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Deoxyhypusine synthase gene is essential for cell viability in the yeast *Saccharomyces cerevisiae***

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Abstract Deoxyhypusine synthase catalyzes the first of two steps in the biosynthesis of hypusine, a modification of a specific lysine residue in the precursor of eukaryotic translation initiation factor 5A. We have purified deoxyhypusine synthase from yeast, and cloned and sequenced the corresponding gene encoding a 387-amino acid protein from *Saccharomyces cerevisiae*. Gene disruption experiments indicated that the deoxyhypusine synthase gene is essential for cell growth in yeast. This gene was shown to be an intron-free, single-copy gene, and its product can catalyze the synthesis of deoxyhypusine equally in two precursor forms of eIF-5A, derived from two distinct genes of yeast.

Key words: Deoxyhypusine synthase; Hypusine; Eukaryotic initiation factor 5A; Post-translational modification; Gene disruption

1. Introduction

Deoxyhypusine synthase catalyzes the first of two steps in the biosynthesis of an unusual amino acid, hypusine, found only in eukaryotic translation initiation factor 5A (eIF-5A) [1]. It catalyzes the transfer of the 4-aminobutyl moiety of spermidine to the ε-amino group of a specific lysine residue in an eIF-5A precursor protein in the presence of NAD^+ [2]. This results in the formation of deoxyhypusine (N^{ε}-(4-aminobutyl)lysine) which is subsequently hydroxylated by a second enzyme to form hypusine [3]. Inhibition of deoxyhypusine synthase activity by chemicals [4] or by limiting the level of the substrate spermidine [5] arrests the growth of eukaryotic cells, suggesting that this enzyme may play a pivotal role in cell growth. It has been shown by mutational analysis that preservation of the site of hypusination of eIF-5A (Lys^{51} in the yeast precursor) is vital for the factor's function(s) [6]. Yeast contains two forms of 5A, designated eIF-5Aa and eIF-5Ab, derived from two distinct genes reciprocally regulated by oxygen [6]. It remains to be elucidated whether the two forms of precursor are modified by different forms of deoxyhypusine synthase.

Very recently, based on the in vitro assay method, the heterologous expression of a yeast cDNA clone of the coding region with deoxyhypusine synthase activity in *E. coli* has been reported [7]. However, questions about the in vivo expression, copy number, and functional importance of the deoxyhypusine synthase gene in yeast remain to be elucidated. We have purified deoxyhypusine synthase from yeast. Based on the partial amino acid sequences of the purified enzyme, we report the cloning and sequencing of the full length deoxyhypusine synthase gene from *S. cerevisiae* genomic DNA and show that it is an intron-free single-copy gene and essential for yeast cell growth. The purified recombinant protein expressed from the cloned gene was shown to have the same level of activity as the yeast enzyme.

2. Materials and methods

2.1. Materials

S. cerevisiae strain 131 (MATa/MATα ade2/ade2) [8] was kindly supplied by Dr. Yasuo Hotta (Nara Advanced Institute of Science and Technology). The diploid yeast strain KA31 (MATa/MATα ura3/ura3 leu2/leu2 his3/his3 trp1/trp1), and the plasmids YIplac128 and YCplac22 [9], were generous gifts from Drs. K. Matsumoto and K. Irie of this department. [^{14}C]Spermidine trihydrochloride (*N*-(3-aminopropyl)[1,4-^{14}C]tetramethylene-1,4-diamine trihydrochloride) with a specific activity of 114 mCi/mmol was from Amersham, UK.

2.2. Enzyme assay

Deoxyhypusine synthase activity was measured based on the incorporation of radioactivity as 3.8% PCA precipitate from [^{14}C]spermidine into the eIF-5A precursor protein, produced by expressing the yeast eIF-5A genes in *E. coli*. Typical assay mixtures contained 4 μM [^{14}C]spermidine, 0.5 mM NAD^+, 3 μM ec-eIF-5A, 1 mM DTT, and purified deoxyhypusine synthase in 50 μl of 0.25 M glycine-NaOH buffer, pH 9.5, and were incubated for 30 min at 25°C. Labeled deoxyhypusine was precipitated onto a Whatman 3 MM paper disk by incubation at 90°C in 3.8% PCA for 10 min. The paper disk was washed with 0.2 N HCl, and ethanol, and dried. Radioactivity was measured with a liquid scintillation spectrometer.

