

# Molecular characterization of a cDNA encoding functional human deoxyhypusine synthase and chromosomal mapping of the corresponding gene locus

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**Abstract** Deoxyhypusine synthase is essentially required for the post-translational formation of hypusine, a modification of a specific lysine residue in eukaryotic initiation factor 5A, which appears to be pivotal for cell proliferation. From a human peripheral blood mononuclear cells cDNA library we isolated two independent sequences encoding biologically active deoxyhypusine synthase. DNA sequence analysis revealed a 369 amino acid protein with a molecular mass of 41.055 kDa. This recombinant deoxyhypusine synthase showed significant catalytic activity in synthesis of deoxyhypusine after *in vitro* transcription and translation as well as upon expression in *Escherichia coli*. Using a panel of somatic rodent–human cell hybrids we localized the deoxyhypusine synthase gene to human chromosome 19.

**Key words:** Eukaryotic initiation factor 5A; Hypusine formation; Post-translational modification; Chromosomal mapping

## 1. Introduction

The eukaryotic initiation factor 5A (eIF-5A) is the only known cellular protein with a unique hypusine modification (for reviews, see [9,10]). This spermidine-dependent posttranslational modification occurs at the Lys-50 residue of eIF-5A in two enzyme-catalyzed reactions, which involve the transfer of the aminobutyl moiety of spermidine to the  $\epsilon$ -amino group of Lys-50 and subsequent hydroxylation of this intermediate. The hypusine modification appears to be required for biological eIF-5A activity.

It has been suggested that eIF-5A plays a role in the initiation of protein synthesis [9,10]. However, more recent data showed that initiation of protein synthesis in eIF-5A-depleted yeast cells is not significantly affected, indicating that eIF-5A might have essential activities in addition to, or instead of, translation initiation [4]. Interestingly, eIF-5A has recently been identified as a cellular cofactor critically required for the activity of HIV-1 regulatory protein Rev [11], which plays a key role in the com-

plex regulation of HIV-1 gene expression and thus, in the generation of infectious virus particles [1].

Deoxyhypusine synthase catalyzes the first step in the hypusine formation on eIF-5A, whereas the second step is mediated by deoxyhypusine hydroxylase. Recently, we described the purification and characterization of the human deoxyhypusine synthase from HeLa cells on protein level [6].

Here we report the molecular cloning of the corresponding cDNA and the expression of active recombinant human deoxyhypusine synthase. Furthermore, we have localized the gene for the human deoxyhypusine synthase to chromosome 19.

## 2. Materials and methods

### 2.1. Generation of human deoxyhypusine synthase-specific PCR products

Total RNA was isolated from human HeLa cells using guanidinium thiocyanate extraction and CsCl gradient centrifugation [8]. Ten  $\mu$ g of total HeLa RNA was used for first strand cDNA synthesis (Amersham, Aylesbury, Bucks, UK). One-tenth of this reaction mixture was subsequently used for PCR amplification. The sequence of the synthetic primers used to amplify partial human deoxyhypusine synthase transcripts were deduced from primary peptide sequences derived from the human deoxyhypusine synthase isolated from HeLa cells [6]. The PCR primers were designed according to the human codon usage bias [7].

The following PCR primers were generated: *sense* 5'-AAGATGATTGCCCGGCTGGGCAAGGAGATCAAC-3' (from the peptide sequence KMIARLGKEIN), and *antisense*: 5'-GAGGTTGGCATTGGCAATGTGGTGCTT (from the peptide sequence KHHIANANL; [6]). Twenty-five cycles of amplification (30 s at 94°C, 30 s at 55°C and 30 s at 72°C) were performed in a 100  $\mu$ l reaction mixture (10 mM Tris-HCl buffer, pH 8.3, containing 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 200  $\mu$ M dNTP, 1  $\mu$ M sense primer, 1  $\mu$ M antisense primer and 3 units of Taq polymerase (Perkin-Elmer)) covered with 100  $\mu$ l of paraffin. A 273 bp fragment specific for the human deoxyhypusine synthase was amplified, and the correct deoxyhypusine synthase nucleotide sequence has been confirmed by DNA sequence analysis (Sequenase 2.0; United States Biochemicals Corp., Cleveland, OH).

