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Molecular cloning of a cDNA encoding calmodulin from *Neurospora crassa*

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A full-length cDNA encoding *Neurospora crassa* calmodulin was isolated from a λZAP II cDNA expression library. The open reading frame encodes a protein of 148 amino acid residues with a calculated M_r of 16,865 Da. Using site-directed mutagenesis, the complete cDNA was ligated into a *trc* promoter-regulated bacterial expression vector to allow expression of *N crassa* calmodulin in *E. coli*. The expressed protein was found to be identical to the native protein on the basis of some of its biochemical properties. Finally, Southern analysis of restriction digests of genomic DNA indicates that calmodulin is encoded by a single-copy gene.

Neurospora crassa; Calmodulin; Nucleotide sequence; cDNA expression

1. INTRODUCTION

Calmodulin belongs to a class of Ca^{2+}-modulated proteins, including parvalbumin, troponin C, S-100 protein and vitamin D-dependent Ca^{2+}-binding protein, among others, which are known to contain homologous EF-hand structures, consisting of two α-helices joined by a Ca^{2+}-binding loop, for the high-affinity binding of Ca^{2+} ions [1].

Like all eukaryotes examined, the filamentous fungus *Neurospora crassa* possesses the small calcium-binding protein, calmodulin [2], which is implicated in mediating the regulatory effects of calcium on a large number of enzymatic activities (i.e. cAMP metabolic enzymes, protein kinases, and a variety of intracellular processes, such as cell cycle control, cellular growth and division (for review see [3,4]).

In addition, the primary structure of the protein appears to be highly conserved throughout the animal and plant kingdoms. These facts reflect the important role of calmodulin as the major intracellular calcium receptor.

In order to improve our knowledge of the mode of action of calmodulin in *N. crassa*, we report here the isolation and sequence determination of a full-length cDNA clone for the fungal calmodulin. We have also expressed this cDNA in an *E. coli* host and identified the expressed protein as calmodulin on the basis of its electrophoretic properties and its ability to induce the phosphorylation of a 47 kDa peptide isolated as previously described [5].

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Abbreviations: CaM, calmodulin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thio-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

The nucleotide sequence reported in this paper has been submitted to the EMBL Data Bank and has been assigned the accession number X70923.

2. MATERIALS AND METHODS

The source of enzymes and chemicals is as follows: Sequenase version 2.0 was purchased from United States Biochemicals, [α-^{35}S]dATP (1,200 Ci/mmol), [γ-^{32}P]dATP (3,000 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) were from Amersham. Restriction enzymes were from Boehringer-Mannheim, Biofinex and Promega Corp. Calf intestinal phosphatase (CIP), T4 DNA ligase and Klenow fragment of DNA-polymerase I were from Stratagene. The pKK233.2 expression vector was from Pharmacia-LKB. Other general reagents were purchased from Sigma, Kontron, Difco and Fluka companies.

2.1. *Strains and cultures*

The wild-type strain, St. Lawrence 74A, of *N. crassa* (FGSC 262) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS, USA. Growth conditions were as described in [2].

E coli strains XL1 Blue [6] and SB1 [7] were grown in LB broth (10% tryptone, 5% yeast extract, and 5% NaCl).

Plasmid pSB6, the products of which greatly facilitate the lysis of the cells, was a kind gift from the laboratory of Dr. T.N. Davis, Department of Biochemistry, University of Washington, Seattle, USA [7].

2.2 *Isolation and analysis of cDNA clones for* N. crassa *CaM*

Clones for *N crassa* CaM were isolated from a λZAP II cDNA

'expression' library prepared with poly(A)$^+$ mRNAs from exponentially growing *N crassa* wild-type cultivated in liquid Vogel's medium [8]. The phage library was screened with a 1:750 dilution of the rabbit polyclonal anti-calmodulin antibody purified as described elsewhere [9]. About 280,000 recombinants were screened, yielding finally 15 positive clones. The 15 pBluescript SK(−) plasmids containing the cDNA inserts were excised from the purified λZAP II clones with filamentous helper phage R408 for complementary analysis by restriction enzyme mapping. Plasmid DNA was isolated by the alkaline lysis miniprep procedure [9] and restriction enzyme digests were performed according to the manufacturer's recommendations. Digests were analyzed on 0.8 or 1% agarose gels.