2.3. Purification of deoxyhypusine synthase from yeast

Frozen *S. carlsbergensis* cells (~400 g) were ground with quartz sand and extracted with buffer A containing 50 mM Tris-HCl (pH 7.5), 30 mM KCl, 10 mM $MgCl_2$, 10 mM 2-ME, 10% (v/v) glycerol, 1 mM PMSF and 5 μg/ml antipain. After ultracentrifugation of the extract at 100 000 *g* for 4.5 h, the ammonium sulfate precipitate (between 50 and 100% saturation) from the supernatant was subjected to conventional column chromatography using DEAE-Sephacel, DEAE-Toyopearl, and hydroxyapatite columns. Two major peaks with enzyme activity (peak 1, 200–235 mM KCl; peak 2, 275–310 mM KCl) were observed on the DEAE-Sephacel column. Both the activity peaks were purified separately. The final column was an aminobutyl-agarose (Sigma) affinity column equilibrated with buffer B (250 mM glycine-NaOH, pH 9.5, 10 mM KCl, 10 mM 2-ME, 0.1 mM EDTA, 0.1 mM PMSF, 0.5 mM NAD^+ and 25% glycerol). After loading, the column was washed with 5.5 column volumes of buffer B, and eluted with

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Abbreviations: ec-eIF-5A, the precursor of eIF-5A expressed in *E. coli* from yeast eIF-5A genes; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

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buffer A. The peak fractions were combined, concentrated, and stored at −70°C.

2.4. Cloning and sequencing of deoxyhypusine synthase gene

On the basis of partial amino acid sequences of the purified enzyme (see Fig. 1; underlined), the oligonucleotides DYS-A: 5′ CCIGAY-GAYTTYGTIAARGTICARGG 3′, and DYS-B: 5′ CYTCRAAIT-TRCAITARTTITCRTTNGG 3′ were synthesized as 5′- and 3′-end PCR primers, respectively (Y = T or C; R = A or G; N = A, G, T or C; I = inosine). Using *S. cerevisiae* genomic DNA as template, PCR was carried out which produced a single DNA fragment (DYS-AB) of ~470 base-pairs corresponding to the region from nucleotide 70 to 550, in Fig. 1. A single 3.7-kb band appeared when DYS-AB was used to probe a Southern blot of *Eco*RI-digested total genomic DNA from *S. cerevisiae* strain 131. This DNA band was eluted and ligated into the *Eco*RI site of pUC19. About 3000 colonies of *E. coli* transformed with this plasmid containing the insert were replicated onto nitrocellulose filters, and colony hybridization was performed with the probe DYS-AB as described [10]. One of ten positive clones, referred to pE724-7, was subjected to sequencing on both strands of the insert. All the following PCR products were confirmed by sequencing on both strands.

2.5. Gene disruption and complementation

A 621 bp long DNA fragment (starting from position 1) was prepared by PCR using DYS1 cloned in pE724-7. This DNA fragment, DYS-621, contained the *Kpn*I site at position 291, and was used as an internal fragment for integrative disruption of DYS1 as described [11]. DYS-621 was cleaved with *Eco*RI and *Hin*dIII (built in the primers, respectively) and inserted into YIplac128 (LEU2). Leu$^-$ diploid yeast strain KA31 was transformed [12] with this ~4.9-kb plasmid linearized by digestion with *Kpn*I (Fig. 2B, a). For complementation, the resulting heterozygous diploid RAKA31 (DYS1/dys1::LEU2) was transformed with the plasmid pRA1 which was constructed by ligation of the 3.7-kb *Eco*RI fragment of pE724-7 carrying the full length DYS1 gene into the vector YCplac22 (TRP1).

