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The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*

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The gene encoding the homologue of the catalytic subunit of the Ca^{2+}/calmodulin-regulated protein phosphatase 2B (calcineurin A) has been isolated from *Aspergillus nidulans*. This gene, *cnaA*$^+$, is essential in this fungal system. Analysis of growth-arrested cells following gene disruption by homologous recombination reveals that they are blocked early in the cell cycle. The *cnaA*$^+$ gene encodes a 2.5 kb mRNA and the deduced protein sequence is highly homologous to the calcineurin A subunit of other species. The mRNA varies in a cell cycle-dependent manner with maximal levels found early in G_1 and considerably before the G_1/S boundary. As calmodulin is also essential for *A.nidulans* cell cycle progression and levels rise before the G_1/S boundary, our data suggest that calcineurin may represent a primary target for calmodulin at this cell cycle transition point.

Key words: *Aspergillus nidulans*/calcineurin A/calmodulin/cell cycle progression/*cnaA*$^+$ gene

Introduction

Calmodulin (CaM) is an essential gene in the filamentous fungus *Aspergillus nidulans* (Rasmussen *et al.*, 1990). Creation of a strain conditional for the expression of the unique CaM gene revealed that this Ca^{2+} receptor protein was required at multiple points in the cell cycle (Lu *et al.*, 1992), observations entirely compatible with those made in mammalian cells (Rasmussen and Means, 1987, 1989a; Lu and Means, 1993). Cell cycle analysis suggested primary roles in G_2/M and G_1/S. In the former case, Ca^{2+} and CaM were shown to be required for activation of the $p34^{cdc2}$ and NIMA protein kinases (Lu and Means, 1993), both of which are essential for the G_2/M transition in *A.nidulans* (Osmani *et al.*, 1991). As NIMA is a phosphoprotein and phosphorylation is required for enzyme activity (Lu *et al.*, 1993a), a Ca^{2+}/CaM-dependent protein kinase is the likely CaM target. Supporting this suggestion is the observation that inducible overexpression of a constitutively active calmodulin kinase produces a G_2 arrest (Planas-Silva and Means, 1992). However, nothing is known about the pathway by which CaM regulates G_1/S.

Recent studies have suggested that the Ca^{2+}/CaM-regulated phosphatase 2B plays an important role in early cell cycle progression. This enzyme, also known as calcineurin, is composed of a catalytic subunit (CnA) and a Ca^{2+}-binding regulatory subunit (CnB) (Kincaid, 1993). Two CnA and one CnB genes have been isolated from *Saccharomyces cerevisiae* (Cyert *et al.*, 1991; Kuno *et al.*, 1991; Lui *et al.*, 1991; Cyert and Thorner, 1992). Whereas disruption of all three of these genes did not result in a lethal phenotype, such cells fail to recover from the arrest produced by the α-factor mating pheromone. As this recovery is equivalent to cell cycle re-entry, these observations are compatible with a role for calcineurin in G_0/G_1. Calcineurin is also required in T lymphocytes for the activation of the NFAT transcription factor involved in regulation of the interleukin-2 (IL-2) receptor gene (McCaffrey *et al.*, 1993). This enzyme is also a target for immunosuppressant drugs such as cyclosporin A and FK-506, which prevent activation of NFAT in response to a mitogenic signal (Schreiber and Crabtree, 1992). Since lymphokine gene transcription is an early requisite step in the pathway that leads to lymphocyte proliferation, these observations are also compatible with a G_0/G_1 role for calcineurin.

In the present study, we have cloned and sequenced the *cnaA*$^+$ gene from *A.nidulans*. The structure of the gene and predicted sequence of the protein are remarkably similar to those features of CnA from other species. Gene disruption reveals the *cnaA*$^+$ gene to be essential and analysis of the arrested cells suggests a block early in the nuclear division cycle, probably in G_1. As the CnA mRNA also fluctuates during the cycle and is maximal in G_1, the collective results support a role for CnA in passage through this phase of the cell cycle. This is the first demonstration of a Ca^{2+}/CaM-dependent enzyme being essential for cell growth and division, and suggests that CnA may represent a primary target for Ca^{2+}/CaM in G_1.

Results

Cloning and characterization of the A.nidulans CnA gene

In order to select clones containing the *A.nidulans* CnA gene, we used a hybridization probe obtained by polymerase chain reaction (PCR) amplification of a region of the *Neurospora crassa* CnA gene (Higuchi *et al.*, 1991), highly conserved relative to the murine CnA coding sequence (Kincaid *et al.*, 1990; Higuchi *et al.*, 1991). A positive phage was obtained that contained two *Sac*I restriction fragments of 2.5 and 1.0 kb, each of which hybridized to the CnA PCR probe on Southern blots. These two fragments were subcloned into pUC19 for sequence analysis. Initial analysis confirmed that both *Sac*I fragments contained open reading frames that would encode protein sequences highly homologous to those found in both mouse and *N.crassa* CnA. Sequence analysis

A

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      5'
-150  tgttctgtcttggtgtgttcacgagcagctaggttcttgcgatttgtcttgccagataattctcgccattgcccc

 -75  aagatggatcggaatctagcgcgcgctgtggccgataagcagccggttccggaaattgatttcaccctgcatgtc

  +1  ATG GAA GAT GGC ACC CAG GTG TCC ACC CTA GAA CGT GTT GTC AAA G  gtgtgtgctatt
      Met Glu Asp Gly Thr Gln Val Ser Thr Leu Glu Arg Val Val Lys G

 +61  gattcaacttttggtgataccgcagctgaacattgatgtttcctca  gAG GTG CAA GCA CCC GCC TTG
                                                      lu Val Gln Ala Pro Ala Leu

+126  AAC AAA CCA TCA GAC GAT CAG TTT TGG GAC CCC GAA GAA CCA ACG AAA CCT AAT CTC
      Asn Lys Pro Ser Asp Asp Gln Phe Trp Asp Pro Glu Glu Pro Thr Lys Pro Asn Leu

+183  CAG TTT CTC AAG CAA CAC TTC TAT CGG GAG GGT CGC CTT ACC GAG GAC CAG GCG CTA
      Gln Phe Leu Lys Gln His Phe Tyr Arg Glu Gly Arg Leu Thr Glu Asp Gln Ala Leu

+240  TGG ATT ATA CAG GCG GGT ACT CAA ATC CTG AAG TCG GAG CCC AAC CTG CTG GAA ATG
      Trp Ile Ile Gln Ala Gly Thr Gln Ile Leu Lys Ser Glu Pro Asn Leu Leu Glu Met

+297  GAC GCG CCC ATA ACT GTG TGC GGT GAT GTT CAC GGG CAG TAC TAC GAT CTG ATG AAG
      Asp Ala Pro Ile Thr Val Cys Gly Asp Val His Gly Gln Tyr Tyr Asp Leu Met Lys