2.3. *DNA sequencing and computer analysis*

The DNA used in the sequencing reaction was purified with Qiagen (Kontron, Switzerland) anion-exchange column. Nucleotide sequencing was performed by the dideoxy chain termination method of [11] using [α-^{32}P]dATP as the radioactive label and the Sequenase enzyme system. Sequencing reactions were subjected to electrophoresis on 6% polyacrylamide gels in the presence of 7 M urea, dried, and exposed to Fuji X-Ray film for 1–3 days.

Computer programs from the sequence analysis software package of the University of Wisconsin Genetics Computer Group were used to analyze nucleotide and amino acid homologies

2.4. *Radiolabelling of cDNA probe*

The *PstI–PstI* fragment of the pNC15 plasmid, containing the entire coding region of calmodulin, was recovered from an agarose gel after electrophoresis onto DEAE-cellulose membranes NA-45 (pore size 0.45 μm; Schleicher and Schuell) according to [12]. The cDNA insert was labelled with [α-^{32}P]dCTP and Klenow enzyme to high specific activity using random hexanucleotides primers (Amersham Corp.) as previously described [13]. Unincorporated nucleotides were separated from the probe by column chromatography on Sephadex G-50 (Pharmacia) in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% SDS. The probe was denatured in boiling water for 5 min prior to hybridization.

2.5. *Isolation of genomic DNA and Southern blotting*

2.5 g of fresh mycelium were ground in a precooled mortar in the presence of liquid nitrogen. Genomic DNA for Southern gel [14] was extracted and purified according to [15]