2.6. Construction of a plasmid expressing the DYS1 gene and purification of deoxyhypusine synthase expressed in E. coli

A DNA fragment from pE724-7, containing DYS1 from +1 to +1352, was amplified by PCR. The PCR product was digested at the *Nde*I and *Bam*HI sites (built in the 5′- and 3′-end primers, respectively), inserted into the vector pET-3a and used to transform *E. coli* BL21 (DE3) cells. The transformants were grown in LB medium containing 50 μg/ml ampicillin and induced with 0.5 mM IPTG at an A_{600} of 0.6. The incubation was continued at 37°C for 2 h. The cells (~10 g) were harvested by centrifugation, washed with and suspended in 50 ml of buffer A. After sonication (3 times for 30 s at an interval of 1 min at 60–70 W setting), the disrupted cells were centrifuged at 50000 rpm for 4.5 h (Beckman TLA 100.3 rotor). The recombinant enzyme was purified to homogeneity using only two columns, DEAE-Toyopearl and aminobutyl-agarose, as described in section 2.3.

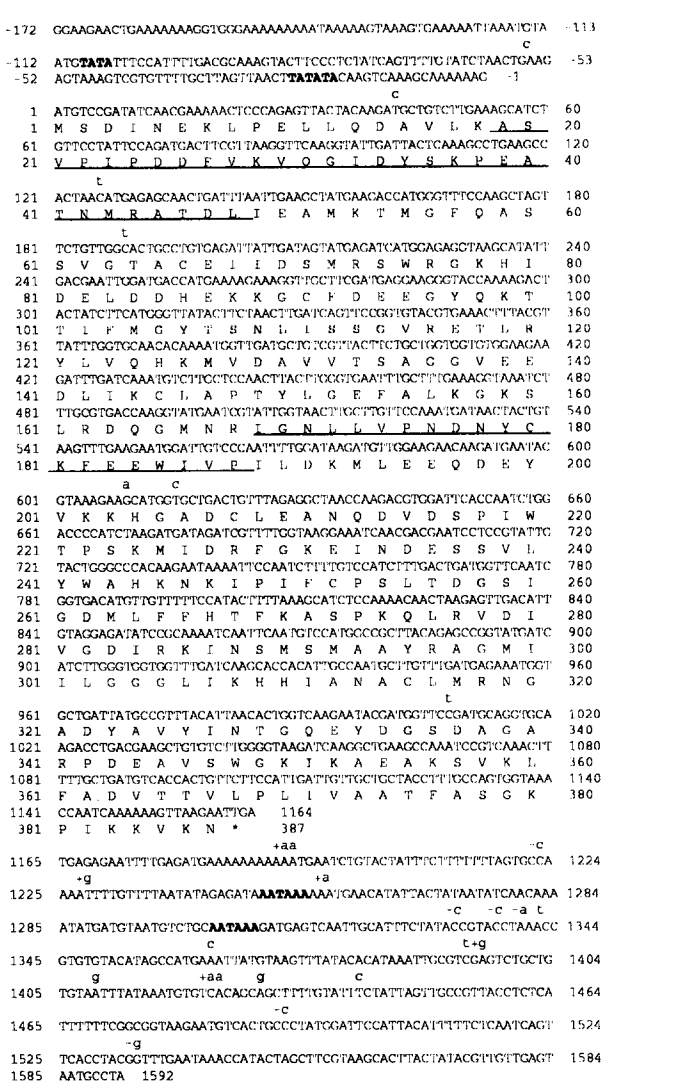

Fig. 1. Nucleotide and deduced amino acid sequences of the DYS1 gene of *S. cerevisiae*. Sequences of tryptic peptides of the purified yeast enzyme that matched with the predicted amino acid sequences of the cloned gene are underlined. The TATA and poly(A) elements are in bold letters. The A of initiator codon is numbered as +1. The nucleotides of YHR068w that mismatch with that of DYS1 are shown in lowercase; + precedes the extra nucleotides found and − precedes the nucleotides not found in YHR068w.

3. Results

3.1. Purification of deoxyhypusine synthase from yeast

Deoxyhypusine synthase was purified from yeast to homogeneity. Enzyme purified from each of the two peaks eluted from DEAE-Sephacel was of same molecular weight and activity. The cause of these two peaks is not known. The partial amino acid sequences were determined using the enzyme purified from the first peak, as indicated in Fig. 1 (underlined). The specific activity after purification was increased ~14000 fold over that of the crude extract. The purified yeast enzyme gave a single band with a molecular weight of 43000 Da when analyzed by SDS-PAGE (Fig. 3A, lane 4).

