PCR-based cloning of the full-length *Neurospora* eukaryotic initiation factor 5A cDNA: polyhistidine-tagging and overexpression for protein affinity binding

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Eukaryotic initiation factor 5A (eIF-5A) is the only cellular protein known to contain a hypusine residue that is formed by transferring the aminobutyl moiety from spermidine to a specific lysine residue, followed by hydroxylation at the aminobutyl group. A simple PCR-based strategy was developed to obtain a full-length cDNA of *Neurospora crassa* eIF-5A. The strategy consists of (i) the design of a pair of key primers (21-mer) based on the highly conserved eIF-5A cDNA domains known in other species, (ii) PCR amplification of *Neurospora* cDNA using the two key primers to obtain the core sequence for the design of core primers, and (iii) combined use of the key primers, core primers and the universal primers, T3 and T7, to amplify the target sequence in a *Neurospora* cDNA library. The longest cDNA obtained was cloned into pBlueScript phagemid, and sequence analysis indicated that it encodes a polypeptide of 163 amino acid residues with a codon usage preference characteristic of abundant *Neurospora* genes. The *Neurospora* polypeptide showed 59% and 67% identity with human and yeast eIF-5A

precursor protein respectively. We subcloned the *Neurospora* eIF-5A cDNA into pQE-30, which introduces six adjacent histidine residues to the N-terminus of the recombinant protein. The resulting plasmid, pQTy21, was overexpressed in *Escherichia coli*, and the soluble polyhistidine-tagged protein was purified by metal chelation chromatography. We obtained about 60 mg of purified eIF-5A precursor from 1 litre of culture in a single step using a Ni(II)-nitrilotriacetic acid (NTA)-agarose column. The histidine-tagged eIF-5A precursor protein could be recognized by anti-*Neurospora crassa* 21 kDa protein serum raised against wild-type eIF-5A precursor and could serve as the substrate protein for deoxyhypusine synthase. Using the histidine-tagged recombinant protein and the Ni(II)-NTA-agarose column, we constructed a protein affinity column and demonstrated an affinity binding between eIF-5A precursor and deoxyhypusine synthase in the presence of NAD⁺. One-step eIF-5A precursor affinity-column chromatography could lead to a 30-fold purification of deoxyhypusine synthase.

INTRODUCTION

Culturing of various mammalian cells in the presence of [³H]putrescine or [³H]spermidine results in radiolabelling of a single protein band with an apparent molecular mass of 18 kDa on SDS/PAGE (Cooper et al., 1982; Chen, 1983; Park et al., 1984). The labelling was found to be due to hypusine formation on the eukaryotic initiation factor 5A (eIF-5A) (previously named eIF-4D) precursor, consisting of two steps: (i) deoxyhypusine synthase catalyses the cleavage and transfer of the butylamino moiety from spermidine to a lysine residue in eIF-5A precursor to form a deoxyhypusine-containing protein and (ii) deoxyhypusine hydroxylase catalyses the addition of a hydroxy group to deoxyhypusine (Cooper et al., 1982; Park et al., 1984). Metabolic labelling experiments using [³H]spermidine in tissue culture showed that hypusine formation in animal cells is enhanced during growth stimulation (Chen and Liu, 1981; Cooper et al., 1982). Subsequently, hypusine-containing protein was also discovered in lower eukaryotic cells such as yeast (Gordon et al., 1987), *Neurospora crassa* (Yang et al., 1990) and *Dictyostelium discoideum* (Sandholzer et al., 1989), and recently in archaeobacteria (Bartig et al., 1992). The highly conserved nature of eIF-5A, the responsiveness to growth stimulation and the specificity of hypusine formation, together with the recognized

importance of polyamines in growth regulation suggest that hypusine formation may have an important role in cell physiology.

Previous studies (Murphey and Gerner, 1987; Park, 1987; Chen and Dou, 1988) have shown that the biosynthesis of eIF-5A precursor and its subsequent modification by spermidine are coupled, and that eIF-5A precursor accumulates only in mammalian cells with prior difluoromethylornithine treatment which suppresses spermidine formation. We have recently described the accumulation of an eIF-5A precursor, with an apparent molecular mass of 21 kDa as estimated by SDS/PAGE, in a mutant strain of *N. crassa* (*arg-12 ota aga*) (Yang et al., 1990). This 21 kDa protein can be radiolabelled with [³H]ornithine *in vivo* or with [³H]spermidine *in vitro*, resulting in deoxyhypusine/hypusine formation. On a two-dimensional gel, the radiolabelled 21 kDa protein exhibits four isoforms with pI values ranging from 3.5 to 6.5, suggesting that further post-translation modifications occur on this protein (Yang et al., 1990). The accumulation of a significant amount of eIF-5A precursor and the wide distribution of pI values of eIF-5A in this *Neurospora* mutant make it an interesting system in which to investigate the physiological function of eIF-5A precursor and its post-translationally modified products. As the amino acid sequences of eIF-5A precursor in several species are known (e.g. Smit-

Abbreviations used: eIF, eukaryotic (translation) initiation factor; IPTG, isopropyl β -D-thiogalactopyranoside; NTA, nitrilotriacetic acid; NC-21K, *Neurospora crassa* 21 kDa protein.

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McBride et al., 1989; Schnier et al., 1991), we decided to clone the full-length cDNA of *Neurospora* eIF-5A, using a PCR-based approach, and to overexpress it in *Escherichia coli*.

