*E^{cyclinB} on inhibition of nuclear division of *nim*T23

Strain	Number of nuclei per cell
R153 (wild type)	6.9 ± 1.10
SO52 (*nim*T23)	1.4 ± 0.50
TR9 (*nim*T23 + *nim*E^{cyclinB})	2.7 ± 0.77

The indicated strains were grown for a period of 12 h at the restrictive temperature of 42°C at which time they were fixed and stained with DAPI to visualize the number of nuclei per cell.

single progenitor nucleus present in the secondary sterigmata (reviewed by Timberlake, 1990).

The observation that the *nim*T23 + *nim*E^{cyclinB} strain is defective in the production of normal primary sterigmata suggests that this phase of differentiation is particularly sensitive to any perturbation of normal cell cycle regulation. As this strain has elevated levels of tyrosine phosphorylated p34^{cdc2} and is likely to have problems making the G_2–M transition, it suggests that lack of nuclear division leads to an inability to developmentally progress from the vesicle cell stage to the primary sterigmata cell stage. A checkpoint may therefore link correct cell cycle regulation to development of primary sterigmata. This would then ensure that enough nuclei were generated in the conidiophore to drive the process of spore formation. Clearly our result linking correct nuclear division control to correct development demonstrates that in *A.nidulans* cell cycle regulation is tightly coupled to development.

*nim*E^{cyclinB}, *nim*T^{cdc25} and the study of cell cycle control in *A.nidulans*

Experiments investigating cell cycle control which have utilized *A.nidulans* as a model system have uncovered two cell cycle regulators that are thus far only identified in this organism, namely NIMA and BIME. As discussed in the Introduction, NIMA is a protein kinase that is required in addition to MPF for advancement into mitosis. The *bim*E gene product is a 229 kDa protein that is a putative negative regulator of the cell cycle (Osmani *et al.*, 1988b; Engle *et al.*, 1990). One limitation to the investigation of the role of these gene products in cell cycle control has been the inability to investigate how they interact with the cell cycle regulators that have been identified in other systems. The identification of the *nim*E and *nim*T genes as encoding positive regulators of the central MPF activation pathway will permit experiments that address the interaction between NIMA, BIME and MPF to be further pursued in *A.nidulans*.

Finally, our results also serve to confirm the importance of *nim*E^{cyclinB} and *nim*T^{cdc25} (and their homologs in other systems) in mitotic regulation. Both have now been identified independently in *A.nidulans* by a mutational screen designed to isolate cell cycle specific genes, only the second time this has been done for a cyclin B type protein.

Materials and methods

Aspergillus strains, libraries and manipulations

The *A.nidulans* strains used in this study were: R153 (*wA3 pyro*A4), SO28 (*nim*E6 *wA2 biA1 paba*A1), SO30 (*nim*T23 *wA2 nicA2 cho*A1 *cnx*E16), SO53 (*nim*T23 *wA2*); SO31 (*nim*E6 *fwA1 nicA2 meth*G1 *paba*A1), GR5 (*pyr*G89 *wA3 pyro*A4) SO25 (*nim*E6 *pyr*G89 *cha*A1 *biA1 meth*G1 *cho*A1 *paba*A1) and SO26 (*nim*T23 *pyr*G89 *wA2 biA1 paba*A1). cDNA clones were isolated from a library constructed in λgt10 (Osmani *et al.*, 1988b).

The chromosome II-specific cosmid library was obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Department of Microbiology) and is described by Brody *et al.* (1991). Standard procedures for the isolation of cosmid DNA and recombinant DNA manipulations were used (Sambrook *et al.*, 1989). Transformation, media for propagation and genetic manipulations of *A.nidulans* were as previously described (Osmani *et al.*, 1987).

PCR amplification of cdc25-related sequences

Amplification of *cdc25*-related sequences by PCR was performed using a Perkin Elmer Cetus GeneAmp PCR kit and a Perkin Elmer Cetus DNA Thermal Cycler. Primers used were: primer 1 (forward primer) AT(TCA)G-A(CT)TG(CT)CGNTT(CT)GA(AG)TA(CT)GA; primer 2 (reverse primer) C(TG)(GA)TGNG(AC)(GA)CT(GA)AA(CT)TA(GA)CA(GA)TG; and primer 3 (reverse primer) TGNA(AG)(GAT)AT(GA)TANA(TC)NTC-NGG(GA)TA. where N = any nucleotide and parentheses indicate alternatives at these positions. Primer 1 is 384-fold degenerate and corresponds to the amino acid sequence IDCRFEY(DE) (amino acids 436–443 in *S.pombe* cdc25). Primer 2 is 1024-fold degenerate and corresponds to the amino acid sequence HCEFS(SA)HR (amino acids 479–486 in *S.pombe* cdc25). Primer 3 is 12288-fold degenerate and corresponds to the amino acid sequence YP(DE)YI(LF)(HN) (amino acids 511–518 in *S.pombe* cdc25). Reactions using 50 ng of DNA from the *A.nidulans* λgt10 based cDNA library (Osmani *et al.*, 1988b) and 1.25 μg of primers 1 and 3 were carried out using the kit reagents under the following conditions: 1 min at 94°C, 2 min at 45°C and 3 min plus 5 s per cycle at 72°C for 30 cycles followed by an additional 7 min at 72°C. The PCR products were resolved on a 2% low gelling temperature agarose gel and a single band specific for both primers of approximately 250 bp was isolated. Approximately 10 ng of this DNA was used as substrate for reactions performed under identical conditions using primers 1 and 2. A strongly amplified band of ~150 bp was isolated, cloned into *Eco*RV digested pBluescriptKS$^-$ (Stratagene) and sequenced. After confirmation of the presence of an open reading frame predicting a cdc25-related protein, this clone was used as a probe for the isolation of *A.nidulans cdc25*/*nim*T cDNAs.

DNA sequencing and sequence analysis

Single-stranded templates for DNA sequencing were rescued from pBluescript based clones using the helper phage M13VCS. Sequencing reactions using standard primers made against pBluescript sequences or specific oligonucleotides made against the *nim*T and *nim*E sequences were performed using a Sequenase Version 2.0 (United States Biochemical Corp.) sequencing kit using the recommended conditions. Computer analysis of sequence data was performed using the University of Wisconsin GCG package (Devereux *et al.*, 1984).

Protein kinase assays and Western blot analysis

Preparation of extracts, p13–Sepharose affinity purification of p34^{cdc2} and Western blotting were as described by Osmani *et al.* (1991b).

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