### 2.2. Isolation of human deoxyhypusine synthase and cDNA sequencing

$2 \times 10^5$  independent colonies of a phytohemagglutinin stimulated human peripheral blood mononuclear cells cDNA library cloned into the  $\lambda$ -GT10 vector were screened with the [ $\alpha$ -<sup>32</sup>P]-labeled 273 bp PCR product of human deoxyhypusine synthase. After hybridization, positive clones were sequenced using a Sequenase kit.

### 2.3. Functional expression of *in vitro* transcribed/translated deoxyhypusine synthase

Sequences encoding human deoxyhypusine synthase, or the antisense strand of human deoxyhypusine synthase cDNA, were cloned into the *Eco*RI site of the pcDNA3 vector (Invitrogen Corp., San Diego, CA) to generate the plasmids pSyns and pSynas used for *in vitro* transcription with T7 polymerase. One  $\mu$ g of either pSyns or pSynas were *in vitro* transcribed and translated using the TNT system (Promega, Madison, WI) with either [<sup>35</sup>S]cysteine (Amersham) or non-radioactive cysteine.

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**Abbreviations:** bp, base pair; eIF-5A, eukaryotic initiation factor 5A; RT, reverse transcription; PCR, polymerase chain reaction.

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number U40579.

The radioactive labeled human deoxyhypusine synthase was resolved using 12% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

#### 2.4. Recombinant protein expression

The full-length coding region for the human deoxyhypusine synthase was amplified by PCR using the cDNA clone described in this paper as template with the introduction of a *NcoI* site at the 5' end and a *BamHI* site at the 3' end (sense oligonucleotide: 5'-GGAAT-TCCATATGGAAGGTTCCCTGGAACGG; antisense oligonucleotide: 5'-CGCGGATCCGCTCAGTCCTCGTTCTTCTCATGC). After amplification, the PCR product was digested with *NcoI* and *BamHI*, subcloned into the expression vector pET-11a [12] and transformed into the *E. coli* strain BL21(DE3). An overnight bacterial culture was diluted 1:10 in fresh LB-medium (supplemented with 100 µg/ml ampicillin) and grown for 1 h at 37°C. Subsequently, IPTG was added (0.5 mM) and culture proceeded for a further 4 h. The cells were pelleted and resuspended in 2 ml of PBS containing 1 mM DTT. After sonication (twice for 30 s at a setting of 10 W) the lysate was centrifuged for 2 min at 14,000 rpm in an Eppendorf centrifuge and the pellet dissolved in 1 ml of 8 M urea in H<sub>2</sub>O and dialysed overnight against 0.3 M glycine-NaOH (pH 9.0)/0.1% Tween 20. After an additional centrifugation step (2 min at 14,000 rpm) the supernatant was analysed for deoxyhypusine synthase activity.

#### 2.5. Deoxyhypusine synthase assay

**2.5.1. Small-scale gel filtration assay.** The deoxyhypusine synthase activity assay is based on the incorporation of radioactivity which is transferred from [<sup>3</sup>H]spermidine to the eIF-5A precursor protein [6]. Briefly, the standard reaction mixture contained 6 µM [<sup>3</sup>H]spermidine (previously diluted with non-radioactive spermidine to a specific activity of 360 mCi/mmol), 1 mM NAD<sup>+</sup>, 2 µM recombinant eIF-5A precursor protein (prepared according to [2]) and various amounts of the recombinant human deoxyhypusine synthase in 0.3 glycine-NaOH buffer, pH 9.0, containing 1 mM DTT. Following an incubation for 2 h at 37°C, 300 µl aliquots of the reaction mixture were passed over Sephadex G-25 gel filtration columns (PD-10 columns, Pharmacia, Uppsala, Sweden) in order to separate the labeled eIF-5A from excess spermidine. The incorporated radioactivity was measured in a liquid scintillation counter. [<sup>3</sup>H]spermidine-HCl was obtained from DuPont-NEN (Dreieich, Germany); the unlabeled spermidine from Calbiochem, and NAD<sup>+</sup> from Boehringer-Mannheim (Germany).