Restriction enzyme-digested *N. crassa* DNA was separated on 0.8% agarose gel, depurinated for 10 min in 0.25 M HCl, and capillary transferred to nitrocellulose filter (BA 85; Schleicher and Schuell)

```
-11                                                                  CG  GCA CGA GAG

      1               5                   10                  15
  1  ATG GCG GAC TCC CTT ACT GAA GAG CAG GTC TCT GAG TTC AAG GAG GCC TTC TCC CTT TTT
     Met Ala Asp Ser Leu Thr Glu Glu Gln Val Ser Glu Phe Lys Glu Ala Phe Ser Leu Phe

     20                  25                  30                  35
 61  GAC AAG GAC GGT GAT GGC CAA ATC ACC ACC AAG GAG CTC GGT ACC GTC ATG CGC TCG TTG
     Asp Lys Asp Gly Asp Gly Gln Ile Thr Thr Lys Glu Leu Gly Thr Val Met Arg Ser Leu

     40                  45                  50                  55
121  GGC CAG AAC CCC TCC GAG TCT GAG CTT CAG GAC ATG ATC AAC GAG GTC GAT GCC GAC AAC
     Gly Gln Asn Pro Ser Glu Ser Glu Leu Gln Asp Met Ile Asn Glu Val Asp Ala Asp Asn

     60                  65                  70                  75
181  AAC GGC ACC ATT GAC TTC CCT GAG TTC CTT ACC ATG ATG GCC AGA AAG ATG AAG GAT ACC
     Asn Gly Thr Ile Asp Phe Pro Glu Phe Leu Thr Met Met Ala Arg Lys Met Lys Asp Thr

     80                  85                  90                  95
241  GAC TCC GAG GAG GAG ATC  CGT GAG GCC TTC AAG GTG TTC GAT CGC GAC AAC AAC GGC TTC
     Asp Ser Glu Glu Glu Ile  Arg Glu Ala Phe Lys Val Phe Asp Arg Asp Asn Asn Gly Phe

     100                 105                 110                 115
301  ATC TCC GCT GCC GAG CTC CGT CAC GTC ATG ACC TCC ATC GGC GAG AAG CTC ACT GAT GAC
     Ile Ser Ala Ala Glu Leu Arg His Val Met Thr Ser Ile Gly Glu Lys Leu Thr Asp Asp

     120                 125                 130                 135
361  GAG GTT GAT GAG ATG ATC CGT GAG GCC GAC CAG GAC GGC GAT GGG CGT ATC GAC TAC AAC
     Glu Val Asp Glu Met Ile Arg Glu Ala Asp Gln Asp Gly Asp Gly Arg Ile Asp Tyr Asn

     140                 145         148
421  GAG TTC GTC CAG CTC ATG ATG CAG AAG TAA ACG GCT TTC TCC TAT ATT CCA ACT CTA CAC
     Glu Phe Val Gln Leu Met Met Gln Lys END

481  GAA GAC AGG TTT TAG CGT TCT CTT TGA ATA CCA CTA TTA GTT GAT ATG CCT CCA TTC GGA

541  TGT GAA GGG ATC GCT TTG TTT CCC ACG CTT GGG ACC ACC AGC AAG ACC GGA GAC CTG TCA

601  ACG AGT GGG GAT GAC CTG CAG GAT GAT TGT GGC AGT GTT GCC TAG CGA CAT CAA CCA TCA

661  AGC CCA GGC CGA ATA TGT GGA TAT CAT CAG CCC TAT AGG GAT TTC CAT ATC GTA CAC ATC

721  CGT ACG GCA CAG CAT CTA GAA AAT CAA CTC ATC TCC CGA TCA CCG TTG AAA AAA AAA AAA

     AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
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Fig. 1. Nucleotide sequence of *N. crassa* calmodulin. The complete sequence of the pNC15 cDNA clone is presented together with the deduced amino acid sequence of the coding region. The initiation codon, ATG, and termination codon, TAA, are shown in bold-face type; the numbers on the left side refer to nucleotide positions.

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                                         25                       50                       75

N.crassa        ADSLTEEQVSEFKEAFSLFDKDGDGQITTKELGTVMRSLGQNPSESELQDMINEVDADNNGTIDFPEFLTMMARK
Vertebrate      --Q-----IA---------------T-----------------T-A------------G----------------
S.cerevisiae    S-N-----IA------A-----NN-S-SSS--A-------LS---A-VN-LM--I-V-G-HQ-E-S---AL-S-Q
A.nidulans      -----------Y---------------------------------------------------------------
C.albicans      -EK-S-Q-IA------------S--K----------------------T-------VNSD-S-------------
A.klebsiana     --Q-----IA-----G---------T-----------------T--------------G----------------
P.tetraurelia   --Q-----IA---------------T------------V----T-A------------G----------SL----

                                         100                      125

N.crassa        MKDTDSEEEIREAFKVFDRDNNGFISAAELRHVMTSIGEKLTDDEVDEMIREADQDGDGRIDYNEFVQLMMQK
Vertebrate      --------------R---K-G--Y-----------NL------E----------I----QVN-E----M-TA-
S.cerevisiae    L-SN---Q-LL-------KNGD-L------K--L---------A---D-L--VS.--S-E-NIQQ-AA-LS.-
A.nidulans      -------------------------------------------------------------------------
C.albicans      -------A--A--------NGD-K--------LL-------S-AD--Q--K---TNN--E--IQ--TL-LAA-
A.klebsiana     ----------L---QG--K-G-----------M--NL------E----------I----Q-NTE---KM--S-
P.tetraurelia   --EQ-----LI---------G--L-----------NL-----------------I----H-N-E---RM-VS-
```

Fig. 2. Comparison of the amino acid sequence of *N. crassa* CaM to the sequences of CaMs from other organisms. The predicted amino acid sequence of *N. crassa* CaM derived from the nucleotide sequence of the cDNA (see Fig. 1) has been aligned manually with the amino acid sequences of CaMs from vertebrate [22], *Saccharomyces cerevisiae* [26], *Aspergillus nidulans* [21], *Candida albicans* [23], *Achlya klebsiana* [27], and *Paramecium tetraurelia* [28]. The predicted EF-hand Ca^{2+}-binding loops [29] are shown in bold-face type. Only those residues that are different from those found in the *N. crassa* protein are given.

