Molecular Cloning and Functional Expression of Neurospora Deoxyhypusine Synthase cDNA and Identification of Yeast Deoxyhypusine Synthase cDNA*

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Deoxyhypusine synthase catalyzes the formation of deoxyhypusine residue on the eIF-5A precursor using spermidine as the substrate. We have purified deoxyhypusine synthase from Neurospora crassa to apparent homogeneity (Tao, Y., and Chen, K. Y. (1995) J. Biol. Chem. 270, 383-386). We have now cloned and characterized the deoxyhypusine synthase cDNA using a reverse genetic approach. Conceptual translation of the nucleotide sequence of the cloned 1258-base pair cDNA revealed an open reading frame containing 353 amino acids with a predicted M, of 38,985. The deoxyhypusine synthase cDNA was subcloned into the expression vector pQE60 to produce a 40,000-dalton recombinant protein on SDS-PAGE which exhibited deoxyhypusine synthase activity. A GenBank search showed that the Neurospora deoxyhypusine synthase cDNA possessed significant sequence homology to a previously uncharacterized yeast sequence. Sequence alignment and hydropathy analysis suggest that the yeast sequence represents deoxyhypusine synthase.

Hypusine formation on the eIF-5A precursor involves (i) NAD⁺-dependent oxidative cleavage of spermidine, (ii) transfer of the aminobutyl moiety derived from spermidine to eIF-5A residue, and (iii) hydroxylation of the deoxyhypusine residue, and (iii) hydroxylation of the deoxyhypusine residue (accession number U00061 (1994)). The yeast sequence encodes a protein of 387 amino acids that shows 69% of total amino acid identity and 80% of total amino acid similarity to the Neurospora enzyme. Sequence alignment and hydropathy analysis suggest that the yeast sequence represents deoxyhypusine synthase.

A general polymerase chain reaction (PCR)² approach has been utilized using a single-sided specific primer in conjunction with nonspecific primers targeted either to the 3' poly(A)° region or to an enzymatically synthesized tail at 5'-end that permits amplifications of the regions upstream and downstream of the core sequence (Frohman et al., 1988; Ohara et al., 1989). Using partial amino acid sequence information obtained from Neurospora deoxyhypusine synthase (Tao and Chen, 1995), we have adopted a simple PCR strategy to clone Neurospora deoxyhypusine synthase cDNA. Here we report the molecular cloning and functional expression of recombinant deoxyhypusine synthase in vitro and in vivo. In addition, we have also identified a hitherto uncharacterized yeast sequence in GenBank that most likely represents the yeast deoxyhypusine synthase cDNA.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains, plasmids, and chemicals were the same as described previously (Tao and Chen, 1994).

Renaturation of Deoxyhypusine Synthase after SDS-PAGE Analysis—Partially purified deoxyhypusine synthase was separated by SDS-PAGE at 4 °C. Gel slices were rinsed with extraction buffer (20 mM phosphate buffer, pH 7.5, 1 m Na dithiothreitol, 0.1 mM EDTA, 10% glycerol, and 0.1% Tween 20) and homogenized. The mixture was spun in a centrifuge, and the supernatant was concentrated using Centricon 30 for 1 h at 4 °C. The concentration step was repeated three times. The concentrated sample was used for the enzyme assay as described (Dou and Chen, 1988).

Amplification of 3'-End Deoxyhypusine Synthase cDNA

Six degenerate primers were synthesized based on the partial amino acid sequences of the deoxyhypusine synthase: P1 (192-fold degeneracy), 5'-dAACAGCATCCTCNGTNTTGTGCCC; P2 (256-fold degeneracy), 5'-AACATATCCNCCNARCCAACCRTC; P3 (32-fold degeneracy), 5'-dCAACTGTGCYCTTTGARGA; P4 (128-fold degeneracy), 5'-dCATGYCGAANCRCART; P5 (128-fold degeneracy), 5'-dTA-CATCAYACNCNGCART; P6 (384-fold degeneracy), 5'-dGTCGAAYTCYTGNGCNGTRTTDAT. (H, Y, T, R, M, K, W, S, N, D; I, L, V, A, N, T, C + G, Y, T + C, R, A + G, D = A + G + T). Other primers used were P8, 5'-dGAGCGGATAACAATTTCACACAGG-3; P9, 5'-dGTCGGCCCTAGCTGGCCCTGAG-3; P10, 5'-dGGCC-CTTG AACGTATGAAAATACAG-3; P12, 5'-dCTTGAGAACCTGGT-CCGCAC-3'; universal primers: MF 5'-dSCCAGGTTTTC- CAGTCAGA-3' and MR 5'-dGACGGGATACAAATTCACACACGG-3'.