Various protocols have been developed that extend the use of PCR for the enrichment of specific but previously uncharacterized sequences (Ochman et al., 1988; Frohman et al., 1988; Loh et al., 1989). These PCR-based approaches rely on the power of PCR for amplification to isolate a particular sequence based on limited or imperfect sequence information. A general approach has been outlined using a pair of specific primers in conjunction with non-specific primers targeted to either the 3' polyadenylated region or an enzymically synthesized tail at the 5' end that permits amplification of the regions upstream and downstream of the core sequence (Ohara et al., 1989).

In the present study, we have adopted a simple PCR cloning strategy to clone the full-length *Neurospora* eIF-5A cDNA. The cDNA was then subcloned into the expression vector pQE-30 in order to introduce a polyhistidine tag to the N-terminus of the recombinant protein. The newly constructed plasmid, pQTy21, can be overexpressed in *E. coli* and the polyhistidine tag at the N-terminus of the recombinant eIF-5A precursor allows its rapid purification using metal-chelation chromatography. The tight binding of histidine-tagged protein and Ni(II)-NTA-agarose resins also permits the use of recombinant eIF-5A precursor or its hypusinated form in protein affinity-column chromatography. As an example, we showed that deoxyhypusine synthase binds to the affinity column in the presence of NAD⁺ and thus can be substantially purified by a one-step protein affinity chromatography.

MATERIALS AND METHODS

Oligonucleotide primers

Oligonucleotides T3 (17-mer) [d(5'-ATTAACCCTCACTAAAG-3')], T7 (17-mer) [d(5'-TAATACGACTCACTATA-3')], P1 (21-mer) [d(5'-CCTTGCAA(G/A)AT(A/T/C)GT(G/A/T/C)-GA(G/A/T/C)ATG-3')], P2 (20-mer) [d(5'-CTTCTT(G/A/T/C)CC(G/A/T/C)GT(G/A)AA(G/A/T)AT(G/A)TC-3')], P3 (21-mer) [d(5'-AAGACCGGCAAGCACGGTCAC-3')], P4 (21-mer) [d(5'-GTGACCGTGCTTGCCGGTCTT-3')] and P5 (23-mer) [d(5'-AAGGATCCACGACGACGCTCAG-3')] were synthesized in our laboratory using a Gene Assembler Plus DNA synthesizer (Pharmacia). Both T3 and T7 are universal primers.

cDNA library

N. crassa cDNA library in the λ ZAP vector was kindly given to us by Dr. J. Lenard, Robert Wood Johnson Medical School, NJ, U.S.A. in *E. coli* strain XL1-Blue.

PCR was performed in a thermal cycler using a Perkin-Elmer Cetus PCR kit. In the first run, two degenerate primers, P1 and P2 (2 μ M each), were used to amplify the *N. crassa* cDNA library. The mixture (50 μ l) was heated to 94 °C for 4 min and cycled 36 times at 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min. A core sequence of size 96 bp was obtained from the PCR product. Two complementary primers, P3 and P4, were designed on the basis of the sequence information of the 96 bp PCR product. In the second run, the mixtures (50 μ l) contained 0.2 μ M primers (T3 and P2 in one tube and T7 and P1 in another tube), 1 μ l of *N. crassa* cDNA library and other reagents provided by the PCR kit. The PCR conditions were 30 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min. The sample containing oligonucleotides T3 and P2 gave PCR product 1 and the other

containing T7 and P1 gave PCR product 2. Both products 1 and 2 were diluted 100 times and used as a separate template in the third run, where T3 and P4 were used as primers to amplify product 1, and T7 and P3 as primers to amplify product 2. The PCR conditions were identical with those used in the second run. The two reactions generated PCR products 3 and 4 respectively. Both products were purified from low-melting agarose gel and dissolved in 100 μ l of TE buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA). In the final PCR run, a mixture of products 3 and 4 (1 μ l each) was used as template and the universal primers T3 and T7 were used to amplify the mixture. The PCR conditions were 30 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 90 s.

Sequence analysis of PCR products

PCR products 3 and 4 were purified from low-melting agarose gel and treated with the Klenow fragment of DNA polymerase I and kinase. PCR product 3 was digested by *Bam*HI and ligated to *Bam*HI-*Small*I-digested pUC19. Product 4 was digested with *Hind*III and ligated to *Hind*III-*Small*I-digested pUC19. The PCR product from the final run was digested with *Eco*RI and ligated to *Eco*RI-digested pBluescript KSII. All of the ligation products were transformed into DH5 α strain of *E. coli* and then plated on agar dishes containing isopropyl β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside using standard procedures (Sambrook et al., 1989). The white clones were picked up and miniprep plasmid DNA was prepared for size determination. The cDNA clones containing inserts of correct size were sequenced by the dideoxy-mediated chain-termination procedure (Sanger et al., 1977) with Sequenase (version 2.0; United States Biochemical, Cleveland, OH, U.S.A.) using double-stranded DNA as template. Oligonucleotides used for sequencing included primers T3, T7, P3, P4 and M13 reverse primer. Sequencing was carried out at least three times from both directions. Storage and manipulation of DNA sequences were carried out using the University of Wisconsin Genetics Computer Group program (Devereux et al., 1984).

