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The *Aspergillus nidulans cnxABC* Locus Is a Single Gene Encoding Two Catalytic Domains Required for Synthesis of Precursor Z, an Intermediate in Molybdenum Cofactor Biosynthesis*

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Shiela E. Unkles‡§¶, Jacqueline Smith‡‖, Ghassan J. M. M. Kanan‡ ‡‡, Lindsey J. Millar‡**, Immanuel S. Heck§§¶¶, David H. Boxer§§, and James R. Kinghorn‡‖ ‖**

From the ‡School of Environmental and Evolutionary Biology, University of St. Andrews, St. Andrews, Fife KY16 9TH, United Kingdom, the §Department of Microbiology, Monash University, Clayton, 3168, Australia, and the §§Department of Biochemistry, Medical Sciences Institute, Dundee University, Dundee DD1 4HN, United Kingdom

The *Aspergillus nidulans* complex locus, *cnxABC*, has been shown to be required for the synthesis of precursor Z, an intermediate in the molybdopterin cofactor pathway. The locus was isolated by chromosome walking a physical distance of 65-kilobase pairs from the *brlA* gene and defines a single transcript that encodes, most likely, a difunctional protein with two catalytic domains, CNXA and CNXC. Mutations (*cnxA*) affecting the CNXA domain, mutants (*cnxC*) in the CNXC domain, and frameshift (*cnxB*) mutants disrupting both domains have greatly reduced levels of precursor Z compared with the wild type. The CNXA domain is similar at the amino acid level to the *Escherichia coli moaA* gene product, while CNXC is similar to the *E. coli moaC* product, with both *E. coli* products encoded by different cistrons. In the wild type, precursor Z levels are 3–4 times higher in nitrate-grown cells than in those grown on ammonium, and there is an approximately parallel increase in the 2.4-kilobase pair transcript following growth on nitrate, suggesting nitrate induction of this early section of the pathway. Analysis of the deduced amino acid sequence of several mutants has identified residues critical for the function of the protein. In the CNXA section of the protein, insertion of three amino acid residues into a domain thought to bind an iron-sulfur cofactor leads to a null phenotype as judged by complete loss of activity of the molybdoenzyme, nitrate reductase. More specifically, a mutant has been characterized in which tyrosine replaces cysteine 345, one of several cysteine residues probably involved in binding the cofactor. This supports the proposition that these residues play an essential catalytic role. An insertion of seven amino acids between residues valine 139 and serine 140, leads to a temperature-sensitive phenotype, suggesting a conformational change affecting the catalytic activity of the CNXA region only. A single base pair deletion leading to an in frame stop codon in the CNXC region, which causes a null phenotype, effectively deletes the last 20 amino acid residues of the protein, indicating that these residues are necessary for catalytic function.

More than 3 decades ago, Pateman, Cove, and co-workers (see Refs. 1 and 2; reviewed in Refs. 3–6) first isolated mutants defective in the synthesis of the molybdenum pterin cofactor. Using the lower eukaryotic model, the ascomycetous fungus *Aspergillus nidulans*, they isolated a class of chlorate-resistant mutants, which were unable to utilize either nitrate or purines such as adenine, hypoxanthine, and xanthine as sole sources of nitrogen. This inability to grow on nitrate and hypoxanthine was due to the pleiotropic loss of NADPH-nitrate reductase and NADH-xanthine dehydrogenase (purine hydroxylase I) activity, respectively. They speculated that such mutants were defective in the synthesis of a cofactor common to both nitrate reductase and xanthine dehydrogenase and accordingly designated the mutants *cnx* (common component for nitrate reductase and xanthine dehydrogenase). Five *cnx* loci (namely *cnxABC*, *cnxE*, *cnxF*, *cnxG*, and *cnxH*) were originally observed by Pateman *et al.* (2) on the basis of complementation tests in heterokaryons grown with nitrate as the sole nitrogen source. Later, a sixth locus, *cnxJ*, was identified, albeit with a different growth phenotype by Arst *et al.* (7).

The *cnxABC* locus showed a complex overlapping pattern of complementation, *i.e.* growth on nitrate in heterokaryon combinations of different pairwise (*cnxA*, *cnxB*, *cnxC*) mutant backgrounds (2, 8). Mutants in *cnxA* and *cnxC* phenotypically complement each other, *vis à vis* growth of *cnxA*/*cnxC* heterokaryons on nitrate as a sole nitrogen source, while *cnxB* mutants fail to complement either *cnxA* or *cnxC* mutants. Since *cnxA*, *cnxB*, and *cnxC* mutants were shown to be genetically tightly linked, it was unclear whether *cnxABC* consisted of one gene in which *cnxA* and *cnxC* mutants exhibited intragenic complementation or two distinct genes, *cnxA* and *cnxC*, with *cnxB* mutants lacking both activities.

Virtually nothing is known about the function of the *A. nidulans cnx* genes and their role in the synthesis of the molybdopterin cofactor. We describe here the molecular and biochemical characterization of *cnxABC*, the first cloned *A. nidulans cnx* locus reported thus far.

EXPERIMENTAL PROCEDURES

A. nidulans Strains, Plasmids, Media, and Transformation—The wild-type strain used was G051 (*biA1*). Strains α8 (*biA1 cnxA9*), G055

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF027213.

¶ To whom correspondence should be addressed: Tel.: 03-9905-4323; Fax: 03-9905-4811; E-mail: shiela.unkles@med.monash.edu.au.

‖ Present address: Roslin Institute, Roslin, Midlothian, EH25 9PS, UK.

** Recipient of a BBSRC Postgraduate Studentship.

‡‡ Recipient of a University of Mu'tah (Jordan) Research Scholarship. Present address: Mu'tah University, P. O. Box 7, Karak, Jordan.

¶¶ Present address: Institute for Plant Biochemistry, Corrensstrasse 41, D-72076 Tuebingen, Germany.

‖‖ Recipient of a Royal Society (London) travel award for study in Australia.

(*biA1 cnxB11*), α23 (*yA2 wA3 cnxC2*), and G832 (*yA2 pyroA4 cnxC3*) were mutants described originally by Pateman and Cove (2). Strains GH140 (*biA1 cnxA140ts*), GH95 (*yA2 pyroA4 cnxA95*), GH1281 (*biA1 cnxB1281*) and GH2O4 (*biA1 cnxB204*) were selected in this study on the basis of resistance to 150 mM chlorate as described by Cove (9).[1] *cnxA95* and *cnxB204* were selected on 10 mM glutamate, *cnxA140*^{ts} with 10 mM proline and *cnxB1281* with 5 mM uric acid all as sole nitrogen sources. The *cnxA95*, *cnx204*, and *cnxB1281* mutations were generated using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, while *cnxA140*^{ts} was synthesized after 1,2,7,8-diepoxyoctane chemical mutagenesis (2). Assignment of mutations to *cnx* loci was carried out by the heterokaryotic complementation test, *i.e.* growth of pairwise heterokaryons on nitrate as the sole nitrogen source against the representative *cnx* mutants, *cnxA9*, *cnxB11*, *cnxC3*, *cnxE3*, *cnxF7*, *cnxG4*, and *cnxH4*, as described by Pateman *et al.* (2). The temperature-sensitive mutant *cnxA140*^{ts} shows mutant phenotypes of resistance to chlorate and nongrowth on nitrate as the sole nitrogen source at 37 °C but near wild type phenotypes of sensitivity to chlorate and growth on nitrate at 25 °C with a mutant phenotype on hypoxanthine at both temperatures. Additionally, this mutant has a wild-type nitrate reductase temperature stability profile from cells grown at the permissive temperature of at 25 °C.[1] Standard *Aspergillus* growth media, handling techniques (10), and transformation (11) were as described previously. For expression analysis, cultures were grown at 30 °C for 16 h in liquid minimal medium (9) containing the sole nitrogen sources stated in the figure legends.

Molecular Methods—Standard procedures were used for propagation of cosmids and for subcloning and propagation of plasmids in *Escherichia coli* strain DH5α. Conditions employed here for *A. nidulans* Southern and Northern blot analysis were as described previously (12). The nucleotide sequence of the wild-type *cnxABC* gene was determined in both strands using a Sequenase version 2 DNA sequencing kit according to the manufacturer's instructions (Amersham Corp.). For primer extension analysis, mRNA was prepared from mycelium grown in minimal medium containing 10 mM sodium nitrate as the sole nitrogen source at 25 °C for 18 h, using a Quickprep mRNA Purification Kit (Pharmacia Biotech Inc.). Messenger RNA (2 μg) was hybridized with 5′ ^{32}P-end-labeled primer PE-1 (5′-GAAGTCGCTTGAGCTGCG-3′, position +47) at 52 °C for 1 h and reverse transcribed using a primer extension system as recommended (Promega). The extension product was compared on a denaturing sequencing gel with a DNA sequence ladder prepared using the same end-labeled primer with pSTA502 as template.

