Molecular cloning and functional expression of human deoxyhypusine synthase cDNA based on expressed sequence tag information

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Deoxyhypusine synthase is an NAD⁺-dependent enzyme that catalyses the formation of a deoxyhypusine residue on the eukaryotic initiation factor 5A (eIF-5A) precursor by transferring an aminobutyl moiety from spermidine to the ϵ -amino group of a unique lysine residue. We have recently cloned and characterized the *Neurospora crassa* deoxyhypusine synthase cDNA using a reverse genetics approach. A GenBank search showed that a stretch of the deduced amino acid sequence (96 amino acids) of *Neurospora* deoxyhypusine synthase matches a short human expressed sequence tag (EST), Z25337, with greater than 70% amino acid identity. Gene-specific primers based on this EST were used together with universal primers to obtain

1219 bp and 1078 bp cDNAs from a human cDNA library. The 1219 bp and 1078 bp sequences, each containing an open reading frame, encode polypeptides of respectively 368 and 321 amino acids. The short sequence is identical to the long one except that it is missing a stretch of 47 amino acids spanning residues 261–307. The 368-amino-acid sequence of human deoxyhypusine synthase shares a high degree of identity (> 50%) and similarity (> 60%) with that of the *Neurospora* and yeast deoxyhypusine synthases. After cloning into an expression vector, the 368-amino-acid recombinant protein exhibits high deoxyhypusine synthase activity. In contrast, the 321-amino-acid recombinant protein shows no detectable activity.

INTRODUCTION

Hypusine formation on the eukaryotic initiation factor 5A (eIF-5A) precursor involves two steps: (i) NAD⁺-dependent oxidative cleavage of spermidine and transfer of the aminobutyl moiety derived from spermidine to the eIF-5A precursor to form a deoxyhypusine [*N*^ε-(4-aminobutyl)lysine] residue, and (ii) hydroxylation of this deoxyhypusine residue [1–3]. The highly conserved nature of eIF-5A [4], its responsiveness to growth stimulation and the specificity of hypusine formation [5–7], together with the recognized importance of polyamines in growth regulation [8,9], suggest that hypusine formation may have an important role in cell physiology. Indeed, disruption of the two eIF-5A genes in yeast has been shown to be lethal [10]. Moreover, inhibition of deoxyhypusine synthase by *N*¹-guanyl-1,7-diaminoheptane causes growth arrest of CHO cells [11] and differentiation of mouse neuroblastoma cells [12]. Previous studies have also demonstrated that eIF-5A may serve as target for the HIV Type I Rev [13] and human T-cell leukaemia virus Type I Rex [14], suggesting that eIF-5A may be involved in mRNA processing. Since deoxyhypusine synthase, the key enzyme for hypusine formation, catalyses the first step in this process, this enzyme may prove to be a useful target for pharmacological manipulation.

We have recently purified *Neurospora crassa* deoxyhypusine synthase [15] and cloned its cDNA using a reverse genetics approach [16]. A GenBank search has identified a hitherto uncharacterized yeast sequence (accession no. U00061) that most likely represents the yeast deoxyhypusine synthase cDNA [16]. In addition, we found that translation of a short human expressed

sequence tag (EST; Z25337) produces an amino acid sequence that shows 74% identity and 85% similarity to an amino acid stretch spanning residues 83–178 of *Neurospora* deoxyhypusine synthase. This finding suggests strongly that EST Z25337 may represent a partial sequence of human deoxyhypusine synthase. Using EST Z25337 as a guide to gain entry into a human cDNA library, we have cloned and expressed two human deoxyhypusine synthase cDNAs of different sizes. The strategy described here provides an example of direct cloning of the target gene from a human cDNA library based on the relevant EST information without resorting to protein isolation, a step generally required for either a reverse genetics approach or a conventional antibody screening approach. The strategy can be applied to any species provided that their EST information is available. The availability of deoxyhypusine synthase cDNA from different species allows sequence alignment for structural and domain analysis. It also facilitates large-scale production of the functional recombinant enzyme for biochemical and crystallographic studies.