+354  CTG TTT GAG GTG GGA GGA GAC CCT GCT GAG ACG CGT TAT CTT TTC CTG GGC GAC TAT
      Leu Phe Glu Val Gly Gly Asp Pro Ala Glu Thr Arg Tyr Leu Phe Leu Gly Asp Tyr

+411  GTC GAT CGA GGC TAC TTC AGT ATT GAG gtgagttccctgcctctaatcactcggcctgtctgacct
      Val Asp Arg Gly Tyr Phe Ser Ile Glu

+477  tttcag  TGT GTC CTG TAC CTA TGG GCA CTG AAG ATC TGG TAT CCG AAT ACA CTC TGG
               Cys Val Leu Tyr Leu Trp Ala Leu Lys Ile Trp Tyr Pro Asn Thr Leu Trp

+534  TTG CTT CGC GGC AAC CAC GAA TGT CGA CAC TTG ACA GAT TAT TTT ACT TTC AAG TTG
      Leu Leu Arg Gly Asn His Glu Cys Arg His Leu Thr Asp Tyr Phe Thr Phe Lys Leu

+591  GAA TGT AAG CAT AAA TAT AGC GAG CGC ATC TAT GAA GCC TGC ATT GAG TCG TTT TGC
      Glu Cys Lys His Lys Tyr Ser Glu Arg Ile Tyr Glu Ala Cys Ile Glu Ser Phe Cys

+648  GCG CTG CCG CTG GCG GCG GTT ATG AAT AAG CAG TTC CTC TGT ATT CAC GGT GGT TTG
      Ala Leu Pro Leu Ala Ala Val Met Asn Lys Gln Phe Leu Cys Ile His Gly Gly Leu

+705  AGC CCT GAA CTG CAC ACT TTA GAA GAC ATC AAA TCG gtatgttacgcgatgctgaatacttca
      Ser Pro Glu Leu His Thr Leu Glu Asp Ile Lys Ser

+767  acagggtcaggctaacctgcctag    ATC GAT CGA TTC AGA GAA CCC CCA ACT CAC GGG CTC
                                 Ile Asp Arg Phe Arg Glu Pro Pro Thr His Gly Leu

+828  ATG TGC GAT ATC CTC TGG GCC GAT CCT TTG GAG GAC TTC GGT CAA GAG AAG ACT GGC
      Met Cys Asp Ile Leu Trp Ala Asp Pro Leu Glu Asp Phe Gly Gln Glu Lys Thr Gly

+885  GAC TAC TTT ATT CAT AAT AGC GTT CGA GGG TGC TCC TAC TTT TTC TCA TAC CCT GCC
      Asp Tyr Phe Ile His Asn Ser Val Arg Gly Cys Ser Tyr Phe Phe Ser Tyr Pro Ala

+942  GCG TGT GCT TTC CTC GAG AAG AAC AAC TTG CTC TCA GTC ATT CGA GCT CAC GAG GCT
      Ala Cys Ala Phe Leu Glu Lys Asn Asn Leu Leu Ser Val Ile Arg Ala His Glu Ala

+999  CAG GAC GCG GGA TAC CGC ATG TAC CGC AAG ACG CGG ACT ACA GGA TTT CCC AGT GTC
      Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Thr Arg Thr Thr Gly Phe Pro Ser Val

+1056 ATG ACC ATT TTC AGC GCA CCG AAC TAC TTG GAT GTA TAC AAC AAC AAA GCC GCC GTC
      Met Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val Tyr Asn Asn Lys Ala Ala Val

+1113 CTG AAA TAC GAG AAC AAT GTC ATG AAC ATC CGA CAA TTC AAC TGC ACC CCT CAC CCT
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+1170 TAC TGG CTT CCC AAC TTC ATG GAT GTG TTC ACC TGG TCT CTG CCG TTT GTC GGT GAG
      Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu

+1227 AAG ATT ACC GAC ATC GTT ATT GCC ATT CTC AAC ACT TGC TCC AAG GAA GAG CTT GAA
      Lys Ile Thr Asp Ile Val Ile Ala Ile Leu Asn Thr Cys Ser Lys Glu Glu Leu Glu

+1284 GAC GAG ACA CCC TCT ACC ATC TCC CCT GCC GAG CCG TCT CCA CCG ATG CCG ATG GAC
      Asp Glu Thr Pro Ser Thr Ile Ser Pro Ala Glu Pro Ser Pro Pro Met Pro Met Asp

+1341 ACA GTG GAT ACA GAG AGT ACC GAG TTC AAA CGA CGT GCT ATC AAG AAC AAG ATT CTC
      Thr Val Asp Thr Glu Ser Thr Glu Phe ***Lys Arg Arg Ala Ile Lys Asn Lys Ile Leu***

+1398 GCC ATT GGC CGG TTG TCT CGA GTC TTC CAA GTG CTG CGT GAG GAG TCT GAA CGT GTT
      ***Ala Ile Gly Arg Leu Ser Arg Val Phe Gln Val Leu Arg*** Glu Glu Ser Glu Arg Val

+1455 ACG GAA CTT AAG ACC GCG GCT GGA GGT CGA CTT CCT GCC GGT ACT TTA ATG CTT GGT
      Thr Glu Leu Lys Thr Ala Ala Gly Gly Arg Leu Pro Ala Gly Thr Leu Met Leu Gly

+1512 GCG GAA GGA ATT AAG CAA GCC ATC ACG AAC TTT GAA GAT GCC CGC AAA GTT GAT TTA
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+1569 CAG AAC GAA CGT CTC CCG CCT TCT CAC GAT GAG GTC GTC AGA CGA AGC GAA GAG GAA
      Gln Asn Glu Arg Leu Pro Pro Ser His Asp Glu Val Asp Arg Arg Ser Glu Glu Glu

+1626 AGA CGC ATC GCC CTT GAC CGC GCC CAA CAC GAA GCT GAT AAC GAT ACT GGC CTT GCC
      Arg Arg Ile Ala Leu Asp Arg Ala Gln His Glu Ala Asp Asn Asp Thr Gly Leu Ala

+1683 ACA GTT GCA AGG CGC ATT AGC ATG tgagttttattgtcctcgtgcggctatcaactgctaataaacc
      Thr Val Ala Arg Arg Ile Ser Met

+1750 atag    GTC CGT CGG ATC AGG AAA ATC CCG TCG ACA ACG AGA CGG TAG tcggggactag
              Ser Val Arg Arg Ile Arg Lys Ile Pro Ser Thr Thr Arg

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+1882 ccctcaattgttgtagtctgctcgttttggacctgtatggaatccccatctcataggacgggggttaatgtcagga