Cloning of NC-21K cDNA in pQE-30

The full-length cDNA, termed NC-21K, was digested by *Eco*RI and ligated into pBluescript KSII and designated p800-1. Primer P5, containing the *Bam*HI site, was used together with T3 to amplify plasmid p800-1. The amplified sequence was digested with *Bam*HI and *Hind*III and ligated to *Bam*HI-*Hind*III-treated pQE-30 (Qiagen Inc., Chatsworth, CA, U.S.A.). The resulting plasmid, termed pQTy21, was transformed into *E. coli*, strain M15 (pREP4), according to the manufacturer's procedure. The plasmid pREP4, which was already in the *E. coli* M15, codes for the *lac* repressor and neomycin phosphotransferase (*neo*) which confers kanamycin resistance to *E. coli* cells. The *E. coli* transformants were selected by miniprep and restriction enzyme analysis.

Overexpression of His-NC-21K protein

This vector/host system has a low level of transcription from the cloned insert before induction by IPTG. Host cells, containing plasmid pQE-30 or pQTy21, were incubated overnight and transferred to fresh L-broth containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin. When the cell density in the culture, as measured by A_{600} reached 0.7, IPTG was added to a final concentration of 2 mM. The cells were harvested by centrifugation at 10000 *g* after 5–10 h of induction. The bacterial pellet

collected from 200 ml of cell culture was resuspended in 5 ml of ice-cold lysis buffer (20 mM phosphate, pH 7.0, 0.4 mM phenylmethanesulphonyl fluoride, 0.2 mM pepstatin A, 0.2 μ M leupeptin and 5 mM 2-mercaptoethanol) and subjected to sonication (Heat System; microtip; maximum setting) for 20 cycles of 20 s sonication/20 s swirling at 4 °C. The lysate, usually containing more than 30% of recombinant protein, termed His-NC-21K, was used directly as a source of substrate protein in the assay of deoxyhypusine synthase activity.

Purification of His-NC-21K protein by Ni(II)-nitrilotriacetate (NTA)-agarose affinity chromatography

The cells containing plasmid pQTy21 were induced with IPTG and harvested as described above. The cell lysates, derived from 200 ml of cell culture, were mixed with Ni(II)-NTA-agarose resins which were prepared according to the manufacturer's protocol (Qiagen Inc.) and gently shaken for 30 min at room temperature. The mixture was precipitated by centrifugation at 10000 *g* for 20 s and washed three times with 1 ml of 8 M urea. The recombinant His-NC-21K protein was eluted from the resin with 200 μ l of EDTA solution (100 mM in 8 M urea). For purification under non-denaturing conditions, the column was washed with 1 M NaCl in lysis buffer. The recombinant His-NC-21K protein was eluted from the resin with 20 ml of imidazole-containing buffer (250 mM imidazole in 20 mM phosphate buffer, pH 7.5, 5 mM 2-mercaptoethanol). The eluted fraction was dialysed against lysis buffer.

Immunological and biochemical analysis of the recombinant His-NC-21K protein

The ability of the precursor to serve as a substrate of deoxyhypusine synthase was tested by an *in vitro* labelling assay as described previously (Dou and Chen, 1990). Western-blot analysis was carried out by standard procedures using a monospecific rabbit antiserum (1:500 dilution) prepared against *Neurospora* eIF-5A precursor protein (H. X. Li, and K. Y. Chen, unpublished work; details available on application to the author). The immunoreactive polypeptide bands were detected with a horseradish peroxidase-conjugated goat anti-rabbit serum used at 1:2000 dilution, using 4-chloro-1-naphthol and H₂O₂ as substrates (Bio-Rad Laboratories).

Protein affinity chromatography

Ni(II)-NTA-agarose resins were washed with phosphate buffer (20 mM phosphate, pH 7.0, 5 mM 2-mercaptoethanol). The protein affinity column was prepared by incubating 100 μ l of resin with 30 μ g of purified His-NC-21K for 2 h at 4 °C. The hydroxyapatite fraction of the *N. crassa* lysates was used as a crude source of deoxyhypusine synthase. The crude enzyme was mixed with the His-NC-21K-Ni(II)-NTA resin in the presence or absence of 1 mM NAD⁺ for 2 h at 4 °C. The resin was then washed with 20 mM phosphate buffer and eluted with EDTA (100 mM in 20 mM phosphate buffer, pH 7.4). Each fraction was tested for deoxyhypusine synthase activity as previously described (Dou and Chen, 1990).

RESULTS

PCR cloning

The success of a PCR cloning strategy depends on the design of

specific oligonucleotide primers and the choice of non-specific primers. The specific key primers, designed to hybridize with highly conserved domains of eIF-5A precursor found in other species, allow initial access to the target gene in a *Neurospora* cDNA library. Figure 1a shows the alignment of eIF-5A precursor amino acid sequences from three different species, human, yeast and slime mould. On the basis of sequence conservation and codon usage, we chose two heptamer segments to use to design primers P1 and P2 (Figure 1b). P1 and P2 were synthesized as a mixture of respectively a 92-fold and a 196-fold degenerate oligonucleotide. Figure 2(a) illustrates our PCR-based cloning strategy. We used P1 and P2 to gain entry into the unique *Neurospora* core sequence which allowed us to design a pair of complementary core primers termed P3 and P4. The combined use of P1, P2, P3 and P4, together with the two universal primers, T3 and T7, completed the isolation of full-length target cDNA. Figure 2(b) outlines our PCR amplification protocol. The first run, using a *Neurospora* cDNA library as a template, gave a clear single band of size 96 bp as expected, suggesting that it was a core sequence of *Neurospora* eIF-5A precursor cDNA. The sequence of this 96 bp fragment was determined and the information was used to design primers P3 and P4, whose sequence should be unique for *Neurospora* eIF-

Figure 1 Design of the key primers for PCR cloning

(a) Alignment of amino acid sequences of eIF-5A precursor. The amino acid sequences of the human (Smit-McBride et al., 1989), yeast (Schnier et al., 1991) and mould (Sandholzer et al., 1989) eIF-5A precursor derived from their respective cDNA were compared. The lysine residue that serves as the hypusination site is indicated by an asterisk. (b) Two heptamer fragments in the conserved region (residues 52–58 and 77–83) were chosen for the design of key primers P1 and P2. K indicates the lysine residue used for hypusination. (c) Degenerate base sequences of the key primers P1 and P2.