EXPERIMENTAL

Design of oligonucleotide primers for DNA cloning and sequencing

Four gene-specific primers based on the EST sequence Z25337 were used: P1 (5'-dCTCATCAGTTCAGGCATCCGTG-3'; 22-mer), P2 (5'-dCTTGCAGTAATTCTCATTGGGC-3'; 22-mer), P3 (5'-dCGCTACCTTGTGCAGCACAAAC-3'; 21-mer) and P4 (5'-dCCAGCAGGTTTCCGATCCTAT-3'; 21-mer). Two gene-specific primers containing initiation codon and stop codon were, respectively, P7 (5'-dTAGGCCCATGGAAGGTTCCC-TGGAAC-3'; 26-mer) and P10 (5'-dCGCAAGCTTTCAG-

Abbreviations used: eIF-5A, eukaryotic initiation factor 5A; EST, expressed sequence tag; 6xHis-NC21K, polyhistidine-tagged *Neurospora crassa* 21 kDa eIF-5A precursor protein; 6xHis-h18K, polyhistidine-tagged human 18 kDa eIF-5A precursor protein; ORF, open reading frame.

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The nucleotide sequence data reported in this paper have been submitted to the GenBank[®]/EMBL Nucleotide Sequence Databases with accession no. U26266 for the short human deoxyhypusine synthase cDNA (1078 bp) and U32178 for the long human deoxyhypusine synthase cDNA (1219 bp).

Figure 2 EST-based PCR cloning strategy

Pairs consisting of a gene-specific primer and a universal primer (P1 or P3 with MF and P2 or P4 with MR) were used to obtain 5'-end and 3'-end cDNA fragments. The two fragments were assembled by the GCG program to give a full-length sequence. Two additional gene-specific primers, P7 and P10, were designed based on the full-length sequence (the double-headed arrows for P7 and P10 indicate the relative sequence positions for designing P7 and P10). P7 and P10 were then used to directly amplify full-length cDNAs from the HeLa cell cDNA library.

that the Z25337 sequence represents a portion of the reverse strand of human deoxyhypusine synthase cDNA. Four gene-specific primers based on the Z25337 sequence were designed (Figure 1B) in order to clone human deoxyhypusine synthase cDNA directly. PCR amplification of a human cDNA library using the P1/P2 pair and the P3/P4 pair produced DNA species of the predicted length (results not shown).

PCR amplifications of the 3'-end and 5'-end fragments

Figure 2 illustrates our PCR strategy for cloning the full-length deoxyhypusine synthase cDNA. Amplification of the human cDNA library using the MF/P3 pair and the MR/P4 pair resulted in 3'-end and 5'-end cDNA fragments of 0.8 kb and 0.6 kb respectively (results not shown). The nucleotide sequences of these two fragments were assembled into an entire sequence using the GCG program. The assembled cDNA contained an open reading frame (ORF) encoding 321 amino acids. The predicted amino acid sequence of this human cDNA is much shorter than those of the *Neurospora* or yeast enzymes. Subsequently we noticed the appearance of another EST sequence, R79890 (435 nt long), in the GenBank Database. EST R79890 matches a portion of the combined sequence but contains an additional 141 nucleotides that were not present in the combined sequence. This, together with the consistent finding that there were faint DNA bands with sizes greater than 0.8 kb in the MF/P3 product, prompts us to speculate that more than one cDNA sequence for the human deoxyhypusine synthase gene may exist.