using 20 × SSPE (200 mM NaH_2PO_4, 3.6 M NaCl, 20 mM EDTA, pH 7.4) as transfer buffer [10]. After immobilization of DNA by baking for 2 h at 80°C, the filter was hybridized in 50% formamide, 6 × SSPE, 5 × Denhardt's solution (1% polyvinylpyrrolidone, 1% Ficoll, and 1% bovine serum albumin), 0.5% SDS, 100 μg/ml denatured sonicated herring sperm DNA, and calmodulin cDNA probe (see above for preparation and radiolabelling) for 72 h at 42°C, after prehybridization in the same solution without probe for at least 8 h at 42°C. The filter was twice washed (5 min each) in 2 × SSPE and 0.5% SDS at room temperature, 30 min in 0.1 × SSPE and 0.5% SDS at 37°C, and finally 5 min in 0.1 × SSPE at room temperature before exposure to Kodak XAR-5 film for autoradiography at −80°C with an intensifying screen.

2.6. *Oligonucleotide-directed mutagenesis*

A unique *Nco*I restriction site at the initiation methionine of CaM cDNA, was introduced with the mutagenic primer: 5′-CGGCACGAGCCATGGCGGACTC-3′.

Mutagenesis was carried out as described [16] using a single-strand template obtained with helper phage and Sequenase version 2.0 as the extending polymerase. The position of mutation (underlined) was verified by dideoxy sequencing according to [11] and by mapping with restriction enzymes.

2.7. *Construction of the CaM expression plasmid*

As shown in Fig. 1, a 621-base fragment containing the entire *N. crassa* CaM coding sequence was inserted into the *Nco*I and *Pst*I sites of the vector pKK233.2 (Pharmacia, Inc.). The CaM cDNA excised from pNC15 plasmid and the phosphatase-treated, *Nco*I/*Pst*I-cut pKK233.2 were combined and treated overnight at 16°C with T4 DNA ligase. This ligation mixture was used to transform competent *E. coli* strain XL1 Blue cells using standard procedure [10]. Transformants were selected by plating on LB agar plates containing 100 μg/ml ampicillin and screened for plasmid DNA containing the cDNA insert following digestion with *Nco*I and *Pst*I. One recombinant plasmid, designated as pNCCaM, which had the CaM cDNA sequence placed under the control of the *trc* promoter, was obtained. By DNA sequence analysis, the spacing between the Shine–Dalgarno sequence (AGGA) and the translation initiation codon (ATG) was found to be eight nucleotides as expected. This resulting plasmid was then used to transform *E. coli* strain SB1 carrying the lysis plasmid pSB6 for expression experiments.

2.8. *Expression and purification of the recombinant protein*

Calmodulin over-expressed in *E. coli* was isolated from bacterial lysate by hydrophobic interaction chromatography [17]. 1 l of LB broth containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) was inoculated with 10 ml of a stationary phase culture of *E. coli* strain SB1 transformed with pNCCaM and the lysis plasmid pSB6. The cultures were grown at 37°C with vigorous shaking until an absorbance of 0.6 O.D. at 600 nm was reached. Protein expression was induced by adding IPTG to a final concentration of 1.5 mM, and after 2 h of growth, the cells were harvested by centrifugation at 2,000 × *g* for 20 min. The bacterial pellet was washed and resuspended in 30 ml of buffer A containing 25 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM benzamidine and 0.5 μg/ml leupeptin. The cells were chilled on ice for 10 min and subjected to 3 freeze/thaw cycles. The lysis of the cells was greatly facilitated by the products of the plasmid pSB6 [7] which contains the lysis genes *R* and *Rz* of bacteriophage λ under the control of *lac* promoter. The crude extract was then cleared by the addition of DNase and the supernatant was recovered after ultracentrifugation at 150,000 × *g* for 30 min in

a Kontron TI 7038 rotor at 4°C. The soluble fraction was made 0.5 M in NaCl, placed in a boiling water bath for 3 min and then cooled in an ethanol ice bath for 5 min. Precipitated material was removed by centrifugation at 20,000 × *g* for 15 min in a Sorvall SS 34 rotor at 4°C. The supernatant was adjusted to a final concentration of 5 mM $CaCl_2$ and then loaded on a 2 ml phenyl-Sepharose CL 4B (Pharmacia) column which had been equilibrated in buffer A containing 0.5 M NaCl. After washing with the equilibration buffer until the absorbance at 280 nm was lower than 0.01 O.D., the recombinant calmodulin was eluted with the same buffer containing 5 mM EGTA instead of Ca^{2+}. The fractions containing calmodulin were pooled, dialysed exhaustively against 10 mM NH_4HCO_3, and concentrated by lyophilization. This procedure takes 1 day to perform and yields 5–6 mg of calmodulin from 1 l of bacterial culture.