Figure 2 Protocol of the PCR-based full-length cloning of eIF-5A

(a) The full-length PCR cloning strategy. The *Neurospora* cDNA library was constructed in the λ ZAP vector with SK direction using T3 for the 5' end and T7 for the 3' end for the insert. The first run yielded a band of 96 bp which was sequenced for the design of the core primers P3 and P4 which are complementary with a unique *Neurospora* sequence. (b) Schematic representation of the PCR cloning protocol. *Neurospora* cDNA library was used as the template in the first and second run. Products 1 and 2 were used separately as templates in the third run. The mixture of products 3 and 4 was used as the template in the final run. The detailed PCR conditions are described in the Materials and methods section. (c) Detection of PCR products. Products 1 and 2 gave mixtures of cDNA as the cDNA library was used as the template. Products 3 and 4 gave single bands at 300 bp and 700 bp respectively (lanes 1 and 2). The final run gave a single band at about 1000 bp which contained full-length eIF-5A cDNA (Lane 3). Lane M, molecular-mass standards.

5A. In addition to gaining access to the core sequence, P1 and P2 were also used, in conjunction with T3 and T7, to directly enrich the full-length target sequence from the *Neurospora* cDNA library. The target-sequence-enriched samples, products 1 and 2, were used as the template for core primers, P3 and P4, and universal primers, T3 and T7, for further amplification. The resulting products, 3 and 4, each gave a single band of size 300 bp and 700 bp respectively (Figure 2c, lanes 1 and 2). The final run, using T3 and T7 as primers and the mixture of products 3 and 4 as template, resulted in an amplification of a 1 kb sequence (Figure 2c, lane 3). The 1 kb product was digested by *Eco*RI and cloned into pBlueScript KSII and the resulting plasmid was designated pBS800-1.

Nucleotide and amino acid sequence of *Neurospora* eIF-5A

The sequence of the insert in pBS800-1 was determined by the labelling/termination dideoxy procedure. Figure 3 shows the

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TTTTTTTTTCTCCCGGTGCCGCTCCAGCGTCTTGATCCATCATCTTGTCTCTCTCT 60
TGCCCTTTTCGACGGTTTATCCTTTGTGCAATCCTTTCAAGATGCTGCGGCCACGACGAC 120
      M S A A H D D
GCTCAGCATGAGCACACCTTCGACTCCGTCGACGCTGGTGGTCCGCGCCACCTACCCCATG 180
A Q H E H T F D S V D A G R S A T Y P M
CAGTGCTCTGCTCTGCGCAAGGGTGGCCATGTCGTGATCAAGAACCCTGCTGCAAGATC 240
Q C S A L R K G G H V V I K N R P C K I
GTCGATATGTCGACCTCCAAGACCGGCAAGCAGGTACGCCAAGGTCCATCTCGTCGCC 300
V D M S T S K T G K H G H A K V H L V A
ATCGATATCTTCACTGGCAAGAAGCTCGAGGATCTCTGCCCTCCACCCACACATGGAC 360
I D I F T G K K L E D L C P S T H N M D
GTTCCCAACGTCAAGCGCACCGACTACCACTTCTCTACATCGAGGAGTCTCTTGTGTC 420
V P N V K R T D Y Q F S Y I D E D F L V
CTTATTGACTCCAACGGTGAGGAGAAGCGTGAGCTCAAGATGCCCGAGGGTGAGCTTGCC 480
L I D S N G E E K R E L K M P E G E L A
AAGCGCATTGAGAAGCTCGAGGAGGAGGCAAGGACTTCTTCGTGCGTGTCCAGACCGCC 540
K R I E K L E E E G K D F F V G V Q T A
ATGGTGAGGAGGCTGCCATCGATGTCAAGGAGGCTCTAACAAGGACTAAAGGAAGTTT 600
M G E E A A I D V K E A S N K D *
GAGGAGTTGGAGSACACCGCGTTTGGCATTTAATGATGCTTCATGGGACGGTTCTGTT 660
GGTTTTGGCTTGCGTAAGAGATATCCGCTCTTTTGGCGCTATATCTGTGTTTAAAGGTCA 720
CATATGAATGGTTTACGCTCTCCCGGTTTGTGAGACCGGTAGCTGCTGGAATAGGGCG 780
CGACTTCTGAAGAAGTCGCACGAAGCTAACGCTGCCAACATCTTGAGAGGAATTC 838

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Figure 3 Nucleotide sequence of full-length eIF-5A cDNA and the conceptual translation amino acid sequence

On the basis of the deduced amino acid sequence, the *Neurospora* eIF-5A contains 163 amino acids with a calculated molecular mass of 18148 Da.