AGCCGGCTATAGGCGCATGGAAGGTTCCCTGGAACGGGAGGCCACCGGGGCGTGCCGC	60
M E G S L E R E A P R G R A A	15
CGTGCTAAAGCACAGCTCGACGTGCGGCCGGAAGCACCGGTCGGGGCTACGACTT	120
V L K H S S T L P P E S T Q V R G Y D F	35
CAACCGCGGTGTGAATTACCGCGCACTGCTGGAGGCCTTCGGCACCACGGCTTCCAAGC	180
N R G V N Y R A L L E A F G T T G F Q A	55
AACCACTTCGGGCGCGCTGTACAGCAAGTCAATGCCATGATCGAGAAGAAGCTGGAACC	240
T N F G R A V Q Q V N A M I E K K L E P	75
ACTGTCACAGGATGAAGACCAGCACCGGACCTGACCCAGAGCCGCGCCACTTACCAG	300
L S Q D E D Q H A D L T Q S R R P L T S	95
CTGCACCACTTTCCTGGGATATACATCCAACTCATCAGTTCAGGATCCGTGAGACCAT	360
C T I F L G Y T S N L I S S G I R E T I	115
TCGTACCTTGTGCGACCAACATGGTGGAGCTATTGGTGACCACTGCGCGCTGGA	420
R Y L V Q H N M V D V L V T T A G G V E	135
Z25337	
GGAAGACCTCATCAAGTGCCTGGCGCCCATACTTGGCGGAGTTTAGCTCAGGGGAA	480
E D L I K C L A P T Y L G E F S L R G K	155
GGAGCTCCGGGAGAACGGATCAATAGGATCGGAAACCTGCTGGTGCCCAATGAGAATTA	540
E L R E N G I N R I G I N L L V P N E N Y	175
CTGCAAGTTTGAGGACTGGCTGATGCCCATCTTGAGCAGATGGTGAGCAGAACAC	600
C K F E D W L M P I L D Q M V M E Q N T	195
AGAGGTGTAAAGTGGAGCGCTTCTAAGATGATCGCCCGCTGGGCAAGGAGATCAACAA	660
E G V K W T P S K M I A R L G K E I N N	215
CCAGAGTCCGTGTATTACTGGGCCAGAAACACATCCCTGTGTTTAGTCCCGCACT	720
P E S V Y Y W A Q K N H I P V F S P A L	235
TACAGACGGCTCGCTGGGCGACATGATCTTCTTCCATTCTACAAGAACCGGGCCTGCT	780
T D G S L G D M I F F H S Y K N P G L V	255
CCTGGACATCGTTGAGGACCTGAGGCTCATCAACACAGGCCATCTTTCGAAGTGACAC	840
L D I V E D L R L I N T Q A I F A K C T	275
TGGATGATCATCTTGGCGGGGCGGTGCAAGCACCACTTCCCAATGCCAACCTCAT	900
G M I I L G G G V V K H H I A N A N L M	295
CGGAAACGGGCGGACTACGCTGTTTACATCAACAGCCAGGAGTTTGATGGCTCTGA	960
R N G A D Y A V Y I N T A Q E F D G S D	315
CTCAGGTCCCGACACAGAGGCTGTCTCTGGGCAAGATCCGGGTGATGCACAGCC	1020
S G A R P D E A V S W G K I R V D A Q P	335
CGTCAAGTCTATGCTGACGCCCTCCCTGGTCTTCCCCTGCTTGTGGTGAAACCTTTGC	1080
V K V Y A D A S L V F P L L V A E T F A	355
CCAGAAGATGGATGCTTCATGCATGAGAAGAAGGAGTCTGAGCGGCTCGCGTCCCAGG	1140
Q K M D A F M H E K N E D	368
AAGGTCTTACCCCTCTCTATTATTATTATTTGAGACCCAGCCCTCCCTACTTTTGTG	
GTGAGCTACGCTCTAGAATAAGATGGTATCTGAAGTCC	

Figure 3 Nucleotide sequence and predicted amino acid sequence of human deoxyhypusine synthase

The ORF, defined by assigning the initiation codon ATG at position 17, encodes a sequence of 368 amino acids. The translation stop codon (TGA) is indicated by an asterisk. A 141 nt stretch from nucleotide 797 to nucleotide 937 is missing from the short 1078 bp cDNA. This 141 nt sequence and its corresponding amino acid sequence are indicated in bold. The amino acid sequence is shown in single-letter code.