+1957 agaagggcgtttgtctagg

      3'
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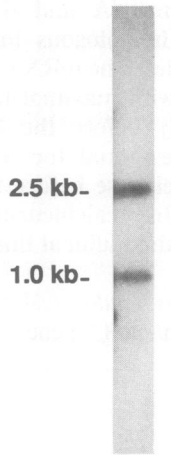
B



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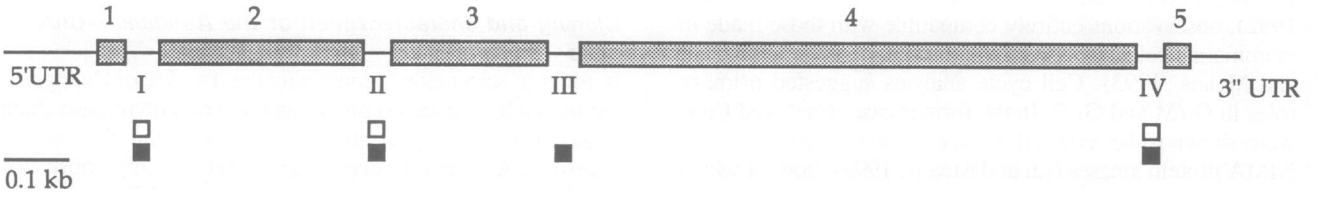

Fig. 1. The *cnaA*$^+$ gene encoding the *A.nidulans* homologue of the CaM-dependent protein phosphatase catalytic subunit (calcineurin A). **(A)** Sequence of the *A.nidulans cnaA*$^+$ gene. The complete nucleotide sequence of the *cnaA*$^+$ gene is shown. Protein coding regions are shown in upper case letters, while non-translated regions are shown in lower case type. Amino acid residues corresponding to the putative CaM-binding domain are indicated by bold-face italic type. **(B)** Southern blot analysis of the *cnaA*$^+$ gene. Genomic DNA from the strain GR5 was digested with the restriction enzyme *Sac*I, and resolved by agarose gel electrophoresis. Southern blotting was performed according to Materials and methods. The data show two hybridization bands corresponding to adjacent *Sac*I restriction fragments and are consistent with a single copy of the *cnaA*$^+$ gene being present in *A.nidulans*. **(C)** Organization of the *cnaA*$^+$ gene. The introns and exons of the *cnaA*$^+$ gene are represented to scale. The open boxes show introns present in the *N.crassa* CnA gene, while the filled boxes correspond to introns present in the murine CnA gene.

A

```
A. nidulans     1  MEDGTQVSTLERVVKEVQAPALNKPSDDQFWDPE.....EPTKPNLQFLKQHF
N. crassa       1  ----s----m---c-d-----mf----e--fed---.....---di-------
Human           1  -aapeparaapp-pppppp-pgadrvv-avpfpp--rl-seevfdldgi-rvdv--nkl
S. cerevisiae   1 mskdlnssrikiik-ndsyikvdrkkdlt-yelengkvis-kdrpiasvpaitgkipsdee

A. nidulans    49 YREGRLTEDQALWIIQAGTQILKSEPNLLEMDAPITVCGDVHGQYYDLMKLFEVGGDPAET
N. crassa      49 --------e------re--kl-ra----------------------------------
Human          60 vk---vd-ei--r--ne-aa--rr-ktmi-ve--------ik--ff----------s--n-
S.c.(CNA1)     62 vfdsktglpnhsflrehffhegrlskeqaikilnmstvalskepnllkl-apiticgdihg

A. nidulans   110 RYLFLGDYVDRGYFSIE.................CVLYLWALKIWYPNTLWLLRGNHECR
N. crassa     110 -----------------.................----------h--k------------
Human         121 -----------------kvlgtedisinphnnine------v---l--s--f---------
S.c.(CNA1)    123 q-yd-lklfev-gdpa-idylflgdyvdrgafsfe-li--ys--lnnlgrf-m-------k

A. nidulans   153 HLTDYFTFKLECKHKYSERIYEACIESFCALPLAAVMNKQFLCIHGGLSPELHTLEDIKSI
N. crassa     153 -----------------a-----m----c------------------------d--rn-
Human         182 ---e-----q---i----rv----m-a-ds-----ll-q----v-------i-------rl
S.c.(CNA1)    184 ---s-----n-mlh--dme--d--cr--nv--------g-yf-----i----ksv--vnk-

A. nidulans   214 DRFREPPTHGLMCDILWADP.......LEDFGQEKTGDYFIHNSVRGCSYFFSYPAACAFL
N. crassa     214 --------q----------.......---------t-f-v--h----------s---h--
Human         243 ---k---af-p---l--s--.......s----n---qeh-sk-t-------yn-p-v-e--
S.c.(CNA1)    245 n----i-sr-----------venyddardgsefdqse-e-vp--l----fa-tfk-s-k--

A. nidulans   268 EKNNLLSVIRAHEAQDAGYRMYRKTRTTGFPSVMTIFSAPNYLDVYNNKAAVLKYENNVMN
N. crassa     268 -------i-----------------------------------------------------
Human         297 qn---------------------sq------li-------------------------
S.c.(CNA1)    306 ka-g---i--------------kynkv-----li-m--------t-h---------e----

A. nidulans   329 IRQFNCTPHPYWLPNFMDVFTWSLPFVGEKITDIVIAILNTCSKEELEDETPST.......
N. crassa     329 ---------------------------------ml----s---e---redsat-spgsasp
Human         358 ------s-----------------------v-emlvnv-si-sdd--mt-gedqfdgsa
S.c.(CNA1)    367 ----hms-------d---------------v-smlvs---i--eq--.....dpesepkaa

A. nidulans   383 .........ISPAEPSPPMPMDTVDTESTEFKRRAIKNKILAIGRLSRVFQVLREESERVT
N. crassa     390 alpsaa.................nq-pd-i-------------------------------
Human         417 ....aar....ke.....................i-r---r---kma---s-------s-l
S.c.(CNA1)    423 eetvkaranatketgtpsdekassailed-tr-k-lr------akv--m-s-------k-e
                                                                      Δ
A. nidulans   435 ELKTAAGGRLPAGTLMLGAEGIKQAITNFEDARKVDLQNERLPPSHDEVVRRSEEERRIAL
N. crassa     435 ----vs-----------------n--ss---------------------kmqd---aq--
Human         449 t--gltptgmlpsgvla-grqtl-.............sg-dvmqlavpqmdwgtphsfanns
S.c.(CNA1)    484 y---mna-v--r-a-ar-t--lnetlst--k---e--i--k----ls--eqekikyyeki-

A. nidulans   496 DRAQHEADNDTGLATVARRISMSVRRIRKIPSTTRR
N. crassa     496 e--tr-----kk-q-ls--l-t-
Human         498 hn-cr-.....f-lffsscl-s
S.c.(CNA1)    545 kg-ekkpql
```