2.9. *Protein analysis*

Protein concentration was determined according to [18] using bovine serum albumin as standard. SDS gel electrophoresis was performed as described by [19] and protein bands were visualized according to [20]. Native CaM of *N. crassa* was prepared as in [17]. The isolation of the Ca^{2+}-CaM-dependent kinase activity, and the conditions of ^{32}P incorporation in the 47 kDa peptide and its quantification were realized as in [5] with both CaMs.

3. RESULTS AND DISCUSSION

In the initial screening of approximately 280,000 phages of the λZAP II cDNA expression library, 37 positive plaques were picked and rescreened until plates contained only plaques reactive to anti-CaM antibody. Fifteen clones were finally isolated and their inserts were excised with the restriction enzymes *Eco*RI and *Xho*I. All the digests analyzed on a 1% agarose gel indicated that they contained an insert with a size ranging from 450 to 850 nucleotides.

Several clones containing the longest insert were selected for sequencing by the dideoxy chain-termination method [11].

The complete nucleotide sequence is shown in Fig. 1, along with the deduced amino acid sequence. This clone, designated as pNC15, has a cDNA 820 base pairs in length which contains 14 bases of 5′ untranslated region (including the initiation codon ATG), the entire protein coding region of 444 bases, 321 bases of 3′ untranslated region (including the termination signal TAA) terminated with a poly(A)$^+$ tail of about 40 bases. The open reading frame (ORF) encodes a protein of 148 amino acid residues with a calculated molecular weight of 16,865 Da. The primary structure of *N. crassa* CaM showed a high degree of identity with CaMs from other species (Fig. 2). A single substitution (Phe13/Tyr) corresponding to a homology of 99.3% differentiates it from that of the filamentous ascomycete, *Aspergillus nidulans* [21], whereas when compared to vertebrate [22] and the pathogenic yeast, *Candida albicans* [23], the homologies were, respectively, of 85.1% and 83.1%. These differences could explain the high specificity of antibodies obtained against calmodulin from *N. crassa* [9]. The sequence shows that the four putative calcium-binding regions are functional, confirming that each molecule of calmodulin from *N. crassa* can bind four Ca^{2+} [24].

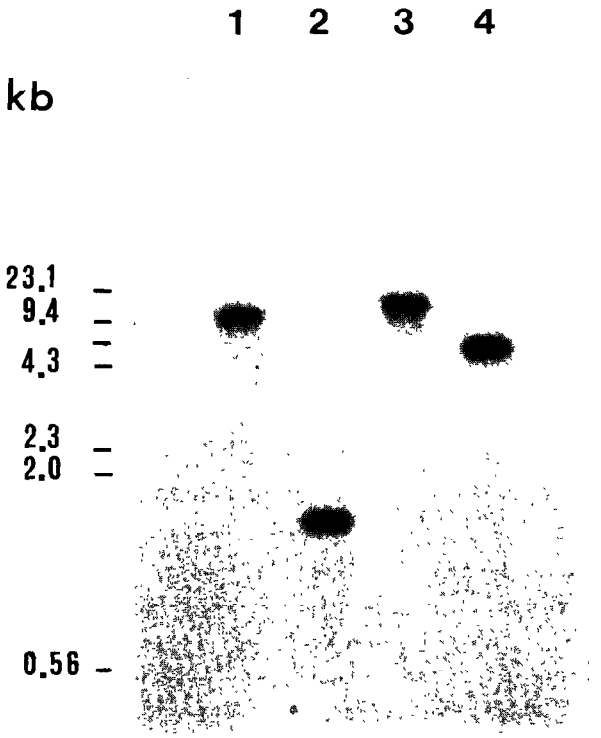

Fig. 3. Southern blot analysis of *N. crassa* genomic DNA. Samples of genomic DNA (5 μg) were digested to completion with the restriction enzymes, *Pst*I, *Hin*dIII, *Pvu*II, *Sca*I (lanes 1–4, respectively), and subjected to electrophoresis in 0.8% agarose gel. The fragments were capillarily transferred to a nitrocellulose membrane and hybridized with ^{32}P-labelled pNC15 cDNA.