PCR cloning of full-length cDNA using gene-specific primers

In order to amplify by PCR all full-length sequences potentially present in the cDNA library, we designed another two gene-specific primers, P7 (sense) and P10 (antisense), using the sequence information for the 5'-end and the 3'-end fragments. P7 contains the initiation codon and P10 contains the stop codon. PCR amplification using P7 and P10 as primers yielded two DNA bands, a faint one (~1.2 kb) and an intense one (~1.0 kb), on an agarose gel after electrophoresis of the PCR product (results not shown). The two DNA bands were subcloned for DNA sequencing. The two sequences are almost identical except that the shorter cDNA has a deletion of a 141-nucleotide stretch. Figure 3 shows the nucleotide sequences of the long (1219 bp) and the short (1078 bp) cDNAs together with their deduced amino acid sequences. The 1219 bp sequence contains one ORF that encodes 368 amino acids with a predicted molecular mass of 41055 Da. The 1078 bp sequence has an

Figure 4 Alignment of the amino acid sequences of human, yeast and *Neurospora* deoxyhypusine synthases

DS.human1 refers to the long 368-amino-acid sequence, and DS.humans refers to the short 321-amino-acid sequence. The amino acid sequence is shown in single-letter code. Gaps (—) were introduced for maximal alignment of the polypeptides. Numbering of amino acids begins with the first residue of the predicted deoxyhypusine synthase sequence. Identical amino acid residues are boxed.

ORF encoding 321 amino acids with a predicted molecular mass of 35750 Da. A stretch of 47 amino acids (residues 261–307) in the long sequence is missing from the 321-amino-acid sequence.

Human deoxyhypusine synthase sequence assembled with homologous ESTs

During the initial phase of this study we could only find one EST (Z25337) homologous to *Neurospora* deoxyhypusine synthase. With the rapid growth of the EST database, the number of homologous EST sequences continues to increase. At present, we have found a total of 23 EST sequences that cover almost 97 % of the entire length of the human deoxyhypusine synthase cDNA sequence (results not shown). This suggests the possibility of using EST information alone to assemble the entire human cDNA sequence simply based on cDNA sequences obtained from other species as diverse as fungi. There are two EST sequences that cover the region where the long (1219 bp) and short (1078 bp) cDNAs differ. The EST sequence R79890 contains the 141 nt region and matches the long cDNA, whereas EST T68101 lacks this 141 nt and matches only the short cDNA. Furthermore, PCR analysis using three different human cDNA libraries (HeLa cells, IMR-90 fibroblasts and B-lymphocytes) shows the presence of both 1.2 kb and 1.0 kb species in all three libraries but with different proportions (results not shown). Taken together, these findings support the notion that there may be two deoxyhypusine synthase transcripts in human cells.

Amino acid sequence analysis of deoxyhypusine synthases from three different species

Figure 4 shows the alignment of amino acid sequences of deoxyhypusine synthases from human (long and short), *Neurospora* and yeast. The overall amino acid sequence identity

Figure 5 Hydropathy profiles of the amino acid sequences deduced from human (long and short), *Neurospora* and yeast deoxyhypusine synthase cDNAs

The residue-specific hydropathy index was determined according to the method of [24] over a window of nine amino acids, using the GCG Wisconsin Package (version 8).

between the human (long) and *Neurospora* deoxyhypusine synthases is 53 %. The identity between the human and yeast enzymes is 55 %. The overall amino acid sequence similarity is 62 % between the human and *Neurospora* enzymes and 68 % between the human and yeast enzymes. The degree of amino acid identity is highest in three regions: residues 97–181, 200–247 and 276–333 (human numbering). For example, the stretch extending from residue 276 to 333 shows 84 % identity and 92 % similarity between the human and *Neurospora* sequences. Compared with the yeast polypeptide, the long human deoxyhypusine synthase lacks two stretches of peptide, one between residues 77 and 92 and the other between residues 198 and 209 (yeast numbering). The predicted amino acid sequences of deoxyhypusine synthase shown in Figure 4 are not related to any other proteins in the GenBank and EMBL Databases.