**2.5.2. Nitrocellulose membrane assay.** The standard enzymatic reaction mixture (total volume 200 µl) contained 5 µg eIF-5A, 0.5 µCi [<sup>3</sup>H]spermidine (15 Ci/mmol), 1 mM NAD<sup>+</sup>, 1 mM DTT and various quantities of the recombinant human deoxyhypusine synthase in 0.3 M glycine-NaOH buffer, pH 9.0, supplemented with 50 µg/ml BSA. The mixture was incubated at 37°C for 3 h and stopped by adding 100 µl of 20 mM spermidine in PBS. The reaction mixture was then transferred onto a nitrocellulose membrane (Millipore HAWPO2500), which was pre-soaked in 20 mM spermidine/PBS for 30 min, and filtered by applying vacuum using a ten-place filter manifold (Hoefer). After washing with 10 ml, PBS filters were dried for 30 min at 45°C and measured in a liquid scintillation counter.

#### 2.6. RT-PCR and Southern blot

cDNAs generated from HeLa, HMC-1 (mast cell), U937 (monocytic cell), MM6 (monocytic cell), HUT78 (T cell), Jurkat (T cell) and MT4 (T cell) cell lines were amplified by PCR using Taq polymerase (Perkin-Elmer). Following oligonucleotide primer pairs were used: human deoxyhypusine synthase (sense 5'-ATGGAAGGTTCCCTGGAACGG; antisense 5'-GGATGTGGTTCTTCTGGGCC) generating a 688 bp product; human S14 ribosomal protein (sense 5'-GGCAGACCGAG-ATGAATCCTCA; antisense 5'-CAGGTCCAGGGGTCTTGGTCC) amplifying an internal 143 bp control product. An aliquot of the PCR products were size-fractionated on a 1% agarose gels, transferred to Hybond-N filters (Amersham) and hybridized with oligonucleotides specific for human deoxyhypusine synthase (5'-CTGCTCCATCACC-ATCTGATCCAG) and human S14 (5'-CAGGTCCAGGGGTCTTGGTCC). These probes were 3' end labeled using a terminal transferase kit (Boehringer) and [<sup>32</sup>P]dATP prior to hybridization.

#### 2.7. Chromosomal localization

For the chromosomal localization of the human deoxyhypusine syn-

thase gene, we used NIGMS mapping panel 2, obtained from Coriell Institute for Medical Research (Camden, NJ), in combination with Southern blot analysis of restriction enzyme digested hybrid DNAs. DNA (3 µg) from each hybrid as well as from the parental human, murine, and hamster cell lines was digested with the restriction enzyme *NcoI*, separated on a 0.7% (w/v) agarose gel and blotted onto Hybond-N filters (Amersham) in 20 × SSC. Filters were then hybridized with the [<sup>32</sup>P]dATP radiolabelled 273 bp fragment specific for the human deoxyhypusine synthase. After washing, the filters were analysed using a phosphor-imager.

### 3. Results and discussion

RT-PCR was used to generate a deoxyhypusine synthase specific fragment from HeLa cells. A single 273 bp product was obtained by using synthetic oligonucleotide primers, and this fragment was subsequently used to screen a phytohemagglutinin stimulated human peripheral blood mononuclear cell cDNA library. Two positive clones were isolated. The complete nucleotide and deduced amino acid sequence of clone, pdSyn-1, is depicted in Fig. 1. This clone encodes the complete protein sequence (369 amino acids) of the mature human deoxyhypusine synthase. The cDNA insert contains 84 bp of the 5' un-