3.2. Nucleotide sequence of yeast deoxyhypusine synthase gene

A 3.7 kb *Eco*RI fragment of *S. cerevisiae* genomic DNA containing the deoxyhypusine synthase gene was cloned in the plasmid pE724-7. The nucleotide sequence of 1.76 kb of the insert DNA was determined, and an intron-free open reading frame (ORF) was deduced as shown in Fig. 1. The ORF encodes a protein of 387 amino acids with a calculated molecular weight of 42899 Da which coincides well with that (43 kDa) of the purified enzyme from yeast and the protein overexpressed from the cloned gene in *E. coli* (see Fig. 3A, lanes 4,3). The yeast gene was designated DYS1 (deoxyhypusine synthase).

The 5′-untranslated region (UTR) contains two putative TATA elements at positions −109 and −24. In the 3′-UTR of the gene, two potential poly(A) addition signals (AATAAA) were observed at 85 and 135 nucleotides downstream of the termination codon (TGA), each followed by the polyadenylation site Py(A)n [13] (CAAAA at position 1281–1285 and TAAA at position 1339–1342) located 26 and 30 nucleotides downstream of the poly(A) signals, respectively.

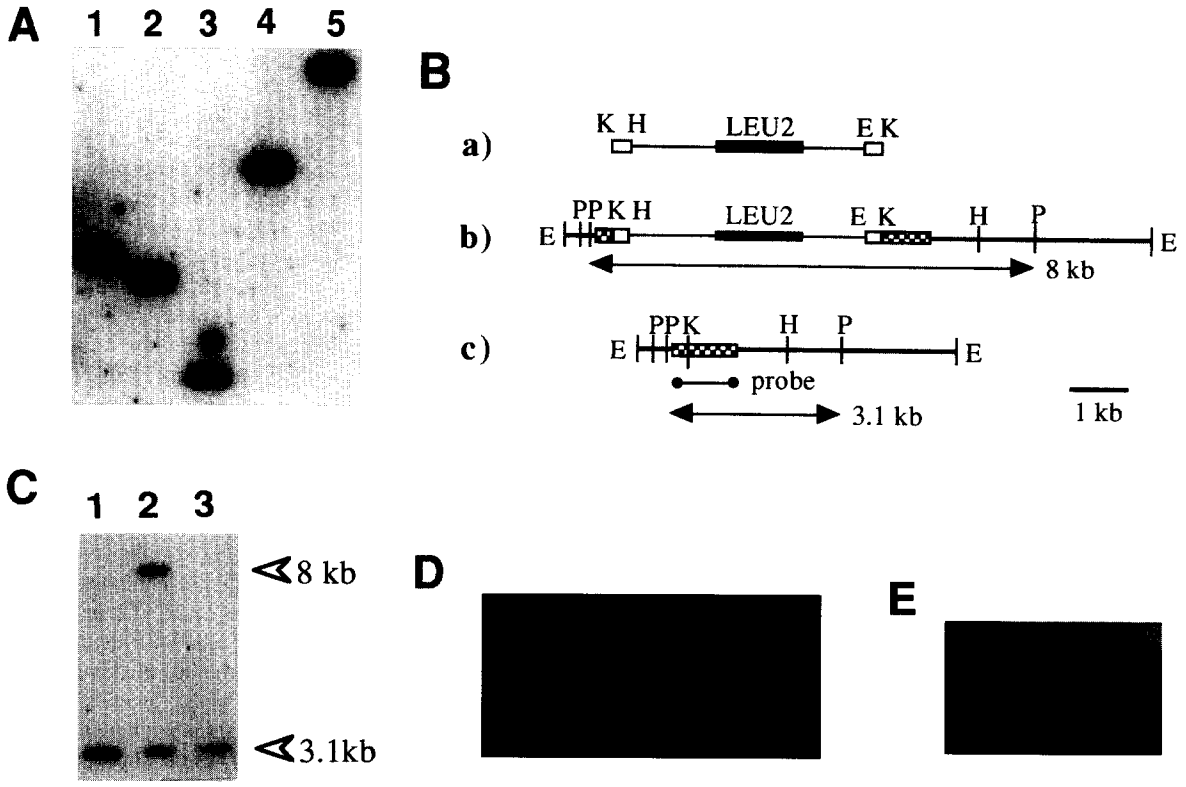

Fig. 2. DYS1 is a single-copy and essential gene. (A) Genomic DNA from strain KA31 was digested with various restriction enzymes and probed with ^{32}P-labeled DYS1. Lanes: 1, *Pst*I; 2, *Eco*RI-*Hin*dIII; 3, *Kpn*I-*Hin*dIII; 4, *Eco*RI; 5, *Bam*HI. (B) Illustration of integrative disruption (white boxes, plasmid-borne coding region of DYS1; stippled boxes, coding region of chromosomal DYS1; thick line, chromosomal DNA; thin line, plasmid DNA): (a) 4.9 kb linearized integrative plasmid YIplac128 carrying 0.3 kb DYS1 fragment at each end; (b) the disrupted dys1 with LEU2 marker; and (c) restriction map of chromosomal DYS1. E, *Eco*RI; P, *Pst*I; K, *Kpn*I; H, *Hin*dIII. (C) Southern blot: *Pst*I digested total DNA from parental diploid KA31 (lane 1), transformed Leu$^+$ diploid RAKA31(lane 2), and Leu$^-$ haploid (lane 3). (D,E) tetrad dissections: after sporulation of a transformed diploid, asci were dissected. Spores from a single ascus were aligned vertically and allowed to geminate at 30°C for 3–5 days. (D) RAKA31 (DYS1/dys1::LEU2); (E) RAKA31 carrying pRA1 (DYS1).