nucleotide sequence of the cloned cDNA which revealed a large open reading frame of 492 nucleotides coding for a polypeptide of 163 amino acid residues with calculated molecular mass of 18148 Da. The conceptual translation of the inserted cDNA indicates that the encoded *Neurospora* polypeptide shares 59% overall amino acid identity with human eIF-5A and 67% overall identity with yeast eIF-5A. The deduced amino acid sequence exhibited the characteristic hypusine consensus pattern: T-G-K-H-G-H with the hypusination site at Lys-57. Thus the sequence information suggests that we had obtained a full-length *Neurospora* eIF-5A cDNA. This notion is further strengthened by the findings that (i) the recombinant protein obtained from the cDNA had the same mobility on SDS/PAGE (see Figure 7a below), and (ii) the recombinant protein was fully active as a substrate for deoxyhypusine synthase (see Figure 7b below). There are three putative casein kinase II consensus phosphorylation sites (residues 99, 111 and 160) as well as four protein kinase C phosphorylation sites at residues 52, 55, 72 and 160. Kang et al. (1993) demonstrated that yeast eIF-5A is phosphorylated on a serine residue. We have previously shown that *Neurospora* eIF-5A exhibits at least four pI isoforms on a two-dimensional gel (Yang et al., 1990). It is likely that phosphorylation at various sites may contribute to the shift of pI values. The codon usage of the cloned sequence was analysed and compared with that of other *Neurospora* genes (results not shown). The results indicate that the cloned eIF-5A cDNA exhibits a pattern of codon preference characteristic of *N. crassa* genes such as *am* (glutamate dehydrogenase), *his* (histone H4) and *bml* (β -tubulin) (Orbach et al., 1986). Thus the use of codon in *Neurospora* eIF-5A encoded by the cloned cDNA was found to be strongly biased in that (i) where a choice between a purine and a pyrimidine in the third position in codon families is allowed, pyrimidine was used with few exceptions, and (ii) adenine is hardly ever used in the third position of the codon. Comparative analysis (Grantham et al.,

Figure 4 Construction of plasmid pQTy21 for expression of *Neurospora* eIF-5A cDNA

The expression vector pQE-30 chosen in this study contains the regulatable promoter/operator element (P/O), the synthetic ribosomal binding site (RBSII), a multicloning site (MCS) with 5 × His tagging for any inserted DNA, gene for β -lactamase (*bla*, confers ampicillin resistance), transcriptional terminator (t_0) of phage λ , and the replication origin (Ori) of plasmid pBR322. The insert NC-21K cDNA was linearized by PCR using pBS800-1 as the template and treated with *Bam*HI and *Hind*III before being used in ligation. details of the construction are described in the Materials and methods section.

1981) of *Neurospora* eIF-5A with more than ten other *Neurospora* genes (results not shown) also suggested that eIF-5A gene is abundantly expressed, consistent with the notion that eIF-5A represents an abundant cellular protein in eukaryotic cells (Cooper et al., 1983).

Construction of plasmid pQTy21

Histidine tagging of a recombinant protein allows rapid purification by metal-chelation chromatography using, for example, a Ni(II)-NTA-agarose column (Hochuli et al., 1988; Hoffmann and Roeder, 1991). Plasmid pQE-30 contains the regulatory *E. coli* phage T5 promoter/operator element, the synthetic ribosomal binding site, and the 6 × His/multiple cloning site which allows attachment of six adjacent histidine residues to the N-terminus in the recombinant protein. The promoter/operator is repressed in the presence of the *lac* repressor and can be induced

Figure 5 Overexpression of *Neurospora* eIF-5A precursor in *E. coli*

Overnight *E. coli* culture (100 μ l) was transferred to 3 ml of fresh Luria-Bertani medium containing ampicillin and kanamycin for 1 h at 37 °C followed by the addition of 2 mM IPTG for another 5 h. Cells were lysed with 8 M urea (150 μ l) and a 10 μ l sample was analysed by SDS/PAGE (15% gel). Lanes 1 and 6, molecular-mass standards; lane 2, *E. coli* transformed with pQE-30, uninduced; lane 3, *E. coli* transformed with pQE-30, induced; lane 4, *E. coli* transformed with pQTy21, uninduced; lane 5, *E. coli* transformed with pQTy21, induced. The arrow indicates the band position of the overexpressed His-NC-21K protein.

for overexpression by the addition of IPTG. The expression vector pQTy21 was constructed by inserting the *Bam*HI and *Hind*III fragment of the cloned cDNA in pBS800-1 into pQE-30. Figure 4 shows the schematic diagram that summarizes the subcloning procedure. To introduce the polyhistidine tag at the N-terminus of the eIF-5A precursor, we designed a 23-base primer, P5, which contains a *Bam*HI restriction site at the 5' end corresponding to the region just upstream of the fourth codon of eIF-5A. Although this led to a deletion of the first four amino acid residues, it avoided undesired duplicate initiation or internal translation. Primer P5 and the universal primer T3 were used to amplify the cDNA insert in the plasmid pBS800-1. The amplified sequence was subcloned into the vector pQE-30 which contains codons for six histidine residues after ATG. The resulting plasmid, named pQTy21, was transformed into the *E. coli* strain M15 (pREP4).