B

CaM Binding Domains of Type 2B Protein Phosphatases

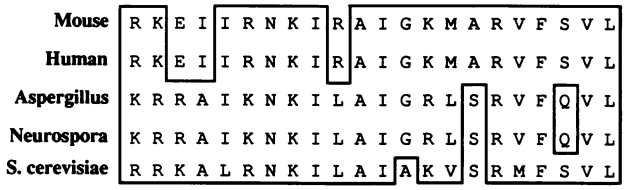

Fig. 2. The predicted *cnaA*$^+$ gene product. (**A**) Alignment of the putative *cnaA*$^+$ gene product with other CnA homologues. The *cnaA*$^+$ protein predicted from the cDNA sequence is compared with other CnA homologues. Sequences were aligned using IntelliGenetics software. The CaM-binding domain is shown in bold-face type. The position of the Ser residue phosphorylated by CaMKII in the vertebrate and yeast sequences is shown by '△'. (**B**) Comparison of CaM-binding domains from CnA homologues. The putative CaM-binding domains from several CnA homologues are compared. Residues identical among these homologues are contained within the boxed regions. Note the remarkable conservation of this sequence among CnA homologues.

also showed that the 2.5 kb fragment contained the 5′ portion of the CnA gene, while the 1.0 kb fragment contained 3′ sequences. The 5′ *Sac*I fragment was then used to screen a λ gt10 cDNA library and full-length clones were obtained. Both the cDNA and genomic clones have been sequenced.

The sequence of the complete gene and the predicted protein product of the gene are shown in Figure 1A. Introns were determined by comparison of the cDNA and genomic clone sequences. The gene contains four introns with sizes typical for *A. nidulans*. All the introns followed the basic rules for intron structure. Each contains a GT at the 5′ donor site and AG at the 3′ donor site (Mount, 1982). Each intron also contains a sequence related to CTRAC which is present in fungal introns (May *et al.*, 1987). All four introns are in locations conserved among CnA genes. Three of the introns (I, II and IV) are in identical positions to the three introns found in the *N. crassa* CnA gene (Figure 1B). Intron 3, not present in the *N. crassa* gene, is in a position conserved in

the murine CnA gene (R.L.Kincaid *et al.*, unpublished observations). Southern blot analysis indicated that *A.nidulans* likely contains a single CnA gene (Figure 1C). Northern blot analysis showed that the internal *SalI* fragment recognized a single 2.5 kb mRNA species, consistent with a single transcription unit for CnA in *A.nidulans* (Figure 3).

Translation of the putative coding sequence of the *A.nidulans* CnA gene yields a 530 amino acid protein with a calculated M_r of 61 kDa. The size is similar to CnA from other species (e.g. mouse, 59 kDa; *S.cerevisiae*, 63–69 kDa; *N.crassa*, 58 kDa). Alignment of *A.nidulans* CnA with *N.crassa*, human and *S.cerevisiae* CnA sequences reveals substantial homology to these other proteins (Figure 2A). *Aspergillus nidulans* CnA was most similar to *N.crassa* CnA, being 84% identical over the entire sequence and 94% identical over a conserved region spanning residues 81–375. The *A.nidulans* CnA protein was 55% identical to human CnA (75% identical in the core region), and 39% identical to the *S.cerevisiae cnaA*$^+$ gene product (48% identical in the core region). The predicted CaM-binding domain shows remarkable conservation among several CnA homologues (Figure 2B). Together, these data show that we have isolated the *A.nidulans* CnA gene. Following accepted designations for *A.nidulans* genes, we have named this gene *cnaA*$^+$.

cnaA$^+$ *mRNA expression during the cell cycle*

Since CaM-dependent processes are required for cell cycle progression, we determined whether the levels of *cnaA*$^+$ mRNA vary during a synchronous cll cycle. To synchronize cells, cultures of exponentially growing *A.nidulans* were incubated for 4 h in medium containing 2 μg/ml nocodazole to arrest cells at metaphase (Nusse and Egner, 1984). Mitotically arrested cells were released by resuspension in medium without nocodazole, and samples removed for isolation of total RNA at 15 min intervals (Figure 3). mRNA levels for both histone H2A (Figure 3A), a control for entry into S phase (May and Morris, 1987; Osmani *et al.*, 1987), and *cnaA*$^+$ (Figure 3B) were determined by Northern blot analysis. As shown, histone H2A mRNA levels varied during the cell cycle as expected, with peak expression from 60 to 90 min, followed by a decrease in mRNA levels and a second peak at 150–165 min after release from nocodazole (Figure 3A). The experiment was not carried out for long enough to observe a second decline in histone H2A mRNA levels. However, the results show that cells pass through two synchronous cell cycles under the conditions used. *cnaA*$^+$ mRNA levels also varied in a cell cycle-dependent manner, but increased much earlier than did H2A mRNA, reaching maximum levels at 30 min, followed by a decline and a second peak around 135–150 min after release (Figure 3B). Determination of the mitotic index after removal of nocodazole indicates that mitosis is complete within 15–20 min. These results indicate that *cnaA*$^+$ mRNA levels increase after the end of mitosis, and before histone H2A mRNA, likely in G_1 prior to the onset of DNA replication in *A.nidulans*.

Effect of cnaA$^+$ *gene disruption*

To disrupt the *cnaA*$^+$ gene, an internal 0.9 kb *SalI* fragment that lacks both 5′ and 3′ sequences was subcloned into the vector pRG3 (May *et al.*, 1989) to produce the plasmid pCnAsp-KO (Figure 4A). pRG3 contains the *N.crassa*

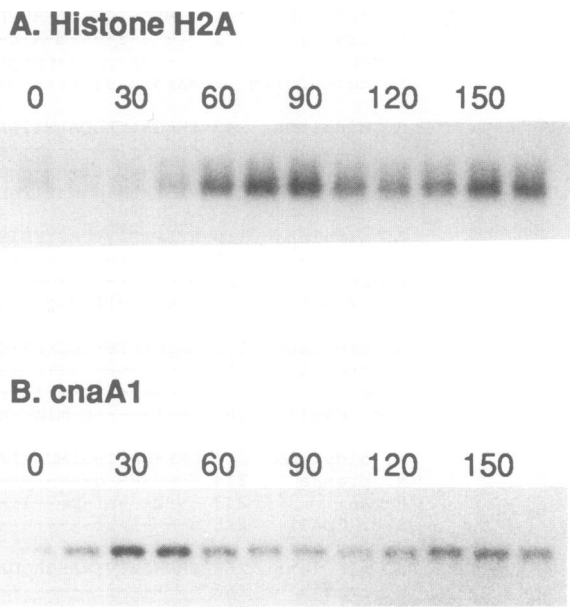

Fig. 3. Cell cycle-dependent expression of *cnaA*$^+$ mRNA. Northern blot analysis was used to analyse *cnaA*$^+$ mRNA levels during the cell cycle. After nocodazole synchronization (see Materials and methods), RNA was prepared from samples taken at the times indicated. Equal amounts of RNA (determined by A_{260}) were resolved by agarose gel electrophoresis in formaldehyde-containing gels, transferred to Nytran filters and probed first for (**A**) histone H2A mRNA (as a control for an mRNA expressed coincident with progression through S phase) and then for (**B**) *cnaA*$^+$ mRNA.