PCR[2] *Amplification and Mutant DNA Sequence Determination*—Genomic DNA was prepared from mycelia grown in liquid culture for 16–18 h at 25 °C using a Nucleon BACC2 Kit (Scotlab). DNA was cleaved with *Eco*RI, and around 100 ng was amplified using 2.5 units/μl Dynazyme (Flowgen) or 2.5 units/μl *Taq* DNA polymerase (Boehringer Mannheim), a 1 μM concentration of each primer, and 100 μM dNTPs. Cycling conditions were 94 °C for 1 min, 50 °C for 20 s, and 72 °C for 50 s for one cycle, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 50 s. The entire *cnxABC* coding region was amplified in four overlapping sections using primers P1 and P2 (5′-CGTTGTCGAGCAGAATC-3′ and 5′-TATGCTCTTCATAACCGC-3′, positions −27 to +687), primers P3 and P4 (5′-GTAAATCTCAGTCTGGAC-3′ and 5′-CATGCCAATAACGTCAAG-3′, positions +601 to +1263), primers P5 and P6 (5′-TGAGGCAGCTCGAACAG-3′ and 5′-GACGTCGGTTGGAGAAG-3′, positions +1176 to +1876), and primers P7 and P8 (5′-ATATGACGCTGATTGATG-3′ and 5′-TGTCATACACATCCAGG-3′, positions +1796 to +2344). Following removal of PCR primers and unincorporated nucleotides by the Glassmax DNA Isolation Spin Cartridge System (Life Technologies), the amplified DNA was sequenced directly in a single strand only by automated DNA sequencing using an ABI 373 A automated fluorescent sequencing apparatus and the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) with the PCR primers at a concentration of 10 μM. Additional primers used for sequencing were PE-2 (5′-CCGGTAGTGACATATCGC-3′, position +140), AR5 (5′-GATTACCTTAGGATCAG-3′, position +280), AR3 (5′-TGGATTCCAAGGCCGAG-3′, position +975), AR8 (5′-TTGTATACATGATTCAC-3′, position +1404), and CR9 (5′-ATGACGCTGAGATGAAG-3′, position +2051). Sequences were compared with the wild type using Sequencher (Gene Codes Corp.). Upon identification of a putative mutation, the relevant primer pair was used to reamplify the digested genomic DNA, and the product was sequenced again to confirm the mutational change.

Phenotypic Complementation—Conidial suspensions of around 10^6 viable cells were made in 0.9% (w/v) sodium chloride, 0.1% (v/v) Tween 80, and 1 μl of the strain under test was spotted on the surface of agar minimal medium containing 10 mM sodium nitrate as the sole nitrogen source. A second 1-μl aliquot of the standard strain was spotted on top of the test strain, and the medium was incubated at 37 °C.

HPLC Analysis of Molybdopterin Form A Dephospho and Compound Z Levels in A. nidulans Oxidized and Dephosphorylated Cell-free Extracts—Form A dephospho and compound Z levels were analyzed, as a measure of precursor Z and molybdopterin, respectively, according to the method described by Johnson and Rajagopalan (13, 14) with modifications. A conidial suspension in 20 ml of 0.9% saline, 0.1% Tween 80 (approximately 5×10^6 conidia/ml) was used to inoculate 400 ml of minimal medium supplemented with the stated sole nitrogen sources after Cove (9). Cells were grown for 16 h at 30 °C and 250 rpm and harvested by filtration through sterile muslin cloth. After washing with 50 ml of sterile distilled water, the cells were pressed dry and frozen in liquid nitrogen. Frozen cell material (1.5 g) was homogenized by sonication (5 × 15 s) in 3 ml of 100 mM Tris-HCl buffer (pH 7.2), and the cell debris was removed by centrifugation (20 min at 14,000 rpm). Of the resulting supernatant, 1 ml was combined with 125 μl of I_2/KI (1%/2%) in 1 M HCl and then left in darkness for 10 h at room temperature. 138 μl of 1% ascorbic acid and 0.5 ml of 1 M Tris-HCl were added before centrifugation at 14,000 rpm for 10 min at room temperature to remove any particulate matter. The supernatant was mixed with 13 μl of 1 M $MgCl_2$ and 2 units of alkaline phosphatase and incubated for a further 12 h in darkness at room temperature. The samples were subsequently applied to QAE-Sephadex A-25 (Sigma) columns (acetate form, 0.5-ml bed volume) and washed with 5 ml of distilled water. Form A dephospho was eluted with 5 ml of 10 mM acetic acid, adjusted to pH 7.0 with NH_4OH, and stored frozen until HPLC analysis. Compound Z was eluted with 8 ml of 10 mM HCl. The HCl eluates were applied to Florisil (Sigma) columns (250 mg of Florisil mesh 100–200, washed with 12 ml of 10 mM HCl; bed volume, 0.6 ml). The columns were then washed with 1 ml of 10 mM HCl, and compound Z was eluted with 1.7 ml of 22.5% acetone. The acetone eluates were rotoevaporated until dry. The compound Z samples were dissolved in 300 μl of H_2O, of which 100 μl was injected for HPLC analysis.

Analysis of form A dephospho and compound Z by reversed phase HPLC was performed using a Hypersil ODS column (250 × 4.6 mm, 5 μm). Form A dephospho (500 μl of the acidic acid eluate) was eluted with 10% methanol, 50 mM ammonium acetate (pH 6.7, 1 ml/min) and detected using a Shimadzu RF-551 fluorescence detector set to 370/450 nm (emission/excitation). Compound Z was eluted with 5% methanol, 50 mM triethylammonium acetate (pH 7.0, 1 ml/min) and detected by fluorescence at 350/450 nm (emission/excitation).

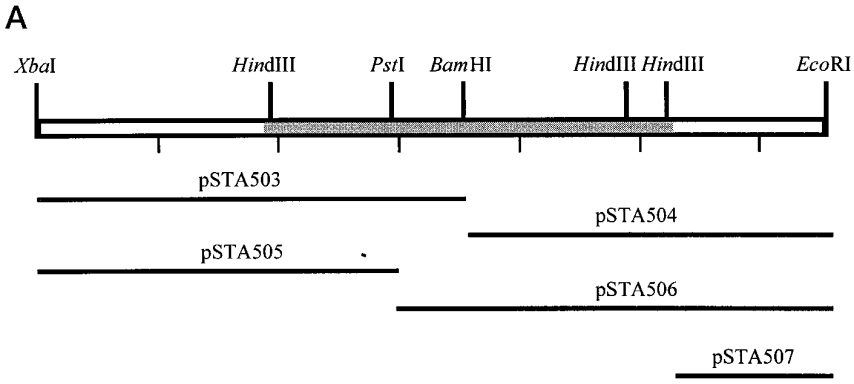
Total protein was estimated using the bicinchoninic acid (BCA) method, with bovine serum albumin as a standard.

RESULTS

Isolation of the cnxABC-complementing Cosmid and Subclones—Previous classical genetics has shown that the *cnxABC* locus is approximately 3 recombination map units from the *brlA* locus on linkage group VIII. Using a 4.8-kb *Bam*HI DNA fragment of pILJ421 (11) containing the *brlA* gene as a hybridization probe, two cosmids were isolated from the chromosome 8-specific cosmid library (15). Since neither of these cosmids complemented phenotypically (*i.e.* growth on nitrate as sole nitrogen source) *cnx* mutant strain *cnxA9*, *cnxB11*, or *cnxC 3* in transformation experiments (see "Experimental Procedures"), the cosmids were used to generate end probes to walk to neighboring cosmids. Eight overlapping cosmids were identified, and one of these, W26G08, was found to complement all three mutant strains, *i.e. cnxA9*, *cnxB11*, and *cnxC3*, at transformation frequencies in excess of 100 transformants/μg of cosmid DNA. Phenotypic complementation of the mutants using *Eco*RI-, *Xba*I-, and *Bam*HI-digested and isolated fragments of cosmid W26G08 indicated that the complementing region was located on a 6.5-kb *Eco*RI-*Xba*I fragment, estimated by restriction endonuclease mapping to be around 65 kb from the *brlA* gene. This DNA fragment, the restriction map of which is shown in Fig. 1*A*, was subcloned into vector pUC18, resulting in recombinant plasmid pSTA502.

[1] G. J. M. M. Kanan, unpublished observations.

[2] The abbreviations used are: PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; kb, kilobase pair.