Overexpression and affinity purification

E. coli M15 cells containing plasmid pREP4 were transformed with the recombinant plasmid pQTy21 and grown in Luria-Bertani medium in the presence of ampicillin and kanamycin. After induction with IPTG for 5 h, cells were harvested and analysed by SDS/PAGE. Figure 5 shows that, after induction with IPTG for 5 h, cells were harvested and analysed by SDS/PAGE. Figure 5 shows that, after induction with IPTG, a single protein band with an apparent molecular mass of about 21 kDa was overexpressed. On the basis of Coomassie Blue staining and densitometric tracing analysis, we estimated that this induced protein accounted for more than 30% of total cellular protein. The polyhistidine tag at the N-terminus of the recombinant protein is known to bind tightly to the Ni(II)-NTA-agarose resin, and is resistant to strong denaturants such as 8 M urea and 6 M guanidine (Hochuli et al., 1987). However, the bound protein can be eluted in the presence of detergents by lowering the pH of the column buffer below the pK_a value of the imidazole moiety. Alternatively, proteins bound to the Ni(II)-

Figure 6 Purification of recombinant His-NC-21K protein by Ni(II)-NTA-agarose affinity chromatography

E. coli M15 transformed with pQTy21 (2 ml) was cultured and induced with 2 mM IPTG for 5 h or overnight. Cell lysates (200 μ l) were mixed with 50 μ l of Ni(II)-NTA-agarose (50% slurry) for 30 min at room temperature. The agarose was washed with 1 M NaCl and eluted with 250 mM imidazole in phosphate buffer (lanes 2–5) or with 8 M urea and eluted with 100 mM EDTA in 8 M urea (lanes 6–8). Proteins in different fractions (10 μ l for each fraction) were analysed by SDS/PAGE in 15% gels and stained with Coomassie Blue R-250. Lanes 1 and M, molecular-mass standards; lanes 2 and 6, *E. coli* cell lysate; lanes 3 and 7, flow-through fraction; lane 4, 1 M NaCl wash; lane 5, 250 mM imidazole eluate; lane 8, 100 mM EDTA eluate. The arrow indicates the band position of purified His-NC-21K protein.

Figure 7 Immunological and biochemical characterization of the recombinant NC-21K protein

(a) Western-blot analysis of the recombinant protein. The anti-NC-21K serum was used at 1:500 dilution. Lanes 1–3, *Neurospora* eIF-5A precursor protein partially purified from *N. crassa* mutant (70, 350 and 700 ng protein); lanes 4–6, recombinant His-NC-21K protein at respectively 12.5, 62.5 and 125 ng. (b) Substrate activity analysis. Protein samples were radiolabelled with [3 H]spermidine by purified deoxyhypusine synthase and analysed by SDS/PAGE and fluorography. Lane 1, wild-type eIF-5A precursor (1.2 μ g) from *N. crassa* mutant; lane 2, cell lysates from uninduced pQTy21 transformants (100 μ l of cells); lane 3, cell lysates from induced pQTy21 transformants (50 μ l of cells); lane 4, cell lysates of induced pQTy18 (human eIF-5A cDNA) transformants (100 μ l of cells). The labelling conditions are described in the Materials and methods section. The arrows indicate the position of recombinant NC-21K protein.

NTA affinity column under native conditions can be eluted by application of imidazole which competes for Ni²⁺ binding and thus replaces the bound fusion proteins (Janknecht et al., 1991). The bound protein can also be eluted with EDTA under either denaturing or native conditions (Hochuli et al., 1988; Janknecht et al., 1991). Figure 6 shows that a one-step Ni(II)-affinity-column chromatography resulted in a more than 99% purification of the recombinant eIF-5A from *E. coli* lysates using either imidazole in phosphate buffer (Figure 6, lane 5) or EDTA in 8 M urea (Figure 6, lane 8) as eluent. When imidazole was used as

eluent, an additional band at a position corresponding to 45 kDa was observed on SDS/PAGE (Figure 6, lane 5). This band could be recognized by anti-NC-21K serum (results not shown), suggesting that it is the dimer of the recombinant eIF-5A. Approx. 10–12 mg of recombinant eIF-5A precursor (> 99% pure) could be recovered from a 200 ml culture of *E. coli* M15 treated with IPTG.

Immunological and biochemical analysis of the recombinant eIF-5A precursor

We have prepared a potent rabbit polyclonal antiserum directed against *Neurospora* eIF-5A precursor protein purified from *N. crassa* (strain *arg-12 ota aga*). Figure 7(a) shows the Western-blot analysis of the recombinant protein using this polyclonal antibody. The results indicate that the recombinant protein was as antigenically potent as the wild-type protein. The recombinant protein was also tested for its ability to serve as substrate for deoxyhypusine synthase. Figure 7(b) shows that the histidine-tagged recombinant protein could be labelled by [3 H]spermidine as effectively as the wild-type eIF-5A precursor, suggesting that other post-translational modification such as phosphorylation may not be a prerequisite for deoxyhypusine formation. Both the immunological and biochemical analysis indicate that the full-length cDNA encodes for the *Neurospora* eIF-5A precursor protein.