pyr4$^+$ gene which complements the *pyrG89* mutation present in the *A.nidulans* strain GR5 used as the recipient in these studies. GR5 germlings were transformed with the plasmid pCnAsp-KO, and *pyr4*$^+$ transformants selected by the ability to grow in medium lacking uridine/uracil. Seven transformants that were *pyr4*$^+$ were analysed by Southern blot to determine if they contained a single integrated copy of the pCnAsp plasmid at the *cnaA*$^+$ gene locus. Genomic DNA from each isolate was digested with *SacI*, resolved on an agarose gel, transferred to a Nytran filter and probed with the 0.9 kb *SalI* fragment used to construct pCnAsp-KO. The expected consequence of a site-specific integration at the *cnaA*$^+$ gene locus is shown in Figure 4A. The probe hybridizes to two *SacI* fragments of 2.5 and 1.0 kb in the normal *cnaA*$^+$ gene. Integration of pCnAsp-KO in heterokaryons was expected to give a hybridization pattern consisting of the 2.5 and 1.0 kb bands from the normal nuclei in the heterokaryon, as well as two additional bands of 4.5 and 0.6 kb from the transformed nuclei. As can be seen, in control DNA from non-transformed GR5 cells, the probe hybridized to the expected 2.5 and 1 kb bands (lane 1; Figure 4B). In the seven transformants, one (isolate #1; lane 2; Figure 4B) showed the predicted pattern for a single integration event. Other isolates have the 4.5 and 0.6 kb bands, but also additional bands indicating multiple, non-homologous integration events. Since we were interested in the effect of specific disruption of the *cnaA*$^+$ gene, isolate #1 (KO-1) was retained for further analysis.

To determine if disruption of the *cnaA*$^+$ gene was lethal, non-transformed GR5 and KO-1 conidia were germinated on non-selective (+uridine/uracil) and selective (−uridine/uracil) media. Because there is dual selection for viability