B

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-1115  TTTGACCTCAAATATCGTGTGGCAAGGAAAGATCATATAAAGACGTCCAAAAAACCGTTT
-1055  AACATAAAGCTTTATAAACTAAGAACTAGGCTTTAGTTATGTCTCTGCTCAAAGAGCGGT
 -995  TTGAGCTACTATCTCGATCAATACTGTTTGTACTTTATGCTGCGTTTCGGGTAGGGTTAA
 -935  ACAGACAACATCGGACGGCATGTTCGGAGCGACGTCACGACGGCGCCCAGAAGAATAGTT
 -875  GGTATCTTGACGTCTTGAAAATCCCATATTTCCGCCTTTGAAAATGTGTTCGCGGTAATA
 -815  CAGCCCTTGGTCTATATCTGGGTCGCAACGTATGCTCGTCAGAAAGGCACAGGCTGCGGG
 -755  CTTGGCGGGCAATGTGAGAACCCCCATGGCAAGCAGAGGAATGATCTGAGGGATTAGATG
 -695  TCATGAAGATTAATCGCTACCTCAAATAGTGGCCGGATATTGAAATATGACGACAAATGC
 -635  ACTTCATGTTCGAGGCATACAGTATGGCCATTGGTTCCTTGGATGAAGCACGCGGGGACA
 -575  TTCGATGATTCCTCGCGCCCTCTGTTTGTGGAGCCATATGACACTAACAAATCACTACAG
 -515  CAGAGATCAATATTAGACTCGAAGCTGACGCGACGGAGGCATCAGACTAAACCTGATCCG
 -455  CGAACTGCACAATTACCAAAGAAGCTATCCAACGAAGTCGACCACGGTTGTTCCCGAGCG
 -395  GTTGATCAGATGCCCTCAAGAAACGGGCGTTGATGCAGTATCAGACAAGAAAACAATCAG
 -335  AGGGCGACACCCAAGCCTAATTTAAATATAGAGTCCATCAGCGTTCTGTTGGGAAATGTC
 -275  AGCATATTGTCTAATATCAGTTGAACATATCTGCTATAATCTTGCTTTTTTAGCGTACGT
 -215  CAGTGCACCGACAGCTATCTTATCGATTAGTAATTGCAGTACTAAGTCAGGTGATGGAGG
 -155  CGACCGAACCCGCGGAGAACAGCGACGATAAGAAATTCCTTATTATCCCATCCTTCCATC
                      ↓
  -95  GTTTGCCTTGGTTTTAGTTGTTAGTGCTTTGTTCCCTCGGGTGATGAAGCGCGTACTTGG
  -35  GATCTGGCCGTTGTCGAGCAGAATCCCTGCAGGAGATGACTGAGCCCATGCTATTTAAGC
                                          M  T  E  P  M  L  F  K  R    9
   26  GGGGCGCAGCTCAAGCGACTTCGTCCTTTTCTCGATTTCCCCGCATCCATACTCGAACTC
         G  A  A  Q  A  T  S  S  F  S  R  F  P  R  I  H  T  R  T  R   29
   86  GACTCCCGACGTGGCGCCGGTTGAGCCCTTCGCCTTCGCGATATGTCACTACCGGTTTGA
         L  P  T  W  R  R  L  S  P  S  P  S  R  Y  V  T  T  G  L  N   49
  146  ACCCGCAAGTGGAAGTTCAGGATGAACCAACTAGGTCTCCATCTCCTACACCGACATCGT
         P  Q  V  E  V  Q  D  E  P  T  R  S  P  S  P  T  P  T  S  S   69
  206  CGACTCGCTGGAACGCGCTGAAAACAGCAAAGCCGTTCTCGGCGTTTCTGACAGACACAT
         T  R  W  N  A  L  K  T  A  K  P  F  S  A  F  L  T  D  T  F   89
  266  TCAACCGCCAGCATGATTACCTTAGGATCAGTGTCACGGAGCGCTGCAATCTGCGCTGTC
         N  R  Q  H  D  Y  L  R  I  S  V  T  E  R  C  N  L  R  C  L  109
  326  TATATTGTATGCCTGAAGAAGGCGTACCGCTATCCCCTCCCGCGCACGTCCTGACGTCGC
         Y  C  M  P  E  E  G  V  P  L  S  P  P  A  H  V  L  T  S  P  129
  386  CGGAGATTGTGTACCTTTCGTCGCTCTTCGTCTCGCAGGGCGTGACCAAGATCCGTCTGA
         E  I  V  Y  L  S  S  L  F  V  S  Q  G  V  T  K  I  R  L  T  149
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FIG. 1. **Restriction endonuclease map of recombinant plasmid pSTA502 and DNA and deduced amino acid sequence of the *A. nidulans cnxABC* locus.** *A*, *shaded area* represents the region of the plasmid in which the DNA sequence has been determined in both strands. The *bars below* the *map* indicate the positions of subclones generated for transformation to localize the complementing region of pSTA502. *B*, *numbers* on the *right* refer to nucleotides relative to the adenosine of the start codon, numbered 1, and *numbers* on the *left* refer to amino acid residues. The *vertical arrow* indicates the transcriptional start point. Potential CCAAT and TATA motifs in the promoter region are *underlined*. A possible receptor site for the NIRA control protein is in *boldface type*. The intron is shown in lowercase with the 5′ and 3′ consensus sequences in *boldface type* and *underlined* and potential lariat motifs *underlined*.

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446  CTGGCGGAGAACCGACTGTTCGGAAAGACATCGTTCCTCTGATGCAGTCAATTGGAGAAT
       G  G  E  P  T  V  R  K  D  I  V  P  L  M  Q  S  I  G  E  L   169

506  TACGACATCACGGACTCCGAGAATTATGCTTGACAACCAATGGCATTTCACTCCATCGTA
       R  H  H  G  L  R  E  L  C  L  T  T  N  G  I  S  L  H  R  K   189

566  AGCTCGAGCCTATGGTGGAGGCTGGGTTGACTGGGGTAAATCTCAGTCTGGACACTTTGG
       L  E  P  M  V  E  A  G  L  T  G  V  N  L  S  L  D  T  L  D   209

626  ATCCGTTCCAGTTTCAAATTATGACGAGGAGGAAGGGCTTTGATGCGGTTATGAAGAGCA
       P  F  Q  F  Q  I  M  T  R  R  K  G  F  D  A  V  M  K  S  I   229

686  TAGACCGCATCCAGGAGCTGAATAAGATGGGAGCTGGGATAAAGCTCAAGATCAATTGTG
       D  R  I  Q  E  L  N  K  M  G  A  G  I  K  L  K  I  N  C  V   249

746  TTGTGATGCGGGGCCTCAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCGTGATA
       V  M  R  G  L  N  E  R  E  I  I  P  F  V  E  M  G  R  D  S   269

806  GCCCCATCGAAGTGCGGTTCATTGAGTATATGCCATTTGATGGCAATAAGTGGAGTAAGG
       P  I  E  V  R  F  I  E  Y  M  P  F  D  G  N  K  W  S  K  G   289

866  GAAAAATGGTTTCCTATCAGGAGATGCTGGCCCTTATTCGGGAGAAGTATCCAACATTGG
       K  M  V  S  Y  Q  E  M  L  A  L  I  R  E  K  Y  P  T  L  E   309

926  AGAAGGTGGTGGATCATAAGAATGACACGAGCAAAACTTATCGCATTCCTGGATTCCAAG
       K  V  V  D  H  K  N  D  T  S  K  T  Y  R  I  P  G  F  Q  G   329

986  GCCGAGTTGGCTTTATCACGAGCATGACGCATAACTTCTGCGGCACTTGCAACCGCCTTC
       R  V  G  F  I  T  S  M  T  H  N  F  C  G  T  C  N  R  L  R   349

1046 GCATTACGTGCGATGGGAATCTTAAAGTCTGCCTATTTGGAAACTCGGAAGTCTCGCTGC
       I  T  C  D  G  N  L  K  V  C  L  F  G  N  S  E  V  S  L  R   369

1106 GTGATATAATCCGACAACAGAATAATGGCGAGCCCATTGACGAGACTGCGCTGCAAGAGC
       D  I  I  R  Q  Q  N  N  G  E  P  I  D  E  T  A  L  Q  E  L   389

1166 TAGGTCTCCTTGAGGCAGCTCGAACAGCAGCCCGCGTTCATGACGAAGGTGGAGTAGTCA
       G  L  L  E  A  A  R  T  A  A  R  V  H  D  E  G  G  V  V  S   409

1226 GTCAAAGAGAAAGAGAGCTTCTTGACGTTATTGGCATGGCAGTGAAGCGGAAAAAGGCCA
       Q  R  E  R  E  L  L  D  V  I  G  M  A  V  K  R  K  K  A  K   429