Amplication of 3'-End and 5'-End Deoxyhypusine Synthase cDNA Fragment by PCR—The reaction mixture in the first PCR run contained 0.5 µl of cDNA library, as well as a degenerate sense primer and a universal primer (0.2 µM each). The first run PCR product was then used as template and amplified by another sense degenerate primer and a universal primer in the second PCR run. In both runs, the "touchdown" protocol (Don et al., 1991) was followed. The amplified 3'-end cDNA fragments were subcloned into a Smal-EcoRI-digested pBlueScript KS vector for sequence determination. Two gene-specific antisense primers, P8 and P10, based on the sequence of the 3'-end cDNA were used to obtain the 5'-end cDNA fragment. Primer P10 and

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1 Z. P. Chen and K. Y. Chen, unpublished data.

2 The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; 6xHis-NC21K, polyhistidine-tagged Neurospora 21-kDa eIF-5A precursor protein; RACE, rapid amplification of cDNA ends; bp, base pair(s); ORF, open reading frame; kb, kilobase pair(s).

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**RESULTS**

Renaturation of Deoxyhypusine Synthase from SDS-PAGE—To examine whether the 40-kDa polypeptide alone is sufficient to account for the deoxyhypusine synthase activity, we have tested the activity of the 40-kDa polypeptide after renaturation from SDS-polyacrylamide gel. Fig. 1A shows that only the 40-kDa band exhibited deoxyhypusine synthase activity after renaturation. The overall recovery of the original enzyme activity was about 10%. Other protein bands recovered from gel slices did not have any effect on the renatured deoxyhypusine synthase, suggesting that the 40-kDa polypeptide represents the only subunit for Neurospora deoxyhypusine synthase. Based on the partial amino acid sequence information, we have designed three pairs of degenerate primers, P1-P6, to gain entry into the Neurospora cDNA library (Fig. 1B).

**PCR Cloning of 3'-End and 5'-End cDNA Fragments and Ligase Free Recombination**—Fig. 2A illustrates the PCR strategy for cloning the 3'-end and 5'-end cDNA fragments. The use of different combination of nested primers not only significantly enhanced the specificity of amplification, they also provided information on the order of these sequences. Fig. 2B shows that for 3'-end cDNA amplification, only R31 and R35 gave prominent DNA bands, indicating the order of primers is P3–P1 and P3–P5. The most prominent bands in R31 and R35...
had the size of about 650 and 450 bp, respectively. Sequence analysis demonstrated that these 650 bp contained one open reading frame (ORF) and a poly(A) tail. The translation of this ORF matched the amino acid sequences of peptide T51 and T35, confirming that we have obtained the 3'9-end cDNA fragment for deoxyhypusine synthase.

The sequence information obtained for the 3'9-end cDNA of deoxyhypusine synthase enabled us to design two gene-specific antisense primers, P8 and P10, for cloning the 5'9-end cDNA of the enzyme. Fig. 2C shows the agarose gel analysis of the PCR products after the second PCR run. The most prominent band has the size of about 700 bp (lane 2). Sequence analysis revealed that the 700-bp fragment contained an ORF with sequences matching tryptic peptides T53 and T4.

The 3'9-end and 5'9-end cDNAs were amplified (Fig. 2D, lanes 3 and 4) and combined by using two universal primers in a ligase-free PCR reaction. The combination of the 700-bp 5'9-end cDNA and 600-bp 3'9-end cDNA produced a 1.3-kb fragment (Fig. 2D, lane 2). This 1.3-kb fragment was subcloned into pBluescript and sequenced.