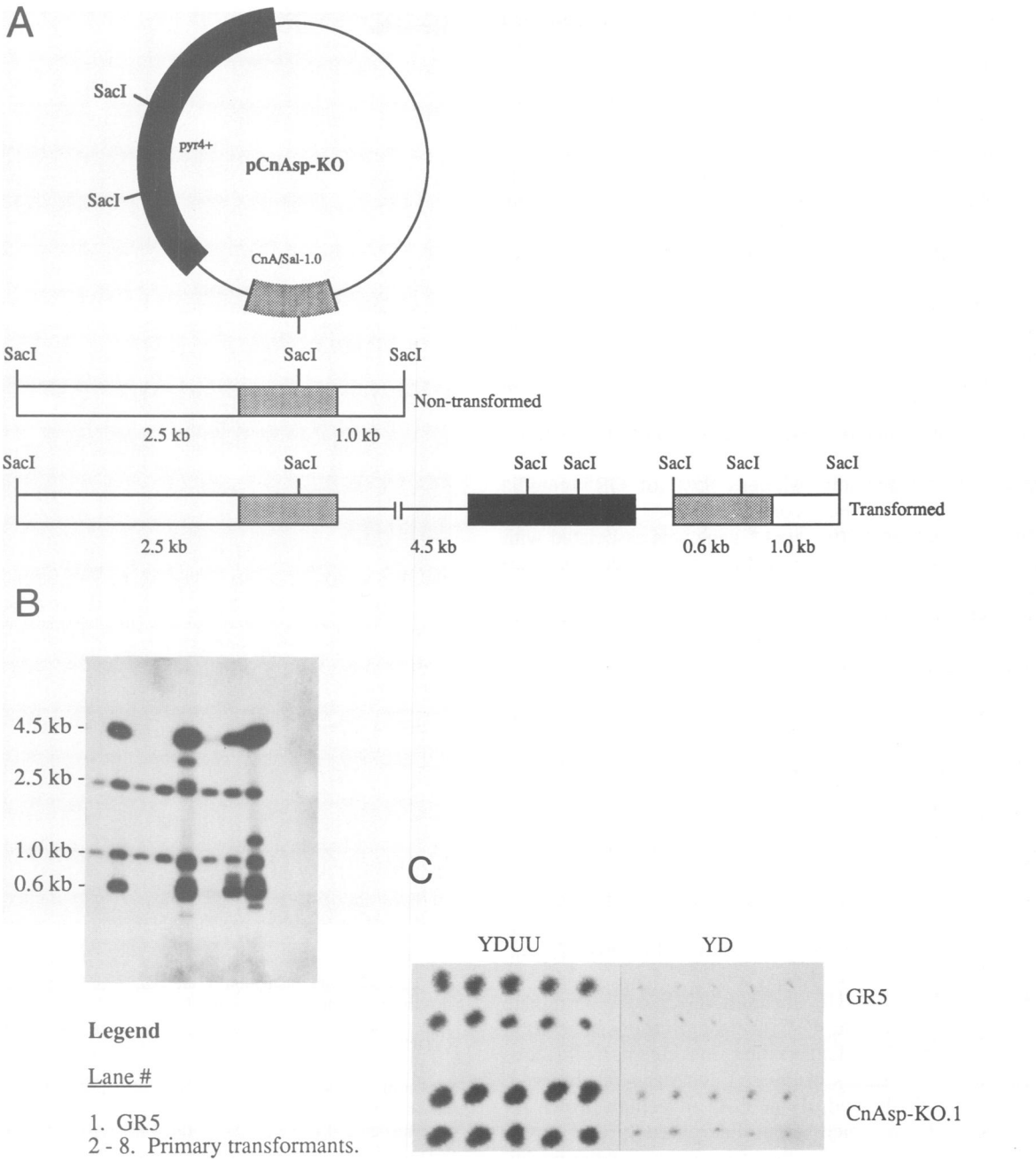

Fig. 4. Disruption of the *cnaA*$^+$ gene. (**A**) Design of disruptor plasmid and expected consequences of site-specific integration of the pCnAsp-KO plasmid at the *cnaA*$^+$ gene locus. The shaded regions correspond to sequences recognized by the *Sal*I restriction fragment from the *cnaA*$^+$ gene used as a hybridization probe. (**B**) Southern blot analysis of genomic DNA from primary pCnAsp-KO transformants. Genomic DNA was isolated from seven different primary *pyr4*$^+$ transformants, digested with *Sac*I and processed for Southern blotting as described in Materials and methods. The 0.9 kb *Sal*I fragment used in the pCnAsp-KO disruptor plasmid was used as the hybridization probe. The normal hybridization pattern is shown in lane 1 using DNA from non-transformed GR5 cells. Lanes 2−8 are from transformants. Lane 2 shows the expected hybridization pattern for a single site-specific integration of the pCnAsp-KO plasmid at the *cnaA*$^+$ gene locus. (**C**) Growth phenotype of *A.nidulans* strains carrying a site-specific disruption (*cna*A1) of the *cnaA*$^+$ gene. Conidia from either the GR5 or KO-1 strains were spotted onto agar plates containing YD medium with (YDUU) or without (YD) added uridine/uracil as described in Materials and methods. GR5 conidia only grow in YDUU medium due to the presence of the *pyrG89* mutation in this strain. KO-1 conidia, derived from a heterokaryon able to grow in YD medium, fail to grow on this medium. This failure to grow is genetic proof that the *cnaA*$^+$ gene is essential for cell growth and division in *A.nidulans*. The appearance of growth in KO-1 conidia spotted onto YDUU medium is due to the normal, non-transformed nuclei present in the KO-1 heterokaryon.

and the marker gene, only heterokaryons carrying both normal and transformed nuclei will survive. The phenotype of disruption is tested by determining whether or not spores are viable on selective medium that requires the presence of the marker. Since *A.nidulans* conidia (haploid spores) are derived by mitotic division, two classes of conidia, (i) non-transformed/non-disrupted and (ii) transformed/disrupted, are produced by heterokaryons. If the disrupted gene is essential, neither type of conidium is viable since non-transformed nuclei lack the selectable marker, and transformed nuclei, while those carrying the marker lack the product encoded by the essential gene being examined. In

contrast, on non-selective medium, the non-transformed conidia are viable and will form mycelia, while the transformed nuclei are still non-viable. The effect will be a reduction in the percentage of conidia able to germinate with the degree of reduction dependent on the relative ratio of normal and transformed nuclei.

As shown in Figure 4C, the non-transformed GR5 conidia are viable and form a mycelial colony on non-selective medium, but were not viable on selective medium due to the *pyrG89* mutation present in the strain. In the KO-1 strain, conidia were not viable on selective medium, despite the fact that the heterokaryon carries the *pyr4*$^+$ gene which complements the *pyrG89* allele (Figure 4C). As explained above, this indicates that the *cnaA*$^+$ gene we have isolated is essential for normal growth of *A.nidulans*. Also consistent with the interpretation that disruption of *cnaA*$^+$ is lethal, analysis of germination frequencies revealed that even on non-selective medium only 46% of KO-1 conidia were capable of germination, whereas 95% of GR5 conidia germinate under identical conditions.

Since disruption of the *cnaA*$^+$ gene was associated with a lethal phenotype, we examined germinating conidia from the KO-1 strain to determine if failure to proliferate occurred at a specific cell cycle stage. Conidia from the parental GR5 and KO-1 strains were germinated on minimal medium + uridine and uracil (MMUU) or minimal medium (MM) for 11 h. Nuclei were then stained with the fluorescent dye DAPI, and nuclear morphology and size used as the criteria to determine cell cycle position. Previous studies have shown that mitotic nuclei have a distinct condensed appearance, and lack a nucleolus, while nuclei in interphase are less condensed and possess a nucleolus (Osmani *et al.*, 1988). In addition, distinction between pre- and post-mitotic nuclei may be made on the basis of nuclear size (Osmani *et al.*, 1991). Since under normal conditions nearly 50% of nuclei are in G_2 during exponential growth, it is especially easy to detect an abnormal excess of small G_1 nuclei (Bergen and Morris, 1983).

After 11 h in MMUU medium, GR5 cells germinate normally and complete 4–5 nuclear divisions, and have between 16 and 32 nuclei/germling. In addition, by this time nuclei at various stages of the cell cycle can be observed. In Figure 5A is the end of one GR5 mycelium grown for 11 h at 32°C in which larger interphase (probably G_2) nuclei can be seen. Notice the even spacing of the nuclei, and the presence of a darker region in the nucleus which is the nucleolus. In Figure 5B, another GR5 mycelium from the same plate is shown in which the nuclei have recently divided, as indicated by the close spacing of each pair of nuclei. The most recently divided (G_1) nuclei are at the tip of the mycelium. Measurement of the size of the nuclei from the photograph reveals that those in Figure 5A are two times longer than those in Figure 5B, but are of similar diameter. Quantitative DNA fluorescence values in arbitrary units are: A: 2.53 ± 0.4; B: 1.33 ± 0.3 ($P < 0.001$). When compared with these controls, the nuclei in KO-1 conidia, germinated under identical conditions, are 1.21 ± 0.3 and thus are likely to be in an early portion of the cell cycle, probably G_1 (Figure 5C). Examination of >100 KO-1 germlings indicated that conidia lacking the *cnaA*$^+$ gene (the Cna A1 strain) are usually able to complete one (two nuclei) or at most two (four nuclei) nuclear divisions. This result confirms that in the absence of a functional *cnaA*$^+$ gene, cell cycle progression is severely inhibited. Together, these data suggest that *cnaA*$^+$ is required for early cell cycle events, prior to DNA replication.