Hydropathy analysis of deoxyhypusine synthase

Figure 5 shows the hydropathy profiles of human (long and short), *Neurospora* and yeast deoxyhypusine synthases as calculated with a window of nine amino acid residues. The patterns of hydrophobic and hydrophilic residues for all four polypeptides are quite similar and reflect a typical structure for a globular protein where the hydrophobic and hydrophilic

Table 1 Functional expression of recombinant deoxyhypusine synthase in *E. coli*

The deoxyhypusine synthase activity of the recombinant protein was assayed using cell lysates from transformed M15 cells as the source of crude enzyme and either 6xHis-NC21K (*Neurospora*) or 6xHis-h18K (human) as the substrate. The enzyme activity was expressed in units/ μ g of lysate protein. Values are the means $\pm$ S.D. of two assays carried out in duplicate. n.d., enzymic activity not detectable; IPTG, isopropyl β -D-thiogalactopyranoside. Plasmid pQhDS-4 and pQhDS-B8 encode polypeptides of 368 and 321 amino acids respectively.

Transformant	Substrate ...	Specific activity (units/ μ g)	
		6xHis-NC21K	6xHis-h18K
pQhDS-B8		n.d	n.d
pQhDS-B8 + IPTG		n.d	n.d
pQhDS-4		0.10 $\pm$ 0.01	0.20 $\pm$ 0.01
pQhDS-4 + IPTG		1.70 $\pm$ 0.08	5.40 $\pm$ 0.20

regions are clustered separately. The hydropathy profiles do not yield any clue as to how the polypeptide subunits assemble into a tetrameric structure. *Neurospora* deoxyhypusine synthase has been shown to bind tightly to a phenyl-Sepharose column in the presence of high-salt buffer [15]. Similarly, human deoxyhypusine synthase has been shown to bind to hydrophobic columns such as 1,12-diaminododecane-agarose (Y. P. Yan and K. Y. Chen, unpublished work) and butyl-Sepharose [20]. It is possible that the hydrophobic regions identified in Figure 5 may represent patches at the enzyme surface that allow the interaction of the enzyme with hydrophobic resins. Helical-wheel representations of both the *Neurospora* and human enzymes reveal hydrophobic clusters (results not shown).

Functional expression of recombinant deoxyhypusine synthase

Recombinant proteins generated from M15 cells after transfection with plasmid containing either a 1.2 kb insert (pQhDS-4) or a 1.0 kb insert (pQhDS-B8) were tested for deoxyhypusine synthase enzyme activity. The recombinant protein from pQhDS-4-transfected cells exhibited an apparent molecular mass of 40 kDa. Table 1 shows that a high level of deoxyhypusine synthase activity could be detected in the cell lysates of the pQhDS-4 transformant after isopropyl β -D-thiogalactopyranoside induction, indicating that the cDNA indeed encodes deoxyhypusine synthase. The recombinant human enzyme can use both human and *Neurospora* eIF-5A precursor proteins as substrate, but exhibits a 3–5-fold higher specific activity with the human substrate. This result indicates that, despite the highly conserved nature of the eIF-5A precursor protein, species specificity in enzyme–substrate interactions is still obvious. In contrast, the recombinant protein generated from the pQhDS-B8 transformant, with an apparent molecular mass of 35 kDa, exhibited no detectable enzyme activity.

DISCUSSION

Deoxyhypusine synthase is a bifunctional enzyme that catalyses the NAD^+ -dependent oxidative cleavage of spermidine and the subsequent transfer of an aminobutyl moiety to a lysine residue on the eIF-5A precursor [1,2]. We have purified *Neurospora* deoxyhypusine synthase to homogeneity and demonstrated that the enzyme consists of four identical 40 kDa subunits [15]. Similar findings have recently been reported for human [20] and rat testis [21] deoxyhypusine synthases. We have also cloned and functionally expressed the *Neurospora* deoxyhypusine synthase

cDNA [16]. The finding that the conceptual translation of a human EST sequence, Z25337, matches a stretch of the amino acid sequence of *Neurospora* deoxyhypusine synthase (Figure 1) prompted us to test the possibility of using this EST information to clone the human deoxyhypusine synthase cDNA (Figure 2).