Substrate protein affinity column chromatography

Protein affinity chromatography has been used to identify and purify proteins transiently associated with known polypeptides (Sopta et al., 1985). The strong binding between a histidine-tagged system and a metal-based resin offers the possibility of construction of an affinity-chromatographic system for protein-interaction studies (Hoffman and Roeder, 1991). Kinetic measurement indicated that the K_m value for the substrate protein was in the submicromolar range (Wolff et al., 1990). Furthermore, we have previously found that the eIF-5A precursor and the deoxyhypusine-forming enzyme can form a complex in the absence of spermidine (Dou and Chen, 1990). We therefore considered the possibility of using the polyhistidine-tagged eIF-5A precursor to construct a substrate protein affinity column which could be used in the purification of deoxyhypusine synthase. As deoxyhypusine synthase activity depends on NAD⁺ (Chen and Dou, 1988), we also examined the possible effect of NAD⁺ in this affinity interaction. The *Neurospora* lysate [hydroxyapatite fraction (Figure 8a, lane H)] was loaded on to the column in the presence (Figure 8, lanes 1–5) or absence (Figure 8, lanes 6–10) of NAD⁺. The Coomassie Blue-stained protein patterns of both unbound (Figure 8a, lanes 1 and 4, lanes 6 and 9) and bound (Figure 8a, lanes 2, 3 and 5, lanes 7, 8 and 10) fractions were compared (Figure 8a) and the corresponding deoxyhypusine synthase activities, as measured by *in vitro* labelling assay using exogenously added eIF-5A precursor and [3 H]spermidine, are shown in Figure 8b. After the lysate had been loaded on to the His-NC-21K column, the flow-through sample (unbound fraction) retained more than 95% of total proteins (Figure 8a, lane 1 compared with lane H) whereas almost no protein, except the 21 kDa band, was detectable in the bound fraction eluted by EDTA (Figure 8a, lane 2). The 21 kDa band detected in the bound fraction was His-NC-21K protein eluted by EDTA from the affinity column. When the unbound and bound fractions were tested for deoxyhypusine synthase activity, the bound fraction contained as much enzyme activity as the unbound fraction (Figure 8b, lane 2 compared with lane

Figure 8 Protein affinity chromatography

Ni(II)-NTA resins (100 μ l) were washed with phosphate buffer (20 mM sodium phosphate, pH 7.0, 5 mM 2-mercaptoethanol). The protein affinity column was prepared by incubating 100 μ l of resin with 350 μ g of His-NC-21K protein for 2 h at 4 °C. The hydroxyapatite fraction of *N. crassa* lysates was used as a source for deoxyhypusine synthase. The enzyme fraction was mixed with the His-NC-21K-Ni(II)-NTA resins in the presence (lanes 1 and 2) or absence (lanes 6 and 7) of 1 mM NAD⁺ for 2 h at 4 °C. The mixture was loaded on to a column and washed with 20 mM phosphate buffer (flow-through unbound fraction) and then eluted with EDTA (100 mM in 20 mM phosphate buffer). Each fraction was tested for deoxyhypusine synthase activity as described in the Materials and methods section. Lanes 1 and 6, unbound fraction; lanes 2 and 7, EDTA-eluted fraction (bound fraction). *Neurospora* lysates were also loaded on to a Ni(II)-NTA column, washed with phosphate buffer (flow-through) and eluted with EDTA (bound fraction). Lanes 3 and 8, bound fraction from Ni(II)-NTA column. The flow-through fraction was then mixed with His-NC-21K-Ni(II)-NTA resins in the presence (lanes 4 and 5) or absence (lanes 9 and 10) of 1 mM NAD⁺ for 2 h at 4 °C. The mixture was loaded on to a column and separated into unbound (flow-through) and bound (EDTA eluted) fractions. Lanes 4 and 9, unbound fraction; lanes 5 and 10, bound fraction. (a) Coomassie Blue-stained protein profile of various column fractions. (b) Fluorogram of deoxyhypusine synthase activity profile in each corresponding fraction. The enzyme activity was measured by radiolabelling the purified *Neurospora* eIF-5A precursor protein with [³H]spermidine. For each enzyme assay, an excess of exogenous eIF-5A precursor (1.2 μ g per assay) was added to the reaction mixture to ensure that the substrate was saturated throughout the reaction. Std, Molecular-mass standards; lane H, hydroxyapatite fraction.

1), suggesting that a significant amount of deoxyhypusine synthase did bind specifically to the NC-21K affinity column. To ensure that deoxyhypusine synthase was bound to His-NC-21K protein rather than to the nickel resin, we showed that, when the lysates were loaded on to the Ni(II)-NTA column rather than the His-NC-21K-Ni(II)-NTA column, no enzyme activity was recovered in the bound fraction (Figures 8a and 8b, lane 3). However, when the flow-through fraction from the Ni(II)-NTA column was applied again to the His-NC-21K affinity column (Figures 8a and 8b, lanes 4 and 5), the bound fraction contained very little detectable protein, but retained high enzyme activity (Figure 8b, lane 5), similar to the binding experiments described above using lysates directly on the substrate affinity column. On the basis of these two sets of experiments, we calculated that a one-step eIF-5A substrate affinity chromatography could result in a more than 30-fold increase in the specific activity of deoxyhypusine synthase. Interestingly, when the same experiments were carried out in the absence of NAD⁺ (Figures 8a and 8b, lanes 6–10), the enzyme activity was recovered only in the flow-through fractions and not in any of the EDTA-eluted fractions (Figure 8b, lane 7 compared with lane 6 and lane 10 compared with lane 9). Overall, Figure 8 shows that deoxyhypusine synthase bound to the substrate affinity column, and the enzyme activity could be released by elution with the chelating reagent, EDTA. Moreover, the binding of deoxyhypusine synthase to the affinity column appeared to be dependent on NAD⁺, suggesting that NAD⁺ binding to deoxyhypusine synthase affects its conformation.