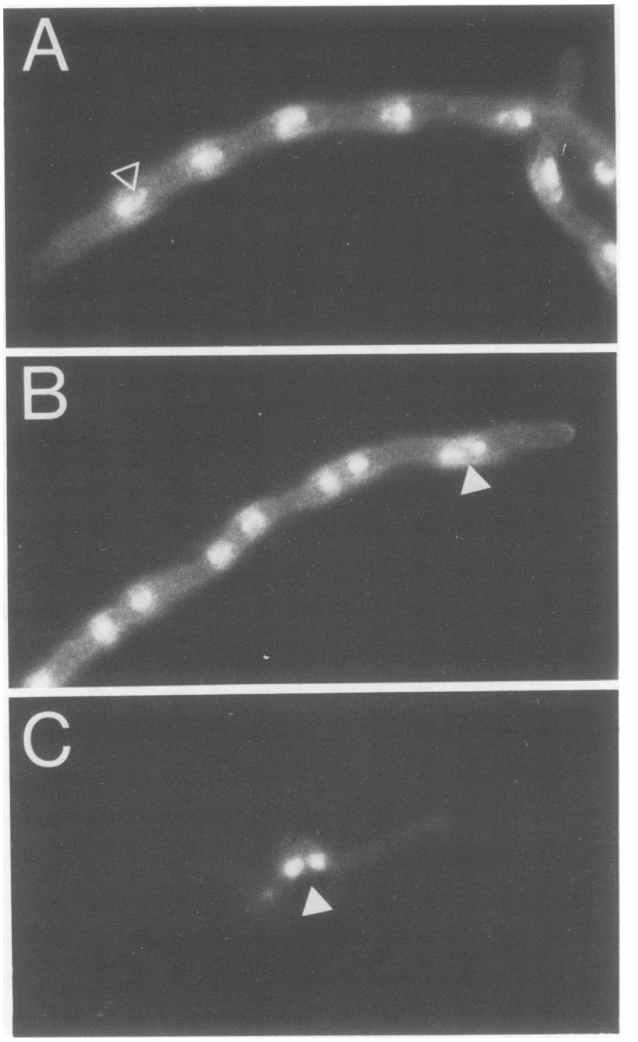

Fig. 5. Nuclear phenotype in *A.nidulans* with a disruption of the *cnaA*$^+$ gene. Gene disruption of the *cnaA*$^+$ gene was accomplished as described in Materials and methods and Results. In (A) and (B) are shown control GR5 germlings grown for 11 h in permissive conditions (YDUU medium). Shown in (**A**) is a germling with representative interphase (likely G_2) nuclei. The open arrow points to a darkened region of a nucleus corresponding to the nucleolus. In (**B**) are shown nuclei in a germling that have recently divided, based on nuclear morphology and previous observations that the most recently divided nuclei are at the tips of germlings (Osmani *et al.*, 1988); the nucleus indicated by the filled arrow is likely late in mitosis (late anaphase or telophase), while nuclei more towards the left have completed mitosis and are early in the next cell cycle. When measured from the photograph, the nuclei in (A) are twice the length of the nuclei in (B), and have the same diameter. This suggests that the nuclei in (A) (late S phase or G_2) are later in the cell cycle than those in (B) (G_1 or early S phase). In (**C**) is shown a typical germling from the *cna*A1 disruption strain KO-1. The arrow points to the only two nuclei present. Also note the apparent failure of nuclei to migrate apart, as normally occurs after mitosis in *A.nidulans*. Based on the comparison of the relative sizes of the nuclei in (A) and (B), it would appear that the nuclei in (C) are more characteristic of nuclei early in the cell cycle. The nucleolus is not present in this particular photograph due to the plane of focus which was intended to give a true representation of the nuclear dimensions. All photographs were printed to the same final magnification.

Discussion

We have isolated a CnA gene from *A.nidulans*, demonstrated that this *cnaA*$^+$ gene is essential and presented evidence that in the absence of this enzyme, cells arrest early in the cell cycle, possibly in G_1. This is the initial report of an essential role for a CaM-dependent enzyme in any organism. The requirement for CnA early in the cell cycle not only supports circumstantial evidence for this possibility obtained in other systems, but also helps to explain why Ca^{2+}/CaM is required for cells to make the transition from G_0/G_1 to S phase (Lu and Means, 1993).

Disruption of the *cnaA*$^+$ gene did not immediately block progression of the nuclear division cycle. Such cells were able to complete either one or two nuclear divisions before the arrest was evident. During the same time period, the normal germlings completed 8−10 cell cycles. Thus, even though some growth occurred in the disrupted strain, this growth was markedly slowed. *Aspergillus nidulans* grows as a multinucleated syncytium, so upon disruption of an essential gene the cells are maintained as a heterokaryon (Rasmussen *et al.*, 1990) and, because of the dual selection of viability and the marker gene, only heterokaryons carrying both normal and transformed nuclei will survive. Since the cytoplasm is common, even spores from a haploid strain containing a single disrupted nucleus will contain an equivalent amount of cytoplasmic constituents, as will those spores that contain a normal nucleus. We suspect that the initial slow growth of cells containing the *cnaA1* disruption is due to the presence of CnA in the spores. When this enzyme is turned over, the cells are arrested at the point in the cycle that requires the enzyme. A similar effect was initially observed upon disruption of the CaM gene in *A.nidulans* (Rasmussen *et al.*, 1990). Subsequent creation of a strain conditional for the expression of CaM allowed the demonstration that even when spores were germinated in media that repressed CaM gene expression, it required 9 h before CaM was completely depleted (Lu *et al.*, 1993b). This was sufficient time for 1−2 nuclear divisions before the cycle was arrested. In the case of CaM gene disruption, some cells (20%) became blocked in G_1 and others (80%) in G_2 (Lu *et al.*, 1992). The phenotype observed upon disruption of the *cnaA*$^+$ gene suggests that the primary target for CaM in G_1 may be CnA. The data necessarily imply that a different CaM-dependent enzyme is required in G_2.

The interpretation that loss of the *cnaA*$^+$ gene product results in an early cell cycle arrest is consistent with studies in other systems suggesting that CnA is required for growth and may act early in the cell cycle. Two genes for CnA and one for the regulatory CnB subunit have been isolated from *S.cerevisiae* (Cyert *et al.*, 1991; Kuno *et al.*, 1991; Liu *et al.*, 1991; Cyert and Thorner, 1992). Deletion of both CnA genes, the CnB gene or all three genes was not lethal. However, such yeast strains were compromised in the ability to recover from the growth arrest produced in response to α-factor (Cyert *et al.*, 1991; Cyert and Thorner, 1992). Foor *et al.* (1992) have also reported that CnA mediates inhibition of recovery from α-factor arrest caused by FK-506 and cyclosporin. As CnA is the primary target for FK-506 and cyclosporin when the drugs are bound to their respective receptor proteins (Schreiber and Crabtree, 1992), these results also show that CnA is required for resumption of growth in yeast. This event is much more sensitive to the immunosuppressive drugs than is inhibition of vegetative growth, the only other effect of these compounds reported in yeast (Foor *et al.*, 1992). Recovery from α-factor arrest in yeast is roughly equivalent to re-entry into the cell cycle from a quiescent state in mammalian cells. Thus, these studies are consistent with a role for CnA in G_0/G_1.

Presentation of antigen to quiescent T lymphocytes initiates a series of events, including lymphokine gene transcription, that are required for mitogenesis. Activation of the transcription factor NFAT requires CnA and is blocked by FK-506 and cyclosporin (Schreiber and Crabtree, 1992). Overexpression of a constitutively active form of CnA markedly increases resistance of the immunosuppressive drugs, while increasing NFAT-dependent transcription (Clipstone and Crabtree, 1992; Okeefe *et al.*, 1992). Finally, NFAT has been isolated from cells as an inactive phosphoprotein that can be dephosphorylated and activated by CnA (McCaffrey *et al.*, 1993). The action of NFAT (and other transcription factors) is required to activate genes, including those for lymphokines such as IL-2 (Schreiber and Crabtree, 1992). It is the autocrine action of such lymphokines that is required for mitogenic activation of T cells. Thus, as in yeast, a strong circumstantial case can be made for the requirement of CnA in G_0/G_1 in T cells. Interestingly, we observed that germlings with a disrupted *cnaA*$^+$ gene were significantly thinner, indicating that growth may also be inhibited in these cells. Since CnA in T cells appears to be important in the regulation of gene expression, it may be that in *A.nidulans* a similar role for CnA exists, and the lack of CnA has a broad effect on growth due to the inability to transcribe other essential genes. Whether this is the reason for the requirement for CnA in *A.nidulans* is unknown.