Two human deoxyhypusine synthase cDNAs of different sizes were cloned from the human HeLa cDNA library; one encodes 368 amino acids, the other 321 amino acids. The 321-amino-acid sequence was identical to the 368-amino-acid sequence except that it lacked a 47-amino-acid stretch. The recombinant protein generated from the long cDNA (1219 bp) exhibited high deoxyhypusine synthase activity (Table 1), indicating that we have obtained functional human deoxyhypusine synthase cDNA. The human recombinant enzyme shows clear substrate specificity, with greater affinity with human eIF-5A precursor than with *Neurospora* eIF-5A precursor. In contrast, the recombinant protein generated from the short cDNA (1078 bp) gave no detectable enzyme activity (Table 1). Since both the long and short cDNAs appear to be present in at least three different human cDNA libraries, it is likely that these two closely related transcripts exist in human cells. However, initial Northern blot analysis revealed only one mRNA species (~ 1.9 kb) in HeLa cells (Z. P. Chen and K. Y. Chen, unpublished work). It should be noted that if these two transcripts differ by only 141 nt in length, Northern blot analysis using agarose gel electrophoresis will not be able to separate them. Since no deoxyhypusine synthase activity could be detected with the short recombinant protein, this also raises questions as to whether the protein encoded by the short transcript does indeed exist in human cells and, if it does, what its functional role could be. Further studies are needed to address these questions and to elucidate the mechanism of regulation of these two transcripts.

The amino acid sequence analogy among human, *Neurospora* and yeast deoxyhypusine synthases is striking (Figure 4). Of particular note are two stretches of amino acids, one spanning residues 97–193 and the other residues 220–352 (human numbering). The amino acid similarity is 85 % in the first stretch (97–193) and over 90 % in the second stretch (220–352), suggesting that these two stretches may contain highly conserved functional domains. Consistent with the high degree of amino acid similarity, the hydropathy profiles of the four proteins appear to be superimposable (Figure 5).

Although NAD^+ -dependent dehydrogenation of spermidine is the first step in the action of deoxyhypusine synthase [2], the deduced amino acid sequences of human, *Neurospora* and yeast deoxyhypusine synthases fail to show any similarity with other known dehydrogenases. The typical nucleotide binding domain, Gly-Xaa-Gly-Xaa-Xaa-Gly, present in many dehydrogenases [22] is also missing from deoxyhypusine synthase. Deoxyhypusine synthase is very sensitive to thiol reagents [23]. However, NAD^+ can protect the enzyme from the inhibitory action of such reagents [15]. Moreover, we have found that deoxyhypusine synthase can bind to its unmodified substrate only in the presence of NAD^+ [18,23]. These results suggest that critical cysteine residues near or within the active site of deoxyhypusine synthase may be involved in substrate binding. There are four cysteine residues in human and *Neurospora* deoxyhypusine synthases and six in the yeast enzyme. Of these, two (positions 141 and 176 in the long human sequence) are conserved in all three species. In addition, these two cysteine residues are located within the most conserved region in deoxyhypusine synthase (Figure 4). It is therefore tempting to speculate that these two cysteine residues are close to the binding sites for NAD^+ , spermidine and the eIF-5A precursor, and are essential for enzyme activity. Disulphide bond formation involving these two cysteine residues is unlikely,

since dithiothreitol or β -mercaptoethanol is required for enzyme activity *in vitro*.

Note added in proof (received 29 January 1996)

A paper describing the cloning of human deoxyhypusine synthase using an antiserum screening approach has recently appeared [25].

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