To determine the number of CaM genes in the *N. crassa* genome, the CaM cDNA was labelled and used to probe *N. crassa* DNA digested with a variety of restriction enzymes (Fig. 3). When the genomic DNA was digested with endonucleases that do not cut in the CaM coding region, one single restriction fragment was detected, indicating that in *N. crassa*, CaM is encoded by a single copy gene. It should be noticed that this is also the case for *Dictyostelium discoideum* [25] and *Aspergillus nidulans* [21].

In order to examine the functional properties of *N. crassa* CaM, the complete cDNA was ligated into a bacterial expression vector containing the *trc* promoter, as summarized in section 2. Fig. 4 illustrates the procedure used to construct the expression vector, pNCCaM, which has the entire coding sequence for *N. crassa* CaM.

Calmodulin from *N. crassa* over-expressed in *E. coli* was analyzed, after purification on SDS-PAGE [19], using native CaM as a reference. As shown in Fig. 5, a band with an apparent size of 16.5 kDa comigrated with *N. crassa* CaM and exhibited, in the presence or absence of calcium, the same characteristic electrophoretic shift. Thus, the expressed protein appears to be identical to native protein with respect to its molecular weight and electrophoretic properties.

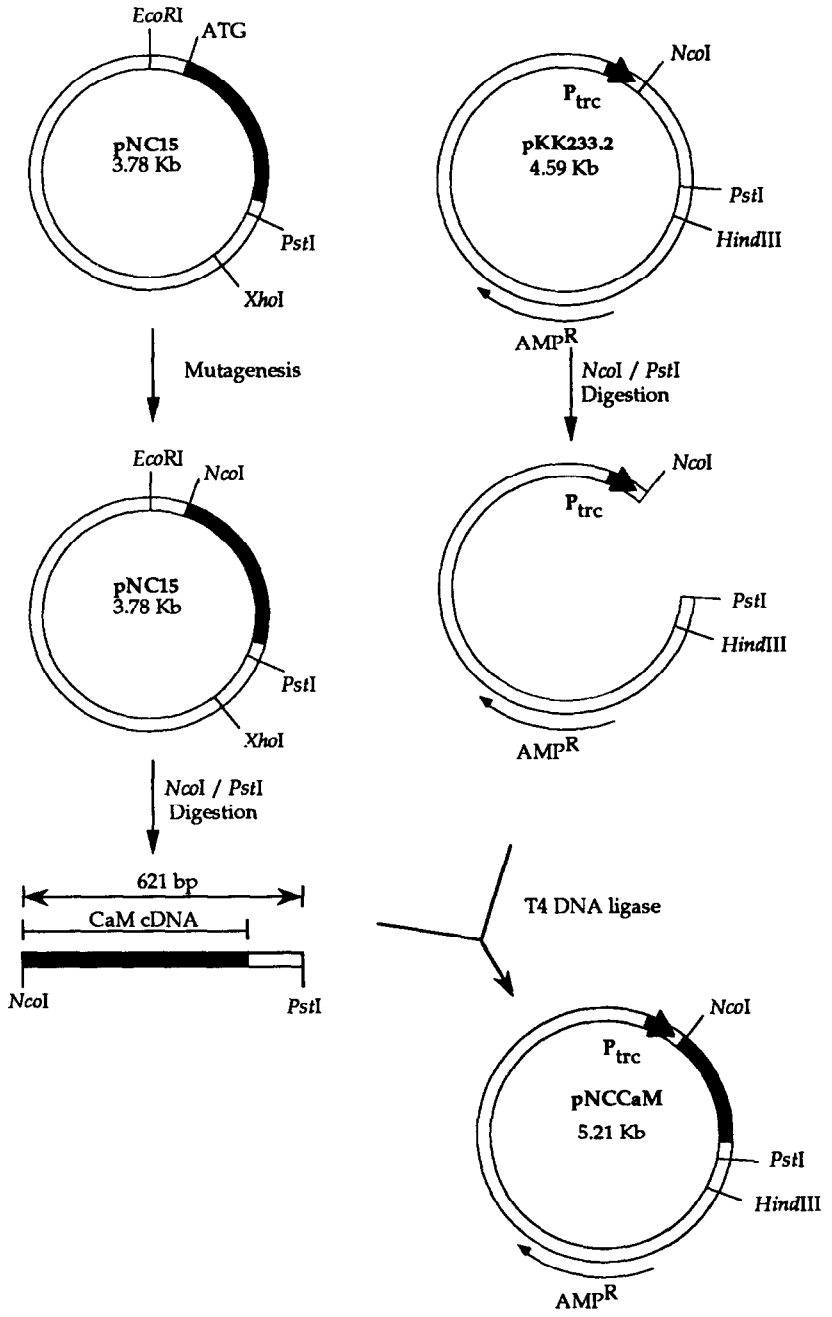

Fig. 4. Oligonucleotide-directed mutagenesis was used to construct a *Nco*I site at the ATG start codon of the *N. crassa* CaM. The 621-base pairs (bp) *Nco*I–*Pst*I fragment containing the entire *N. crassa* calmodulin coding sequence was inserted into pKK233.2, producing pNCCaM. Restriction sites for *Eco*RI, *Nco*I, and *Pst*I are indicated. Kb, kilobase pair(s).