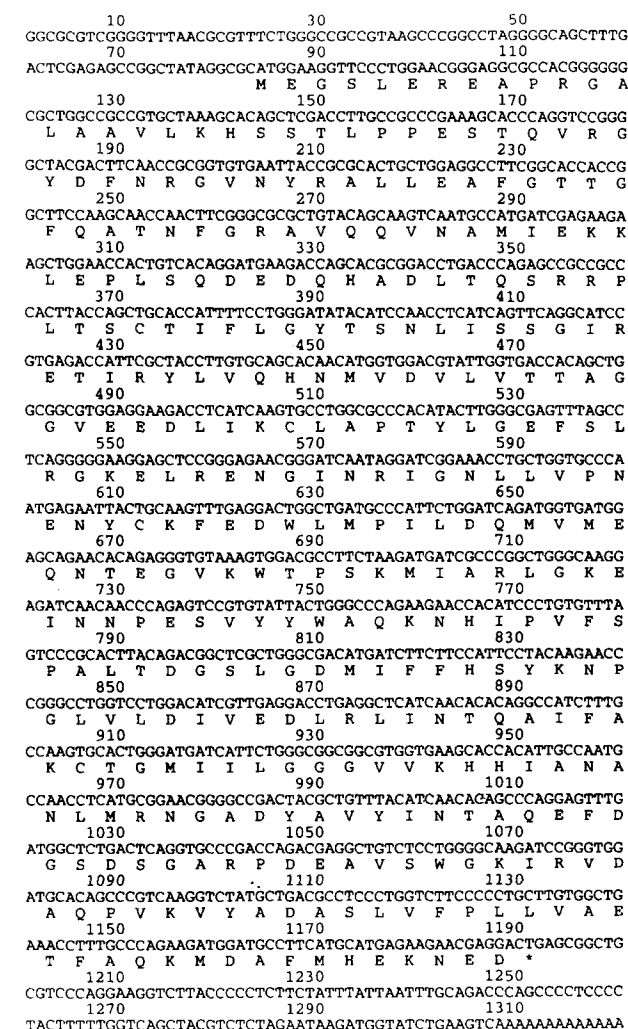


Fig. 1. Nucleotide and predicted amino acid sequence of the cDNA clone pdSyn-1 encoding human deoxyhypusine synthase. The deduced amino acid sequence is shown below the nucleotide sequence. The putative polyadenylation signal is underlined.

translated region and a 114 bp long 3' untranslated region followed by a poly(A) tail. The 3' untranslated region contains a putative polyadenylation signal (AATAA) 93 bp downstream from the stop codon. Comparison of the published protein sequence of the yeast deoxyhypusine synthase [5] with the deduced amino acid sequence from clone pdSyn-1 revealed nearly 60% identity, indicating that the human deoxy-hypusine synthase may have functional similarity with the enzyme present in yeast.

The cell specific synthesis of human deoxyhypusine synthase in a number of human cell lines was investigated by RT-PCR using cDNAs generated from HeLa, HMC-1, U937, MM6, HUT78, Jurkat and MT4 cells. Using PCR primers specific for the human deoxyhypusine synthase a product of 688 bp was amplified from all cell lines (Fig. 2). In order to show the specificity of these amplification products, the DNA was blotted and hybridized with an internal, deoxyhypusine synthase specific oligonucleotide. A specific signal was identified in each of the tested cell lines. The highest transcription rate was identified in HMC-1, U937 and MM6 cells.

Coupled in vitro transcription/translation of the human deoxyhypusine synthase cDNA in presence of [ $^{35}$ S]cysteine resulted in the synthesis of a radiolabeled protein of the expected molecular mass of approx. 41 kDa (Fig. 3A, lane 2). In addition, two signals corresponding to proteins of lower molecular mass were also detectable. These translation products presumably originate from the usage of internal Met-codons within the deoxyhypusine synthase coding region. In contrast, no signal was detectable when the corresponding control antisense sequences were transcribed in this in vitro reaction (Fig. 3A, lane 1).

The reticulocyte extract containing the in vitro translated recombinant human deoxyhypusine synthase possessed deoxyhypusine synthase activity. As shown in Fig. 3B, this activity was dose dependent and reaching a distinct maximum value. In sharp contrast, the reticulocyte extract carrying the human deoxyhypusine synthase antisense construct did not show any significant deoxyhypusine synthase activity.