3.3. *DYS1 is a single-copy and essential gene*

A single band of the expected size was observed when yeast genomic DNA was digested with *Pst*I, *Eco*RI, *Bam*HI, and a combination of *Eco*RI and *Hin*dIII enzymes, and probed with the coding region of DYS1 (Fig. 2A). Unexpectedly, digestion with *Kpn*I (having its restriction site in the coding region) combined with *Hin*dIII (Fig. 2A, lane 3) gave a single band. This may be due to the absence of any *Kpn*I and the second *Hin*dIII sites outside the coding region in the close vicinity of the DYS1 gene. These findings indicated that the DYS1 gene is a single-copy gene in yeast.

The essentiality of the DYS1 gene for yeast growth was examined by gene disruption and its rescue with the normal gene as shown in Fig. 2. A Leu$^+$ diploid strain RAKA31, heterozygous at the DYS1 locus, was obtained (section 2). It carried normal DYS1 (panel c) on one allele, and the disrupted dys1::LEU2 (panel b) formed by homologous recombination at the *Kpn*I site of the chromosomal gene on the other allele. Gene disruption was confirmed by southern hybridization as shown in Fig. 2C (lane 2). Upon sporulation, tetrads were dissected (Fig. 2D). Only two of the four spores of each tetrad were viable (7 out of 9 dissected asci) and all viable spores were Leu$^-$, indicating that the disruption of DYS1 gene is lethal in haploid. Furthermore, RAKA31 (DYS1/dys1::LEU2) was transformed with the centromeric plasmid pRA1 (section 2). One of the Leu$^+$Trp$^+$ transformants was allowed to sporulate for tetrad analysis (Fig. 2E). All the viable spores were either Leu$^+$Trp$^+$ or Leu$^-$Trp$^+$, indicating that the disrupted DYS1 on a chromosome was rescued by an intact DYS1 on pRA1. No Leu$^+$Trp$^-$ spores were obtained. The Leu$^+$Trp$^+$ haploid cells were stable for many generations. These results confirm that the cloned DYS1 is expressed in yeast and the gene is essential for yeast cell viability.