To compare the biochemical activity of both CaMs we tested the activation of the Ca^{2+}-CaM-dependent protein kinase activity. The apparent half-maximal activation of ^{32}P incorporation in the 47 kDa peptide was obtained, in the presence of saturating concentration of Ca^{2+}, with 0.2 μM of either calmodulin (data not shown), values which were in perfect agreement with our first report [5]. These results suggest that no post-translational modification of calmodulin, affecting the stimulation of the protein kinase, occurs in *N. crassa* [24].

In conclusion, we have cloned and characterized a full-length cDNA of calmodulin from *N. crassa*, which, after over-expression in *E. coli*, gave rise to a protein that was identified as calmodulin by several biochemical criteria. This full-length clone, as well as the sequence information, will enable us to produce site-specific mu-

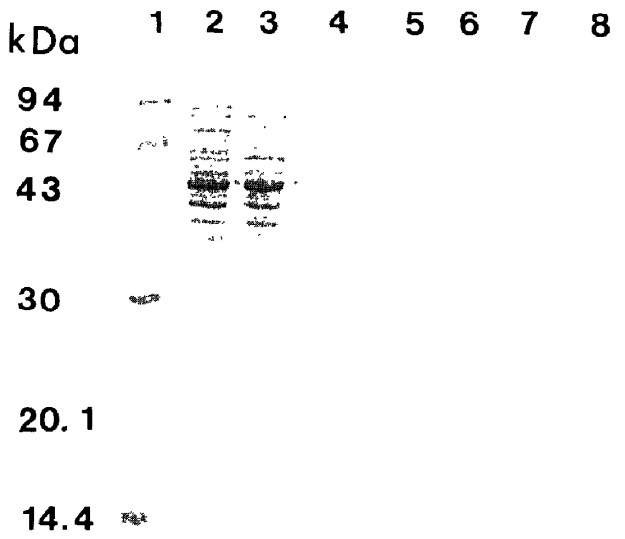

Fig. 5. Electrophoretic analysis of expressed *N. crassa* CaM on 15% SDS-PAGE. Lanes: 1, low molecular weight standards; 2, lysate (40 μg) of *E coli* SB1 cells containing only the lysis plasmid pSB6; 3 and 4, lysate (40 μg) of *E coli* SB1 cells transformed with expression vector pNCCaM, respectively, before and after heating, 5 and 6, purified recombinant CaM (3 μg) in the presence of either 5 mM EGTA or 5 mM $CaCl_2$; 7 and 8, native CaM (3 μg) in the presence of, respectively, 5 mM EGTA or 5 mM $CaCl_2$.

tants of CaM in order to systematically analyze the structure and function of CaM.

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