DISCUSSION

The cDNAs and genes for eIF-5A of several eukaryotic species, including *D. discoideum* (Sandholzer et al., 1989), yeast (Schnier et al., 1991), alfalfa (Pay et al., 1991) and tobacco (Chamot and Kuhlemeier, 1992) have been cloned. Recently a hypusine-containing protein and its gene were also isolated from the

archaeobacterium *Sulfolobus acidocaldarius* (Bartig et al., 1992). All these studies involve screening of cDNA libraries using either antibodies or oligonucleotide probes. PCR provides a powerful means of cloning a target gene of unknown sequence based on limited information of the sequence for this gene obtained from other species (Ohara et al., 1989). Thus initial entry into the cDNA sequence can be achieved by using imperfect oligonucleotides derived from related organisms. The approach used in the present paper relies on sequence information of the eIF-5A precursor previously reported for human, yeast and slime mould (Sandholzer et al., 1989; Smit-McBride et al., 1989; Schnier et al., 1991) and on the availability of a *Neurospora* cDNA library (Figure 1). The *Neurospora* cDNA library was constructed on a λ Zap vector which contains T3 and T7 promoters adjacent to the cloning sites (Short et al., 1988). As the cDNA library itself is used directly as template, there is no need to add synthetic adaptors or tails to the cDNA (Ohara et al., 1989; Frohman and Martin, 1989). Thus, instead of anchors or adaptors added to the amplified sequences as described in other PCR-based cloning procedures (Frohman et al., 1988; Troutt et al., 1992), we used a pair of universal primers, T3 and T7, to amplify specifically the eIF-5A sequence from the *Neurospora* cDNA library (Figure 2). The method is simple and straightforward because it does not require screening of the cDNA library. The PCR-based cloning procedure described here should be generally applicable for cloning eIF-5A cDNAs or their genes from other species provided that the cDNA or genomic library is available.

Both the conceptual translation (Figure 3) and the codon usage of the cloned cDNA (results not shown) suggest that we have obtained the *Neurospora* eIF-5A cDNA. To demonstrate conclusively that we have obtained the correct cDNA, we proceeded to construct a recombinant vector, pQTy21, using the full-length cDNA as the insert (Figure 4). The strong promoter in the expression vector, pQE-30, allowed the overexpression of the recombinant protein, accounting for about 30% of total cellular proteins (Figure 5). The apparent molecular mass of the

recombinant protein on SDS/PAGE is similar to that of *Neurospora* eIF-5A (Figure 5). The use of pQE-30 also introduces six contiguous histidine residues to the N-terminus of the resulting recombinant protein, termed His-NC-21K. As expected, the overexpressed His-NC-21K protein can be rapidly purified in one step using Ni(II)-NTA-agarose column chromatography under either native or denaturing conditions (Figure 6). Despite a slight modification at the N-terminus, the recombinant His-NC-21K appears to be biologically active because (i) it can be recognized by rabbit anti-NC-21K serum raised against eIF-5A precursor purified from *N. crassa* (Figure 7a), (ii) it can serve as substrate for deoxyhypusine synthase *in vitro* (Figure 7b) and (iii) it can bind to deoxyhypusine synthase in the presence of NAD⁺ (Figure 8). Taken together, we conclude that we have obtained the full-length cDNA. However, further work is needed to determine the transcription initiation site.

In order to develop a procedure for purifying deoxyhypusine synthase, it is necessary to have a dependable source of substantial amounts of eIF-5A precursor to serve as substrate for the assay of deoxyhypusine synthase activity. Construction of the vector pQTy21 and overexpression of this plasmid in *E. coli* fulfil this requirement. This is particularly important in view of the fact that eIF-5A precursor does not accumulate in living cells or organisms unless spermidine biosynthesis is suppressed by drugs such as difluoromethylornithine (Gerner et al., 1986; Park, 1987).

Current assay methods for deoxyhypusine synthase activity rely on (i) separation of radiolabelled eIF-5A, in either the deoxyhypusine or hypusine form, from free [³H]spermidine by SDS/PAGE and liquid-scintillation counting of radioactivity associated with the labelled eIF-5A (Dou and Chen, 1990) or (ii) direct quantification of labelled deoxyhypusine/hypusine in labelled eIF-5A by h.p.l.c. after acid hydrolysis of the protein (Park et al., 1982). Both methods are tedious and time consuming. The tight binding between His-tagged NC-21K protein and Ni(II)-NTA-agarose resin, which resists high-salt (> 1 M NaCl) or denaturant (e.g. 8 M urea) washing (Figure 7), permits us to develop a convenient assay method for determining deoxyhypusine synthase activity. On the basis of the observation that free [³H]spermidine can be easily separated from radiolabelled His-NC-21K protein bound to a Ni(II)-NTA-agarose column by washing, we have now developed a quick and reliable assay method for measuring deoxyhypusine synthase activity (Y. Tao, H. M. Skrenta and K. Y. Chen, unpublished work).

The fact that eIF-5A is the only cellular protein known to contain hypusine that is derived from spermidine strongly suggests that this post-translational modification may mediate some of the growth-regulatory actions of polyamines. Although eIF-5A has been shown to stimulate methionyl puromycin bond formation *in vitro* (Benne et al., 1978), its biological function and physiological significance *in vivo* remain unclear (Park et al., 1993). One approach to discovering the function and significance of hypusine formation is to examine the possible transient interactions of different forms of eIF-5A (precursor form, deoxyhypusine form, hypusine form and possibly phosphorylated forms) with other cellular proteins. Thus the demonstration that deoxyhypusine synthase can bind to the recombinant His-tagged NC-21K protein (Figure 8) is significant in three regards. (i) It suggests the possibility of using the protein affinity column constructed with His-tagged NC-21K for purifying deoxyhypusine synthase. (ii) Given the ease with which large amounts of His-NC-21K protein can be expressed and purified, the system described here permits conversion of His-NC-21K protein into various forms of eIF-5A for construction of different protein affinity columns for protein-interaction studies. (iii) It also

permits a direct mutational analysis of protein-interaction domains that will improve our understanding of the molecular basis for the association of eIF-5A proteins involved in regulatory networking.

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