1286 AGCATGCCGGCATGGGAGAGTTAGAGAACATGAAGAACCGGCCGATGATCCTTATTGgtg
       H  A  G  M  G  E  L  E  N  M  K  N  R  P  M  I  L  I  D      448

1346 ggtaggcactttccaccacctatatatatgactgcgacgagagactatgatcgatgagtt

1406 gtatacatgattcacttggttttttttttctttttttttttttccttttcctttttcttttctt

1467 gacgcgggcgttcaaatcacattcttgtatatacggttatgcatgacactgagagatgct

1527 gataggcggttcagATAAAACGAGCGATGCCCAGAGAAATATAAGGTACTTCGCCTCCAT
                    K  T  S  D  A  Q  R  N  I  R  Y  F  A  S  M   463

1587 GTCGTCAATGATGTCCAAGGGACAAGTAATGAACGTATCGACGCATTCACTCGGTCTCGG
       S  S  M  M  S  K  G  Q  V  M  N  V  S  T  H  S  L  G  L  G   483

1647 GATGCCAATGGCTACCCAAGTCCGACTTTACCATCGCGAACGTACCACAACTTCGTGCGA
       M  P  M  A  T  Q  V  R  L  Y  H  R  E  R  T  T  T  S  C  E   503

1707 GGAACCCAGCAACAAGGACTCAAAATTCGCTTCGCTCCCTACATCCGACGACCCCGATCT
       E  P  S  N  K  D  S  K  F  A  S  L  P  T  S  D  D  P  D  L   523

1767 GCCTCATCTCAATCGCTCGCAAAACGTCCATATGACGCTGATTGATGAAAAGCCCATTTC
       P  H  L  N  R  S  Q  N  V  H  M  T  L  I  D  E  K  P  I  S   543

1827 AAAACGCCTCGCAACAGCCACCTGTCACGTCCGCTTCTCCAACCGACGTCCCTGGGAACT
       K  R  L  A  T  A  T  C  H  V  R  F  S  N  R  R  P  W  E  L   563

1887 CCTCAGACAAGGCCCCGGCAGCCGCAAAGGCGACGTATTTGGCATCGCTCGTATTGCCGG
       L  R  Q  G  P  G  S  R  K  G  D  V  F  G  I  A  R  I  A  G   583

1947 TATCACCGCCGCGAAGAAGACGCCGGATATTGTACCGCTTTGTCATCCTGGGCTGGGGTT
       I  T  A  A  K  K  T  P  D  I  V  P  L  C  H  P  G  L  G  L   603

2007 GACGGGGGTGGAGGTGGATGTGAAGCTTCTTGACCCGTCCGCCGATGACGCTGAGATGAA
       T  G  V  E  V  D  V  K  L  L  D  P  S  A  D  D  A  E  M  K   623
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FIG. 1.—*continued*

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2067  GCATGGGGCAATGCATGTCACGGCCACGGTGGGCTGTGTTGGAAGAACGGGGGTGGAGAT
       H  G  A  M  H  V  T  A  T  V  G  C  V  G  R  T  G  V  E  M    643

2127  GGAAGCCATGACGGCGACGATGGGGGCTGCATTGACGGTGTATGATATGTTGAAAGCGGT
       E  A  M  T  A  T  M  G  A  A  L  T  V  Y  D  M  L  K  A  V    663

2187  TGACAAGGGGATGGTGATTGGGGGAGTGAAACTGCTAGAGAAGATGGGGGGCAAGAGCGG
       D  K  G  M  V  I  G  G  V  K  L  L  E  K  M  G  G  K  S  G    683

2247  GCATTGGGTAAGAGAGGAGAACGTGAAGGATGAGTAGATGTGGTTGACACACTGGGTTGA
       H  W  V  R  E  E  N  V  K  D  E  *                           694