Human deoxyhypusine synthase was also expressed as a recombinant protein in *E. coli* using the expression vector pET11-

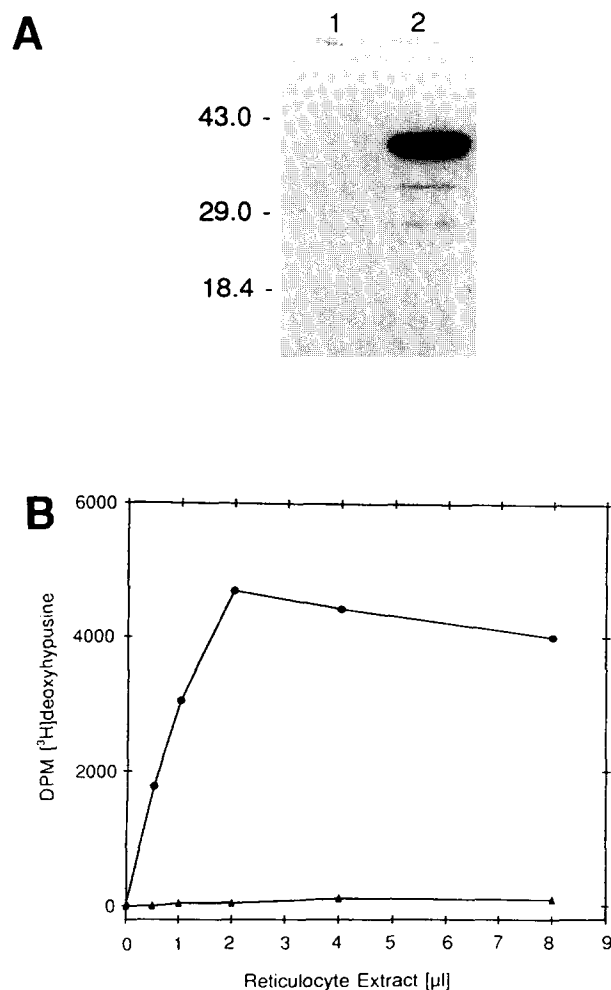


Fig. 3. (A) Autoradiography of reticulocyte extracts expressing anti-sense (lane 1) or sense (lane 2) human deoxyhypusine synthase sequences. The reaction products were separated on 12% SDS-PAGE. The triangle indicates the position of the deoxyhypusine synthase. (B) Biological activity of sense (circles) and antisense (triangles) sequences of human deoxyhypusine synthase after coupled in vitro transcription and translation.

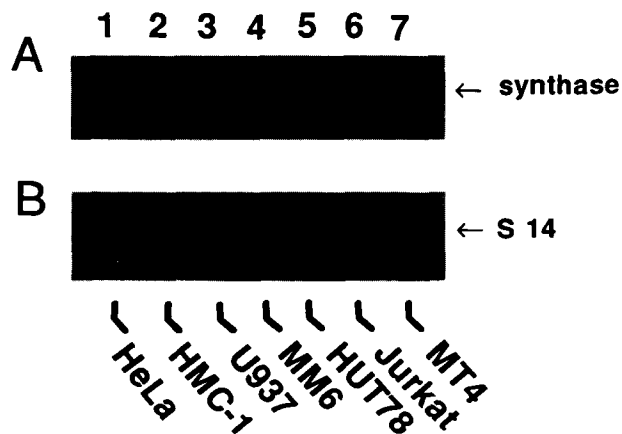


Fig. 2. RT-PCR of total RNA from different human cell lines. (A) Amplified bands were hybridized with a human deoxyhypusine synthase specific oligonucleotide as described in section 2. (B) As an internal loading control, the same blot was also hybridized with an oligonucleotide specific for the human S14 ribosomal protein.

a, as shown in Fig. 4. The recombinant deoxyhypusine synthase, which was prepared from the pellet fraction was >90% pure as judged by 2D gel electrophoresis and subsequent staining with silver nitrate and shows an approx. molecular weight of 41 kDa (not shown). A specific activity of 30,000 units/mg of protein was determined for the recombinant human deoxyhypusine synthase using the small-scale gel filtration assay (one unit is defined as the amount of enzyme forming 1 pmol of product in 2 h at 37°C).