Compelling evidence exists for the involvement of CaM in cell cycle progression (Davis *et al.*, 1986; Takeda and Yamamoto, 1987; Rasmussen and Means, 1989a; Rasmussen *et al.*, 1990). A variety of studies in cells from several species have shown CaM requirements for re-entry of cells from a G_0 quiescent phase, the rate of progression through G_1, execution of the G_1/S transition, movement from G_2 into M and the poleward separation of chromosomes that occurs between metaphase and anaphase (Rasmussen and Means, 1989a,b; Lu and Means, 1993). However, as CaM is required for so many essential cellular processes and has therefore been suggested to serve as a 'housekeeping' protein, the specificity and rate-limiting nature of these multiple cell cycle requirements can be questioned. The findings that CnA is required for early post-mitotic events, whereas CaM kinase may be important for G_2 and is clearly involved in the metaphase/anaphase transition (Lorca *et al.*, 1993), begin to address this crucial issue. Collectively, the available data suggest that different CaM target enzymes may be required at different times during the cell cycle. Both CnA and CaM kinase genes appear to be unique in *A.nidulans* based on Southern analysis of genomic DNA (data not shown), and the mRNAs are regulated during the cell cycle with CnA mRNA maximal in G_1 and CaM kinase mRNA maximal in G_2 [Figure 3 and Kornstein *et al.* (1992)]. On the other hand, CaM is present throughout the cell cycle and its content doubles between G_1 and G_2 (Lu *et al.*, 1992). The K_{cam} of CaM kinase is considerably greater than that of calcineurin (Bartelt *et al.*,

1988; Higuchi *et al.*, 1991). Thus, the precise role of CaM in G_1 and G_2 may be determined both by the presence of the appropriate enzyme and an optimal concentration of CaM required for activation of the enzyme. Since both CaM-dependent enzymes are essential, creation of strains conditional for the expression of CnA or CaM kinase will allow us to answer these questions and to identify substrates relevant for CaM-dependent cell cycle progression.

Materials and methods

Strains and culture of A.nidulans

For all experiments, the *A.nidulans* strain GR5 (*pyrG89, wA3, pyroA4*) was used. Standard conditions for growth and media recipes have been described previously (Rasmussen *et al.*, 1990). Techniques for gene disruption in *A.nidulans* heterokaryons have also been described in detail (Rasmussen *et al.*, 1990). For synchronization with nocodazole, early log-phase cultures were incubated with 2 μg/ml nocodazole for 4 h. To release cells, they were washed three times with fresh medium, then resuspended in fresh medium without nocodazole. For examination of nuclear morphology, germlings were fixed in 3.7% formaldehyde and stained with 1 μg/ml DAPI (Bergen and Morris, 1983). Photographs were taken using a Zeiss Axiophot microscope with epifluorescence. All micrographs are printed to the same net magnification.

Cloning of the cnaA$^+$ gene and cDNA

Genomic clones were obtained by screening a λ gt11 genomic DNA library using a probe derived by PCR from an *N.crassa* CnA cDNA using standard procedures (Maniatis *et al.*, 1982). Subgenomic fragments were subcloned into the plasmid pUC19 for sequencing. cDNA clones were obtained using *A.nidulans* genomic clones as hybridization probes with which to screen a λ gt11 cDNA library. DNA sequence analysis was performed with a Sequenase 2.0 kit (US Biochemical).

Molecular biology

DNA and RNA were isolated from cultures as described previously (Rasmussen *et al.*, 1990). For Southern blotting, genomic DNA was digested with the indicated restriction enzyme and resolved by agarose gel electrophoresis. DNA was denatured with 0.5 N NaOH, 1.5 M NaCl prior to transfer to Magnagraph nylon filters by capillary action (Maniatis *et al.*, 1982). For Northern blots, equal amounts of RNA, as determined by A_{260}, were resolved on formaldehyde-containing agarose gels and transferred to filters as described previously (Maniatis *et al.*, 1982). Size estimates of the *cnaA*$^+$ mRNA were determined using rRNA as an internal size standard.

For detection of *cnaA*$^+$ by Southern or Northern blot hybridizations, an internal *Sal*I fragment (0.9 kb) was used. For detection of histone H2A mRNA, the 2 kb *Eco*RI/*Pst*I fragment in pH1.1 was used (May and Morris, 1987). Probes were labelled by the oligolabelling method (Feinberg and Vogelstein, 1983). Unincorporated [^{32}P]dCTP was separated from the probe by gel filtration using Sephadex G-100 in a buffer of 10 mM Tris−HCl (pH 8.0), 1 mM EDTA, 0.1% SDS. Hybridizations were performed in hybridization buffer (5 × SSPE, 50% formamide, 5 × Denhardt's, 100 μg/ml denatured herring sperm DNA; Maniatis *et al.*, 1982) at 42°C for 16 h. Filters were washed five times for 5 min each wash in 1 × SSC, 0.1% SDS and twice for 15 min each wash in 0.1 × SSC, 0.1% SDS at 55°C. Hybridization was detected by autoradiography using Kodak XAR film at −70°C with intensifying screens.

DNA sequence comparisons were performed using IntelliGenetics Suite software package (Intelligenetics Inc., Mountainview, CA).

Gene disruption

The *cnaA*$^+$ gene was disrupted in heterokaryons using standard procedures (Rasmussen *et al.*, 1990). GR5 germlings were transformed with a plasmid containing an internal fragment of the gene cloned into the plasmid pRG3 (May *et al.*, 1989) which contains the *N.crassa pyr4* gene, able to complement the *pyrG89* mutation present in GR5 (Rasmussen *et al.*, 1990). Primary heterokaryon transformants able to grow on selective medium were then tested by Southern blot analysis for integration of the plasmid at the *cnaA*$^+$ gene locus. For phenotype testing, conidia from GR5 and a gene disruption strain were spotted onto selective or non-selective plates and cultured for 2 days at 32°C to test for growth. For microscopic examination of nuclei, conidia were germinated at 32°C for 12 h prior to fixation and staining. Under these conditions, normal germlings complete eight cell cycles (Bergen and Morris, 1983).

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