2307  GTCTGTACCTGTACCTATTTACCTGGATGTGTATGACAACGGGACCTGGCGGTGTCTTAC

2367  TAAGAAATCTCCCGTCCCCTCGTAGGGCTGACCCAAGCTTTTGCCATGCCTTTTCTCGCA

2427  CCAAAATCATGGAGACTACGCCTTGTCATCTCCTCTCAAGCCAGCTGCCAGAGCAGAAAA

2487  CAGCCCCTGGGCACTAAA
```

FIG. 1.—*continued*

To define more precisely the regions of pSTA502 responsible for the complementation of the three *cnx* mutant alleles, further subclones of pSTA502 were obtained (Fig. 1*A*). Subclones pSTA503, pSTA505, and pSTA507 did not complement *cnxA9*, *cnxB11*, or *cnxC3* alleles. Subclones pSTA504 and pSTA506 complemented the *cnxC3* mutation at high frequency, but only pSTA506 complemented *cnxB11* and *cnxA9*, although at an efficiency much reduced compared with the original recombinant plasmid, pSTA502. Therefore, the minimum region necessary for complementation resides on the stretch of pSTA502 to the right of the *Pst*I site (Fig. 1*A*).

Molecular Characterization of cnxABC—The DNA sequence of a 3619-base pair stretch of pSTA502 encompassing the complementing region was determined in both strands (Fig. 1*B*). A long open reading frame is interrupted by a single intron 198 base pairs long (shown in lowercase), the precise location of which was determined by sequencing a reverse transcriptase-PCR product generated by primers P5 and P6 straddling the proposed intron position.[3] The intron is bounded by canonical 5′- and 3′-splice sequences and has an internal motif corresponding to that required for lariat formation in fungi (16). A single transcriptional start point was determined to be at position −73 relative to the proposed translational start,[3] which is the first ATG in frame following the transcriptional start point and is surrounded by a recognized translational initiation sequence context for *A. nidulans* (17). Perusal of the noncoding region upstream of the ATG suggests potential TATA motifs at positions −241 and −315, and CCAAT motifs at positions −447 and −509. Additionally, a potential receptor site for NIRA (CCGCGG) (18), the product of the *nirA* gene, which mediates nitrate induction of systems required for nitrate assimilation including nitrate reductase, nitrite reductase (13), and nitrate uptake (19), is present at position −146.

Comparison of the *cnxABC* deduced amino acid sequence to the Swissprot data base indicated that the inferred *A. nidulans* protein was homologous to two *E. coli* proteins (Fig. 2), encoded by the *moaA* gene (similar to the first section of the *A. nidulans* sequence) and the *moaC* gene (similar to the second segment) (20). The *cnxABC* intron lies between the two regions.

The *A. nidulans* CNXA domain has an 80-residue N-terminal extension in comparison with the *E. coli* MOAA protein. This N-terminal extension has no similarity to other proteins in the data base. The start of the CNXC domain is not clear from comparison with the MOAC protein, since there is a stretch of around 120 amino acid residues following the MOAA-similar region without similarity to any other proteins,[3] including *E. coli* MoCo biosynthetic proteins (21). Conserved cysteine residues of potential functional significance in CNXA are in ***boldface type*** (Fig. 2) and are discussed below under "Determination of Mutant *cnxA* Sequences."

Equivalence of A. nidulans cnxA, cnxB, and cnxC and E. coli moaA and moaC—Fragments PCR1, PCR2, PCR3, and PCR4 were amplified using pSTA502 as the template and used in genetic transformation experiments in attempts to repair phenotypically the growth defects of mutant strains *cnxA9*, *cnxB11*, and *cnxC3* on nitrate as the sole source of nitrogen (Fig. 3). Such transformation experiments were carried out using one of the above amplified DNA fragments with and without the presence of the vector pHELP, an autonomously replicating *A. nidulans* plasmid that greatly enhances transformation frequencies of co-transformed plasmids (see Ref. 21 and "Experimental Procedures"). It was observed, not unexpectedly perhaps, that the frequency of nitrate utilizing complementers was at least 100-fold greater when PCR fragments were co-transformed with the vector pHELP. Since the frequencies using PCR fragments alone tended to be low, the inclusion of pHELP sharpened the differences between positive and negative phenotypic complementation, although the overall trend with the different PCR fragments was similar. PCR1 complemented phenotypically each recipient mutant strain, *i.e. cnxA9*, *cnxB11*, or *cnxC3*, at high frequency using vector pHELP (1000–3000 nitrate utilizing transformants/μg of PCR fragment). PCR2 complemented only the *cnxA9* mutation, while PCR3 complemented the *cnxC3* mutation only. No complementing transformants were obtained for the *cnxB11* mutation with either fragment PCR2 or PCR3. Fragment PCR4 did not complement the *cnxC3* mutation. The results indicate, in conjunction with the homologies observed above, that the *cnxA* section of the *cnxABC* locus is equivalent to *moaA* and that the *cnxC* locus is equivalent to the *E. coli moaC* gene, but phenotypic complementation of the *cnxB11* mutant requires the presence of both *cnxA*- and *cnxC*-encoding DNA fragments. Finally, the observation that transformation with pHELP permits complementation of the *cnxC3* mutant by the apparently promoterless fragment PCR3 encoding CNXC domain suggests that the transcriptional activity may initiate from within pHELP itself. Complementation by PCR3 is less likely to be due to integration at the homologous locus by double crossover, since fragment PCR4, which covers the region of the *cnxC3* mutation but does not contain the entire CNXC domain, does not complement the *cnxC3* mutant following transformation with pHELP.

Determination of Mutant DNA Sequences—In an attempt to resolve conclusively the question of how three genetically complementing classes of mutants could be obtained in a single gene (*i.e.* the *cnxABC* gene), the DNA sequence of the original

[3] S. E. Unkles, unpublished observations.

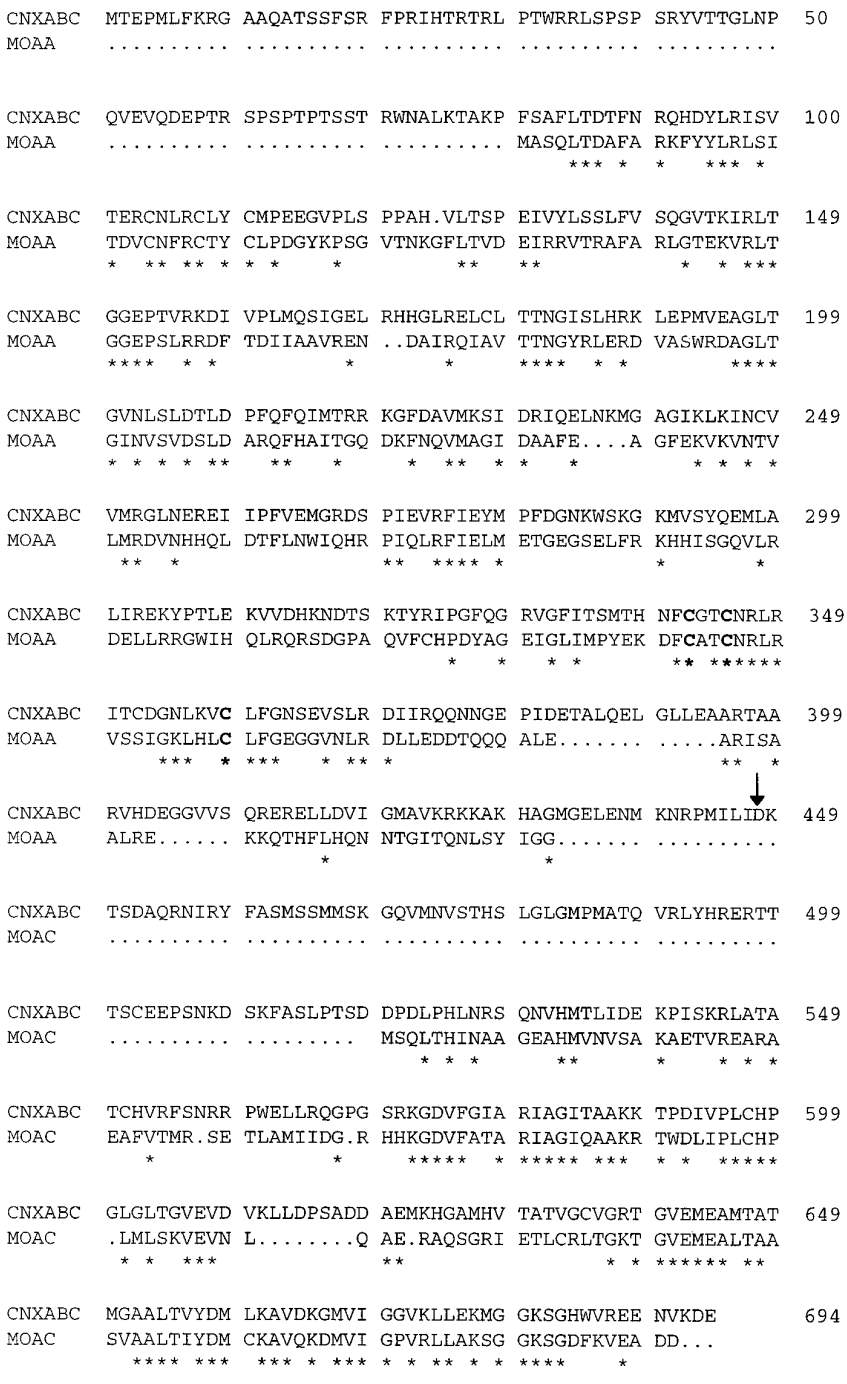


FIG. 2. **Comparison of *A. nidulans* CNXABC with *E. coli* MOAA and MOAC proteins.** *Numbers* to the *left* indicate amino acid residues of CNXABC. The *asterisks* indicate identical residues, and the *vertical arrow* shows the intron position. Conserved cysteine residues thought to be involved in binding of an iron-sulfur prosthetic group in *E. coli* (22) are shown in *boldface type*.

Pateman *et al.* mutants (2) (*i.e. cnxA9*, *cnxB11*, *cnxC2*, and *cnxC3*) as well as *cnx* mutants isolated and characterized during this study (*cnxA95*, *cnxA140*^{ts}, *cnxB204*, and *cnxB1281*) were determined following PCR amplification. (The origin of the mutants is cited under "Experimental Procedures.") The positions of the mutational changes and the DNA sequence of the wild type compared with the mutant are shown in Fig. 4. The *cnxA9*, *cnxA140*^{ts}, and *cnxA95* mutations are the result of either single base pair changes or duplication of short stretches of nucleotides in multiples of three in the *cnxA*-encoding domain. Therefore, neither type of event changes the reading frame, *inter alia* grossly affects the sequence of the protein. One of the original Pateman and Cove mutants, *cnxA9*, is an insertion of three amino acids (between residues 345 and 346) into a region highly similar between *A. nidulans cnxA* and *E. coli moaA* protein products and in which Rajagopalan and colleagues have recognized cysteine residues (highlighted in *boldface type*, Fig. 2), which are involved in the binding of an iron-sulfur prosthetic group in the *E. coli* MOAA protein (22). The mutant *cnxA95* is a single base pair alteration resulting in the change of one of these conserved cysteine residues, cysteine 345, to tyrosine, supporting the suggestion that this is an important functional site. The phenotypically temperature sensitive allele, *cnxA140*^{ts}, is a duplication resulting in the addition of seven amino acids between residues 139 and 140, just preceding a short homologous amino acid stretch at residue 147 (Fig. 2).

The DNA sequence changes in the *cnxB11*, *cnxB1281*, and *cnxB204* mutants all occur within the *cnxA*-encoding domain of the gene and are either duplications (not in multiples of three) or single base pair deletions. The result of these duplications or deletions is a change in reading frame, positioning a stop codon

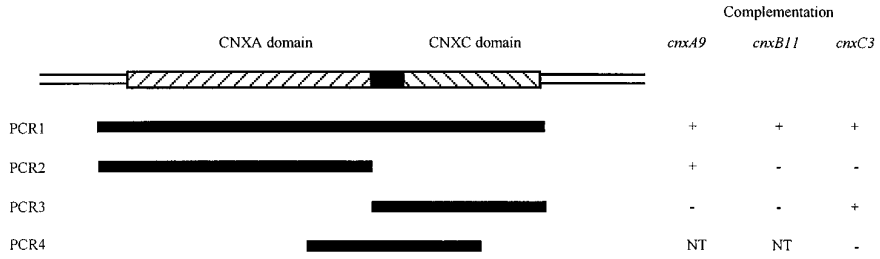
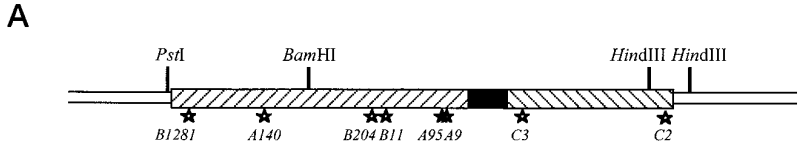


FIG. 3. **Phenotypic complementation of *A. nidulans cnxA*, *cnxB*, and *cnxC* mutants transformed with PCR-generated fragments encoding the *cnxA* and *cnxC* domains.** The *hatched bar* to the *right* represents the *moaA*-similar sequence, the *hatched bar* to the *left* shows the *moaC*-similar sequence, and the *solid bar* shows the intron. *Below*, *solid rectangles* show the position of PCR fragments used for phenotypic complementation, *i.e.* growth on nitrate as the sole source of nitrogen (see "Experimental Procedures"). For complementation, + indicates high frequency transformation (300 to >1000 transformants/μg), and − indicates no transformants obtained or less than 10 colonies. *NT*, not tested.



B