In order to determine the chromosomal localization of the human deoxyhypusine synthase gene, we used a human-rodent somatic cell hybrid mapping panel consisting of 24 single human chromosome hybrids. The parental human, hamster and murine cell lines were digested with the restriction enzyme *Nco*I. After hybridization with the [ $\alpha$ - $^{32}$ P]-labeled 273 bp PCR product of human deoxyhypusine synthase, a unique *Nco*I restriction band was identified for the human specific deoxyhypu-

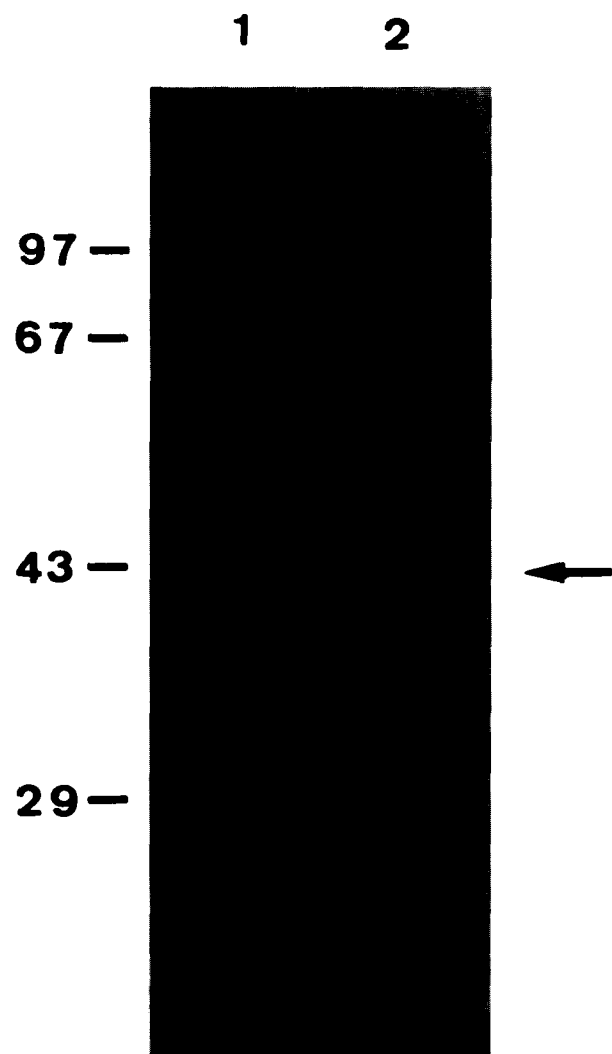


Fig. 4. Recombinant expression of the deoxyhypusine synthase in *E. coli*. Lane 1 shows non-induced bacterial cell lysates. Lane 2 represents the total cell lysate after 4 h induction with IPTG. The arrow indicates the position of the recombinant deoxyhypusine synthase.

sine synthase. This *Nco*I fragment was clearly distinguishable from the hamster and mouse hybridization signals (data not shown). Using the human–rodent mapping panel this human

specific *Nco*I-fragment was only detected in the somatic cell hybrid containing chromosome 19.

The cloning of an active human deoxyhypusine synthase will allow to perform functional as well as structural studies in order to elucidate the mechanism of deoxyhypusine synthesis, and in addition to generate possible inhibitors which could be exploited for the regulation of cell proliferation. Furthermore, the knowledge of the chromosomal localization of the gene coding for the human deoxyhypusine synthase provides a potentially valuable genetic marker, which may be useful for genetic linkage studies.

At the final stage of the completion of this work, another group reported the cDNA cloning of the human deoxyhypusine synthase from the HeLa cell line, showing one amino acid exchange at position 11 [3]. The here-described clone codes for CGG (Arg) at this position, whereas the HeLa sequence encodes GCG (Ala).

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