Sequence Analysis of Deoxyhypusine Synthase cDNA—Fig. 3 shows the entire nucleotide sequence of the cDNA, including the 5'- and 3'-untranslated regions of 36 and 160 bp, respectively. The cDNA contained one ORF, encoding a protein of 353 amino acids with molecular mass of 38,985. All four tryptic peptides previously sequenced can be identified within this ORF. The predicted amino acid sequence of *Neurospora* deoxyhypusine synthase is not related to any other known proteins in the GenBank and EMBL databases. However, we found that the *Neurospora* deoxyhypusine synthase cDNA shows a high homology to a hitherto uncharacterized yeast sequence (YHRO68w, accession number U00061 (1994), located on chromosome VIII). The yeast sequence contains one ORF encoding a protein of 387 amino acids. Fig. 4 shows the alignment of this 387-amino acid sequence with that of *Neurospora* deoxyhypusine synthase. The overall amino acid sequence identity between the *Neurospora* deoxyhypusine synthase and the putative yeast enzyme is 69%, and the overall amino acid sequence similarity is 80%. The degree of amino acid identity is not distributed equally, being highest in the middle of the polypeptide but not at the N terminus. For example, a 132-amino acid sequence extending from residues 220 to 352 (yeast numbering) shows 81% identity and 94% similarity between these two polypeptides. Such high homology suggests that the U00061 sequence represents the yeast deoxyhypusine synthase cDNA.

We also found a short human expressed sequence tag (Z25337 (1993)) that bears considerable homology to *Neurospora* deoxyhypusine synthase cDNA. The amino acids encoded by this 312-bp human sequence covers from residues 93 to 196 (Fig. 4). It can be noted that a striking homology exists in the amino acid sequence extending from 101 to 196 for human, yeast, and *Neurospora* polypeptides, with amino acid similarity as high as 85%, suggesting that deoxyhypusine synthase is a highly conserved enzyme. The identification of this expressed sequence tag as a partial human deoxyhypusine synthase sequence proves invaluable in our cloning of two full-length human cDNAs for deoxyhypusine synthase.

The hydropathy profiles of *Neurospora* and yeast enzyme are fragments previously sequenced can be identified within this ORF. The predicted amino acid sequence of *Neurospora* deoxyhypusine synthase is not related to any other known proteins in the GenBank and EMBL data bases. However, we found that the *Neurospora* deoxyhypusine synthase cDNA shows a homology to a hitherto uncharacterized yeast sequence (YHRO68w, accession number U00061 (1994), located on chromosome VIII). The yeast sequence contains one ORF encoding a protein of 387 amino acids. Fig. 4 shows the alignment of this 387-amino acid sequence with that of *Neurospora* deoxyhypusine synthase. The overall amino acid sequence identity between the *Neurospora* deoxyhypusine synthase and the putative yeast enzyme is 69%, and the overall amino acid sequence similarity is 80%. The degree of amino acid identity is not distributed equally, being highest in the middle of the polypeptide but not at the N terminus. For example, a 132-amino acid sequence extending from residues 220 to 352 (yeast numbering) shows 81% identity and 94% similarity between these two polypeptides. Such high homology suggests that the U00061 sequence represents the yeast deoxyhypusine synthase cDNA.

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SDS-PAGE. Cell lysates were prepared as described (Tao and Chen, 1995). Lane 1, protein standard; lane 2, lysate (2 μl) from the pQE60-transfected cells; lane 3, lysate (2 μl) from pODS-transfected cells. B, deoxyhypusine synthase activity of the recombinant protein. The radio-labeled 6xHis-NC21K substrate protein was detected by autoradiography after SDS-PAGE. Lane 1, lysates from pODS transformant (1 μl); lane 2, lysates from pODS transformant (5 μl); lane 3, lysates from pQE60 transformant (1 μl); lane 4, lysates from pQE60 transformant (5 μl).

nearly superimposable (data not shown), consistent with their high sequence homology. In the case of Neurospora deoxyhypusine synthase, both the N and C termini of this enzyme appear to be highly hydrophobic. Whether this may be related to the hydrophobic chromatographic behavior of the Neurospora enzyme remains to be examined.

Functional Expression of Recombinant Deoxyhypusine Synthase In Vitro and In Vivo—We first tested functional expression of recombinant protein in vitro. A significant incorporation of radioactive spermidine into 6xHis-NC21K was detected (1,000 cpm over the control for 1 μg of plasmid) when the reticulocyte lysate was programmed with plasmid containing deoxyhypusine synthase cDNA insert (data not shown). We then examined the enzyme activity of recombinant protein expressed in E. coli. Fig. 5A shows the isopropyl-1-thio-β-o-galactopyranoside-induced induction of expression of the 40-KDa protein in transfected M15 cells (Fig. 5A, lane 3 versus lane 2). The lysates from pODS-transfected cells were directly used to test for the presence of deoxyhypusine synthase activity. Fig. 5B shows that deoxyhypusine synthase activity could be detected in cell lysates transfected by pQDS after isopropyl-1-thio-β-o-galactopyranoside induction (Fig. 5B, lanes 1 and 2 versus lanes 3 and 4). Taken together, these results confirm that the cloned 1258-bp cDNA represents the gene for deoxyhypusine synthase from N. crassa.