```
wild-type   1012  ACGCATAACTTCTGCGGCACTTGC.....AACCGCCTTCGCATTACGT
cnxA9             ACGCATAACTTCTGCGGCACTTGCcgcacttgcAACCGCCTTCGCATTACGT
             338  T  H  N  F  C  G  T  C  R  T  C  N  R  L  R  I  T  C

wild-type   1012  ACGCATAACTTCTGCGGCACTTGCAACCGCCTTCGCATTACGT
cnxA95            ACGCATAACTTCTGCGGCACTTaCAACCGCCTTCGCATTACGT
             338  T  H  N  F  C  G  T  Y  N  R  L  R  I  T  C

wild-type    391  ATTGTGTACCTTTCGTCGCTCTTCGTC .....TCGCAGGGCGTGACCAAGA
cnxA140ts         ATTGTGTACCTTTCGTCGCTCTTCGTCacctttcgtcgctcttcgtcTCGCAGGGCGTGACCAAGA
             131  I  V  Y  L  S  S  L  F  V  Y  L  S  S  L  F  V  S  Q  G  V  T  K  I

wild-type    745  GTTGTGATGCGGGGCCT.............CAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCGTGA
cnxB11            GTTGTGATGCGGGGCCTtgatgcggggcctCAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCGTGA
             249  V  V  M  R  G  L  D  A  G  P  Q  R  T  R  D  Y  S  V  C  R  N  G  A  *

wild-type    688  GACCGCATCCAGGAGCTGAATAAGATGGGAGCTGGGATAAAGCTCAAGATCAATTGTGTTGTGATGCG.............
cnxB204           GACCGCATCCAGGAGCTGAATAAGATGGGAGCTGGGATAAAGCTCAAGATCAATTGTGTTGTGATGCGtgaataagatggga
             230  D  R  I  Q  E  L  N  K  M  G  A  G  I  K  L  K  I  N  C  V  V  M  R  E  *
wild-type         .....................................GGGCCTCAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCG
cnxB204           gctgggataaagctcaagatcaattgtgttgtgatgcgGGGCCTCAACGAACGCGAGATTATTCCGTTTGTCGAAATGGGGCG

wild-type     61  TTTCCCCGCATCCATACTCGAACTCGACTCCCGACGTGGCGCCGGTTGAGCCCTT
cnxB1281          TTTCCCCGCATCCATACT.GAACTCGACTCCCGACGTGGCGCCGGTTGAGCCCTT
              21  F  P  R  I  H  T  E  L  N  S  R  R  G  A  G  *

wild-type    1549  AGCGATGCCCAGAGAAATATAAGGTACTTCGCCTCCATGTCGTCAATGATGTCCAAGGG
cnxC3             AGCGATGCCCAGAGAAA....AGGTACTTCGCCTCCATGTCGTCAATGATGTCCAAGGG
             451  S  D  A  Q  R  L     G  T  S  P  P  C  R  Q  *

wild-type    2200  GTGATTGGGGGAGTGAAACTGCTAGAGAAGATGGGGGGCAAGAGCGG
cnxC2             GTGATTGGGGGAGTGAAACTG.TAG
             668  V  I  G  G  V  K  L   *
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FIG. 4. **DNA sequence analysis of *cnx* mutations and inferred protein changes.** *A*, a description of the representation of *cnxABC* is given in the legend to Fig. 3. The positions of the mutations relative to the coding region of *cnxABC* are shown by *stars*. *B*, DNA sequence of the mutant compared with the relevant section of wild-type sequence is shown. *Numbers* to the *left* of the DNA sequence correspond to the numbering system for the *A. nidulans* wild-type *cnxABC* sequence (Fig. 1*B*). Changes in the sequence relative to the wild type are shown in *lowercase*. *Underlined* are the sequences present in the wild type that are duplicated in the mutant. The amino acid sequence deduced from the mutant DNA sequence is also shown. The *number* to the *left* refers to the amino acid residue position in the wild type. Changes in the deduced amino acid sequence are shown in *boldface type*.

in frame downstream of the change. Therefore, both *cnxA* and *cnxC* domains of the protein are markedly disrupted by the *cnxB* mutations. A further mutant, *cnxB35*, is possibly a translocation within the *cnxA* domain between primers P3 and P4.[3] A PCR product was obtained from this mutant with primer combinations P1 and P2, P5 and P6, and P7 and P8, but not with P3 and P4 or P3 and P6. (See "Experimental Procedures" for primer details).

The *cnxC2* mutation results from the deletion of a single base pair near the end of the CNXC-encoding domain, while the *cnxC3* mutation is the consequence of a four-base pair deletion eight codons after the position of the intron in *cnxABC*. This mutation is the most proximal *cnxC* mutation and hence helps to delimit the start of the CNXC protein at least to before this point. Both *cnxC* mutations, therefore, disrupt only the CNXC domain. Interestingly, the *cnxC2* mutation indicates an essential role in protein function for the C-terminal 20 amino acid residues.

Determination of Precursor Z and Molybdopterin Levels in Wild Type and cnxA, cnxB, and cnxC Mutants—The results

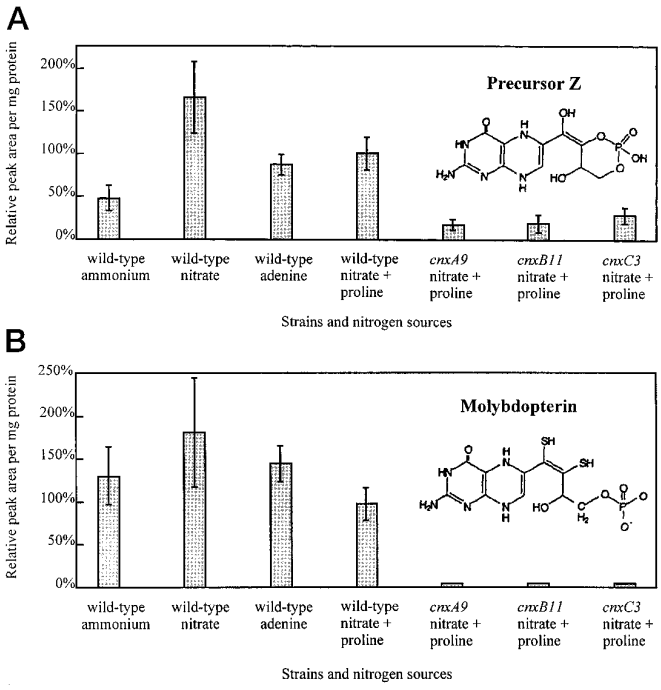

FIG. 5. **Levels of precursor Z and molybdopterin measured by reversed phase HPLC analysis of compound Z and form A dephospho, respectively.** Three independent experiments were carried out for each strain and growth condition. Integrated peak areas were calculated per mg of protein. *Columns* show these values expressed as a percentage of the mean value ± S.D. for the wild type grown with proline plus nitrate. *Error bars* indicate S.D. *A*, fluorescent material co-chromatographing with purified compound Z was detected by excitation at 350 nm and emission at 450 nm. *B*, fluorescent material co-chromatographing with purified form A dephospho was detected with excitation at 370 nm and emission at 450 nm. Form A was not detectable in mutant samples.

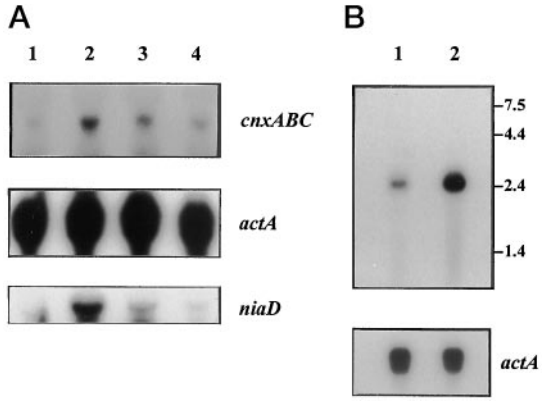

FIG. 6. **Transcript levels of *cnxABC* in *A. nidulans* wild type.** *A*, total RNA was isolated from cells grown on 5 mM ammonium tartrate (*lane 1*), 10 mM sodium nitrate (*lane 2*), 10 mM sodium nitrite (*lane 3*), and 10 mM adenine (*lane 4*). Following development of autoradiographic signal, the blot was stripped and rehybridized using a 0.85-kb *Kpn*I-*Nco*I fragment containing the *A. nidulans actA* encoding actin (23) as a control for RNA transfer and the 2.7-kb *Xba*I fragment of pSTA8 containing the *A. nidulans niaD* gene (24) as a control for the growth conditions. *B*, messenger RNA isolated from wild-type mycelium grown with 5 mM ammonium tartrate (*lane 1*) or 10 mM sodium nitrate (*lane 2*) as sole nitrogen source. In both series of experiments, the probe was the 0.6-kb *Pst*I-*Bam*HI fragment of pSTA502.