**DISCUSSION**

The demonstration that the 40-KDa polypeptide exhibited deoxyhypusine synthase activity after renaturation (Fig. 5A) and the functional expression of the cloned deoxyhypusine synthase cDNA in bacteria (Fig. 5B) strongly suggest that the 40-KDa subunit alone constitutes the active tetrameric enzyme that catalyzes both oxidative cleavage of spermidine and subsequent transfer of the aminobutyln to NAD^+ for deoxyhypusine synthase. The cloned 1258-bp cDNA contains one ORF that covers the entire amino acid sequence of deoxyhypusine synthase (Fig. 3). This conclusion is supported by the following evidence: (i) the molecular mass of the predicted amino acid sequence corresponds to that determined by SDS-PAGE; (ii) the Kazak consensus sequence precedes the initiation codon; (iii) the predicted amino acid sequence contains four tryptic peptide fragments that were previously sequenced; (iv) the predicted amino acid sequences from different species share high homology (Fig. 4); and (v) the recombinant protein exhibits deoxyhypusine synthase activity (Fig. 5).

Based on the amino acid sequence alignment (Fig. 4), it is likely that we have also obtained the complete sequence for yeast deoxyhypusine synthase and about one-third of human sequence. The homology between Neurospora, yeast, and human (partial) protein is striking. Of particular note are two stretches of amino acids, one spans from residue 101 to 196, and the other from 220 to 352 (yeast numbering). Consistent with a high degree of homology, the hydrophathy profiles of both Neurospora and yeast proteins are similar (data not shown). Neurospora deoxyhypusine synthase has been shown to bind tightly to phenyl-Sepharose column in the presence of high salt buffer, suggesting the presence of hydrophobic patches at the enzyme surface (Tao and Chen, 1995). These patches cannot be clearly identified based on hydrophathy plot.

Deoxyhypusine synthase is a bifunctional enzyme that utilizes NAD^+ as co-factor (Chen and Dou, 1988). Surprisingly, the deduced amino acid sequence of Neurospora or yeast deoxyhypusine synthase fails to share any similarities with other known dehydrogenases. We also failed to detect the nucleotide binding domain, Gly-X-Gly-X-Gly (Rossmann et al., 1974) in either Neurospora or yeast sequence. Nevertheless, the binding sites for NAD^+ and for the two substrates, spermidine and eIF-5A precursor, are likely to be located in the most conserved region in the protein. We have shown that the binding of eIF-5A precursor to deoxyhypusine synthase occurs only in the presence of NAD^+ (Tao and Chen, 1994). We have also shown that the presence of NAD^+ protects the enzyme from the inhibitory action of sulfhydryl reagents (Tao and Chen, 1995). These results prompt us to speculate that the two cysteine residues (positions 145 and 180), located within the most conserved region in deoxyhypusine synthase (Fig. 4), may be near or within the active site. The tetrameric structure of deoxyhypusine synthase also raises the possibility that the four subunits of the enzyme may generate two pockets for spermidine binding when they are assembled. Further studies are needed to elucidate the enzyme structure and its interaction with co-factor and substrates. The availability of deoxyhypusine synthase cDNA should prove invaluable in achieving these goals.

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Note Added in Proof—While this manuscript was under review, two papers describing the identification of yeast deoxyhypusine synthase cDNA were published (Klier, H., Csonga, R., Steinaker, A., Wohl, T., Lottspeich, F., and Eder, J. (1995) FEBS Lett. 364, 207–210 and Kang, K. R., Wolff, E. C., Park, M. H., Folk, J. E., and Chung, S. I. (1995) J. Biol. Chem. 270, 18146–18142).

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**FIG. 5. Expression of pQDS in M15 E. coli.** A, protein pattern on a SDS-PAGE. Cell lysates were prepared as described (Tao and Chen, 1994). Lane 1, protein standard; lane 2, lysate (2 μl) from the pQE60-transfected cells; lane 3, lysate (2 μl) from pODS-transfected cells. B, deoxyhypusine synthase activity of the recombinant protein. The radiolabeled 6xHis-NC21K substrate protein was detected by autoradiography after SDS-PAGE. Lane 1, lysates from pODS transformant (1 μl); lane 2, lysates from pODS transformant (5 μl); lane 3, lysates from pQE60 transformant (1 μl); lane 4, lysates from pQE60 transformant (5 μl).