presented in Fig. 5 show the levels of both precursor Z and molybdopterin, *vis à vis* compound Z and form A dephospho (see "Experimental Procedures"), in oxidized extracts from wild-type cells grown on adenine, ammonium, or nitrate as the sole nitrogen source. These nitrogen sources were chosen, since growth on nitrate requires nitrate reductase, and utilization of adenine requires xanthine dehydrogenase, both molybdenum cofactor-containing enzymes, while neither of these enzymes is required for growth on ammonium (3). The levels of precursor Z are substantially higher, *i.e.* more than 3–4-fold, in nitrate-grown compared with ammonium-grown wild-type cells. Additionally, there is a lower but significant increase in adenine-grown cells. Levels of molybdopterin are slightly higher in nitrate-grown and adenine-grown cells than in ammonium-grown wild-type cells, but this may not be significant. In mutants and wild type grown on nitrate plus proline as the nitrogen sources (since the mutants are unable to grow on nitrate alone), precursor Z is markedly reduced in strains *cnxA9*, *cnxB11*, and *cnxC3*, and molybdopterin levels are undetectable in all three mutants.

Analysis of cnxABC Expression—Using either the 0.63-kb *Pst*I-*Bam*HI fragment, embracing the *cnxA*-encoding section, or the 0.35-kb *Hin*dIII, for the *cnxC* portion, both from plasmid pSTA502 (Fig. 1*A*) as a probe in all growth conditions (Fig. 6, *A* and *B*), a single transcript of 2.4 kb was observed. In total RNA, the 2.4-kb transcript was observed at its highest intensity in nitrate-grown (Fig. 6*A*, *lane 2*) and nitrite-grown cells (Fig. 6*A*, *lane 3*). Lower intensity signals were observed when adenine (Fig. 6*A*, *lane 4*) and ammonium (Fig. 6*A*, *lane 1*) were the sole nitrogen sources. However, since the transcript requiring prolonged exposure times is in low abundance, to confirm the trend observed with total RNA, analysis was performed using mRNA from cells grown with ammonium or nitrate as sole nitrogen source. This confirmed that the 2.4-kb transcript was markedly increased in abundance in mRNA isolated from wild-type cells grown with nitrate (Fig. 6*B*, *lane 2*) compared with ammonium as the sole nitrogen source (Fig. 6*B*, *lane 1*). Approximately equal loadings of RNA were present for each condition as judged by the intensity of the control transcript, *actA*, a constitutively expressed *A. nidulans* gene encoding actin (23). Expression of nitrate reductase encoded by the *A. nidulans* structural gene, *niaD*, which was used as a control for the correct growth regimes,[3] was as expected from previous published work (24).

Phenotypic Complementation in cnxA and cnxC Heterokaryons Revisited—During characterization of mutants isolated in this study, it was apparent that, while *cnxA* and *cnxC* mutants completely failed to complement *cnxB* mutants in mixed heterokaryons, as originally reported by Pateman *et al.* (2), growth of heterokaryons during complementation between *cnxA* and *cnxC* mutants was reduced compared with heterokaryons generated between *cnxA* or *cnxC* and any of the other *cnx* mutants (*i.e.* those with mutations in *cnxE*, *cnxF*, *cnxG*, or *cnxH* genes). Growth tests illustrating this feature are shown in Fig. 7, where *cnxA9* in self-heterokaryon combination (*i.e. cnxA9*/*cnxA9*) fails to grow on nitrate as a sole nitrogen source, while strong growth is observed in mixed heterokaryons of *cnxA9* and *cnxF8* (or *cnxE3*, *cnxG4*, *cnxH3*).[3] Mixed heterokaryons of *cnxA9* and *cnxC3*, however, although showing complementation, result in a colony of comparatively reduced size after 3 days of incubation at 37 °C. Similar results were obtained when hypoxanthine was used as the sole source of nitrogen[3] and using different *cnxA* (*cnxA95, cnxA140*^{ts}) or *cnxC* (*cnxC2*) alleles with nitrate as the sole nitrogen source.

DISCUSSION

The finding that the *A. nidulans cnxABC* locus is a single gene comes from two main lines of evidence: a single open reading frame and one transcript of the expected size. The gene consists of two regions, *cnxA* and *cnxC*, separated by an intron, each region encoding an enzyme domain, CNXA and CNXC, which are homologues of *E. coli* MOAA and MOAC proteins,

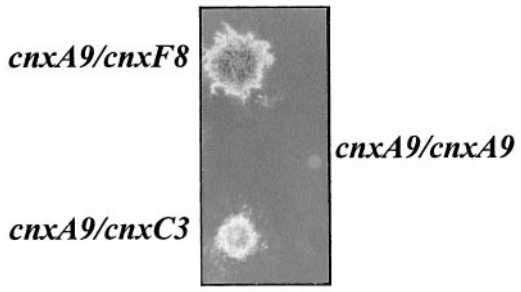

FIG. 7. **Phenotypic complementation of heterokaryons grown on 10 mM sodium nitrate as a sole nitrogen source.** Conidial suspensions were spotted onto the minimal medium in the combinations indicated and incubated for 3 days at 37 °C. The result of the *cnxC3*/*cnxC3* heterokaryon was the same as for *cnxA9*/*cnxA9* and not shown.

respectively. Additionally, no likely fungal promoter-like motifs exist in the intron between CNXA- and CNXC-encoding sequences that would permit transcription of a CNXC-encoding gene.

The *A. nidulans cnxABC* transcript is likely to be translated as a single protein, since *cnxB* frameshift mutations, *i.e. cnxB11*, *cnxB204*, and *cnxB1281*, abolish both CNXA and CNXC functions (as judged by the lack of phenotypic growth complementation in *cnxB*/*cnxA* and *cnxB*/*cnxC* heterokaryons). An operon structure is unlikely, since operons are not known to exist in eukaryotes. Whether the translated protein remains as a single difunctional unit with two domains, CNXA and CNXC, separated by a possible hinge region, or is cleaved to form two individual CNXA and CNXC products remains uncertain. If the former is true, the protein would be expected to exist in dimeric or multimeric form. That heterokaryons of *cnxA9* or *cnxA95* with *cnxC3* grow less well than *cnxA* or *cnxC* heterokaryons with other *cnx* mutants (*cnxE3*, *cnxF8*, *cnxG4*, or *cnxH4*) supports the occurrence of a difunctional protein. We point out, however, that the results of our *cnxA*/*cnxC* complementation tests differ from Cove's original observation (3), but this possibly reflects a difference in the experimental procedure for assessing complementation.

The biochemical results indicate that the CNXA and CNXC activities are required for the synthesis of precursor Z (as measured by its derivative compound Z), an intermediate in molybdenum cofactor biosynthesis, thereby verifying Pateman and Cove's original hypothesis (2). Precursor Z, the chemical structure of which is shown in Fig. 5, is a unique phosphorylated pterin that is probably the first intermediate in the pathway of molybdopterin cofactor biosynthesis (from a guanosine derivative) and is converted to molybdopterin containing a dithiolene group (Fig. 5) (25, 26). Mutant strain *cnxA9*, which has an alteration in the CNXA domain; *cnxC3*, which has an alteration in the CNXC domain; and *cnxB11*, which is a frameshift affecting both domains, all have low levels of precursor Z and undetectable molybdopterin (as determined by form A dephospho measurement), a later intermediate in the pathway. The presence of apparently low amounts of precursor Z in the three mutants may represent background contamination, since the mutants are most likely to be loss-of-function because they have no detectable nitrate reductase activity. Additionally, *cnxB11* and *cnxC3* mutations are the result of an insertion and a deletion, respectively, leading to frameshifts. In *E. coli*, the involvement of the CNXA counterpart has been previously demonstrated by the lack of precursor Z in extracts of the *chlA1* mutant (now redesignated as the *moaA1* mutation) (13, 14). In *Aspergillus*, we show here that the CNXC activity, *i.e.* the MOAC equivalent, is also required for precursor Z synthesis. The two *E. coli* proteins are specified by two separate cistrons, *moaA* and *moaC* (13, 14, 20, 25, 26) and in the higher plant, *Nicotiana tabaccum*, by unlinked genes (27). Both of these protein activities are implicated in the synthesis of molybdopterin precursor Z from guanosine or a guanosine derivative. The metabolic pathway from guanosine to precursor Z is unclear, although progress in identifying intermediates has been made recently (28).

Several of the mutants are instructive with regard to structure/function relationships and should become useful tools with the determination of the structure of the CNXABC product. Mutants *cnxA9* and *cnxA95* disrupt a motif that is common to *E. coli* and *A. nidulans* and thought to be a domain associated with the iron-sulfur prosthetic group in *E. coli* (22). The mutation *cnxA95* changes the conserved cysteine 345 to tyrosine, thus altering a candidate residue for interaction with the prosthetic group. The *cnxA9* mutation results from the insertion of three amino acids, arginine, threonine, and cysteine, immediately after cysteine 345, supporting the notion that this is an important functional motif in *A. nidulans*. The mutation *cnxA140*^{ts} results from the tandem duplication of seven amino acid residues close to a region of high homology between *E. coli* and *A. nidulans*. This alteration confers temperature sensitivity for growth on nitrate but not hypoxanthine. The *cnxA140*^{ts} mutation will complement a *cnxC* mutation such as *cnxC2*, indicating that only the structure of the CNXA region is affected. This would suggest that the *cnxA140*^{ts} defect does not produce a gross change in the folding of CNXABC such that the CNXC domain is affected.

The levels of precursor Z are substantially higher in nitrate-grown *A. nidulans* wild type compared with ammonium-grown cells, implying a degree of metabolic regulation. This is perhaps not unexpected, since more cofactor should be required for cells grown with nitrate. Precursor Z is still present in ammonium-grown cells, which may indicate that the molybdenum cofactor is required for other molybdoenzymes expressed during growth on ammonium. Northern blot studies show that the abundance of the *cnxABC* transcript is higher in cells grown with nitrate than with ammonium as the sole nitrogen sources, indicating that control in part may be at the transcriptional level. However, this is in contrast to the transcript abundance levels for *cnxE*, *cnxF*, *cnxG*, and *cnxH* genes in cells grown under similar conditions.[4] It would appear, therefore, that there is modest nitrate induction of transcription exerted only on the earlier section of the pathway. In this regard, a possible binding motif for NIRA, the product of the *nirA* regulatory gene mediating nitrate induction of the nitrate assimilation genes including nitrate reductase (3), exists in the 5′-region of the *cnxABC* gene, but direct proof of NIRA involvement requires *in vivo* functional analysis. Cells grown with adenine as the sole nitrogen source, which might also be expected to require elevated levels of molybdenum cofactor for xanthine dehydrogenase activity, show only a modest increase in precursor Z and a marginal (if any) increase in *cnxABC* transcript, and no binding motif (5′-TCGGX_6CCGA) for the UAY regulator of purine catabolism (29) can be recognized in the *cnxABC* promoter.

A. nidulans was the first organism in which mutants in this pathway were described. Given that this eukaryotic experimental organism allows easy selection of mutations both forward, by selection for chlorate resistance, and backward, by selection for growth on nitrate, we anticipate that the exploitation of this organism will be invaluable for the elucidation of structure/function and interrelationships between the compo-

[4] L. J. Millar, S. E. Unkles, and V. A. Appleyard, unpublished observations.

nents of the molybdenum cofactor pathway, a pathway found from bacteria to humans.

Acknowledgments—We thank Dr. K. V. Rajagopalan, Duke University, for helpful comments. We are also grateful for the receipt of the *brlA*-containing clone from Dr. A. J. Clutterbuck, University of Glasgow, and to Professor D. J. Cove, University of Leeds, for *cnxA9*, *cnxB11*, *cnxC2*, and *cnxC3* mutants.

Note added in proof—After submission of this article, a comment (H. N. Arst (1997) *Microbiology* **143,** 1437) regarding a temperature conditional *cnx* mutant came to our attention. The note reports that the *cnxC20* cryo-sensitive mutant will grow partially on nitrate but not hypoxanthine at 25 °C as sole nitrogen sources. This report is in addition to temperature-sensitive mutants in the *cnxH* gene characterized many years ago (D. W. Macdonald and D. J. Cove (1974) *Eur. J. Biochem.* **47,** 107–110). The *cnxA140* mutant reported in the current article is a further example of growth differences of temperature-conditional mutants between nitrate and hypoxanthine at the non-permissive temperature. The explanation advanced for this is that the requirement of xanthine dehydrogenase (purine hydroxylase I) is more stringent than nitrate reductase for the molybdenum cofactor.

REFERENCES

1. Cove, D. J., and Pateman, J. A. (1963) *Nature* **198,** 262–263
2. Pateman, J. A., Cove, D. J., Rever, B. M., and Roberts, D. B. (1964) *Nature* **201,** 58–60
3. Cove, D. J. (1979) *Biol. Rev.* **54,** 291–327
4. Scazzocchio, C. (1974) *J. Less Common Metals* **36,** 461–464
5. Scazzocchio, C. (1980) in *Molybdenum and Molybdenum-containing Enzymes* (Coughlan, M. P., ed) pp. 489–515, Pergammon Press, Oxford, UK
6. Tomsett, A. B. (1989) in *Nitrogen, Phosphorus and Sulphur Utilisation by Fungi* (Boddy, L., Marchant, R., and Read, D. J., eds) pp. 33–58, Cambridge Press, Cambridge, UK
7. Arst, H. N., Jr., Tollervey, D. W., and Sealy-Lewis, H. M. (1982) *J. Gen. Microbiol.* **128,** 1083–1093
8. Hartley, M. J. (1970) *Genet. Res.* **16,** 123–125
9. Cove, D. J. (1966) *Biochim. Biophys. Acta* **113,** 51–56
10. Clutterbuck, A. J. (1974) in *Handbook of Genetics* (King, R. C., ed) Vol. 1, pp. 447–510, Plenum Publishing, New York
11. Johnstone, I. L., Hughes, S. G., and Clutterbuck, A. J. (1985) *EMBO J.* **4,** 1307–1311
12. MacCabe, A. P., Riach, M. B. R., Unkles, S. E., and Kinghorn, J. R. (1990) *EMBO J.* **9,** 279–287
13. Johnson, M. E., and Rajagopalan, K. V. (1987) *J. Bacteriol.* **169,** 110–116
14. Johnson, M. E., and Rajagopalan, K. V. (1987) *J. Bacteriol.* **169,** 117–125
15. Brody, H., Griffith, J., Cuticchia, A. J., Arnold, J., and Timberlake, W. E. (1991) *Nucleic Acids Res.* **19,** 3105–3109
16. Gurr, S. J., Unkles, S. E., and Kinghorn, J. R. (1987) in *Gene Structure in Eukaryotic Microbes* (Kinghorn, J. R., ed) pp. 93–139, IRL Press, Oxford, UK
17. Arst, H. N., Jr., and Sheerins, A. (1996) *Mol. Microbiol.* **19,** 1019–1024
18. Punt, P. J., Strauss, J., Smit, R., Kinghorn, J. R., van den Hondel, C. A. M. J. J., and Scazzocchio, C. (1995) *Mol. Cell. Biol.* **15,** 5688–5699
19. Unkles, S. E., Hawker, K. L., Grieve, C., Campbell, E. I., Montague, P., and Kinghorn, J. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 204–208
20. Rivers, S. L., McNairn, E., Blasco, F., Giordano, G., and Boxer, D. H. (1993) *Mol. Microbiol.* **8,** 1071–1081
21. Gems, D. H., and Clutterbuck, A. J. (1993) *Curr. Genet.* **24,** 520–524
22. Rajagopalan, K. V. (1997) in *Nitrogen Assimilation: Fourth International Symposium* (Solomonson, L. P., Barber, M. J., and Cannons, A. C., eds), USF Printing Services, Tampa, FL
23. Fidel, S., Doonan, J. H., and Morris, N. R. (1988) *Gene* (*Amst.*) **70,** 283–293
24. Johnstone, I. L., McCabe, P. C., Greaves, P., Gurr, S. J., Cole, G. E., Brow, M. A. D., Unkles, S. E., Clutterbuck, A. J., Kinghorn, J. R., and Innis, M. A. (1990) *Gene* (*Amst.*) **90,** 181–192
25. Pitterle, D. M., and Rajagopalan, K. V. (1989) *J. Bacteriol.* **171,** 3373–3378
26. Pitterle, D. M., and Rajagopalan, K. V. (1993) *J. Biol. Chem.* **268,** 13499–13505
27. Hoff, T., Schnorr, K. M., Meyer, C., and Caboche, M. (1995) *J. Biol. Chem.* **270,** 6100–6107
28. Wuebbens, M. M., and Rajagopalan, K. V. (1995) *J. Biol. Chem.* **270,** 1082–1087
29. Suarez, T., de Queiroz, M. V., Oestreicher, N., and Scazzocchio, C. (1995) *EMBO J.* **14,** 1453–1467