3.4. *The cloned DYS1 gene can produce highly active deoxyhypusine synthase*

In an attempt to identify the protein product from the cloned DYS1 gene, the DNA clone was expressed in *E. coli* using pET-3a plasmid. As illustrated in Fig. 3A, SDS-PAGE analysis showed that a 43-kDa protein was overexpressed in IPTG-induced cells (amounting to 15–20% of the total soluble protein in the extracts, lane 1) and was subsequently isolated as a single band using two chromatographic steps based on deoxyhypusine synthase activity (lanes 2,3). A polyclonal antibody raised against the recombinant enzyme detected a 43-kDa protein as a single band in yeast cell extracts (data not shown). The recombinant enzyme exhibited the same kinetics as the purified yeast enzyme, and both enzymes equally catalyzed the formation of deoxyhypusine with a specific activity of 250 pmol of product/μg of enzyme per 30 min at 25°C, as shown in Fig. 3B. Similar values were obtained using either one of the two precursors, ec-eIF-5Aa and ec-eIF-5Ab, as an acceptor of the aminobutyl group.

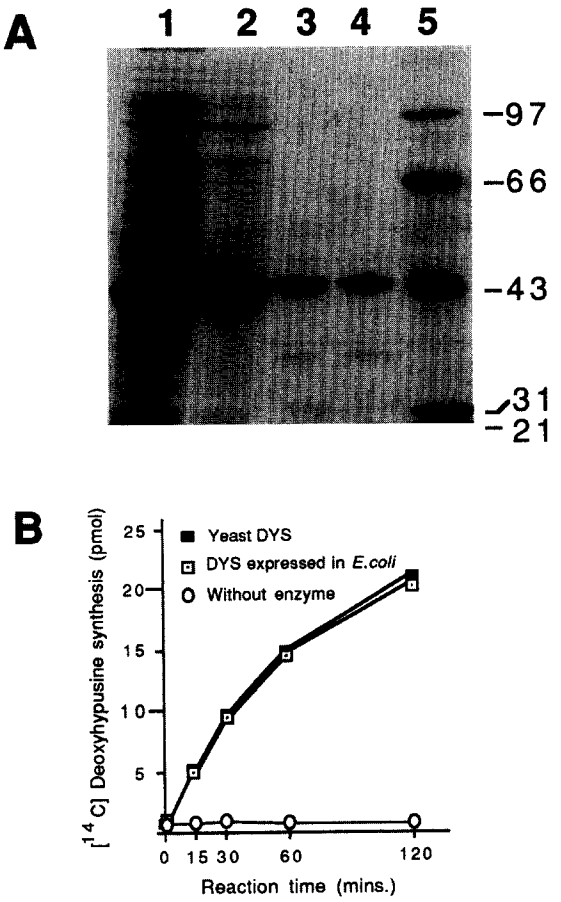

Fig. 3. Purification and kinetics of the recombinant deoxyhypusine synthase expressed in *E. coli*. (A) SDS-PAGE (10% gel). Lanes: 1, extract of *E. coli* expressing recombinant enzyme; 2, an activity peak fraction of the recombinant enzyme on DEAE-Toyopearl column; 3, a pooled fraction of the enzyme eluted from aminobutyl agarose column; 4, deoxyhypusine synthase purified from yeast; 5, molecular weight markers (kDa). (B) Kinetics of synthesis of deoxyhypusine from [^{14}C]spermidine in yeast ec-eIF-5Aa. The amount of the enzyme used in each reaction was 40 ng, either purified from yeast or from *E. coli* (recombinant). DYS, deoxyhypusine synthase.

4. Discussion

We have described here for the first time the purification of deoxyhypusine synthase from yeast. Based on the partial amino acid sequences of the tryptic peptides obtained from the enzyme, we have cloned the corresponding gene. A genomic DNA clone of *S. cerevisiae* encoding a protein of 387 amino acid with deoxyhypusine synthase activity was obtained and characterized. While this manuscript was in preparation, the purification of deoxyhypusine synthases from diverse species, e.g. *Neurospora* [14], rat [15] and human [16], and amino acid sequences of several peptides were reported. Very recently, the region YHR068w on chromosome VIII of *S. cerevisiae* [17] was indicated as a gene for a yeast deoxyhypusine synthase by cloning a cDNA obtained from PCR of the coding region, based on its (YHR068w) homology to deoxyhypusine synthases from other organisms [7,16] and the heterologous expression of its activity in *E. coli* [7]. However, they provided no in vivo evidence to indicate that this YHR068w region expressed the active enzyme within yeast cell. Our amino acid sequence of DYS1 is identical with that of YHR068w except for 6 nucleotide changes in the coding region (all of them at third base of the codons) and 18 nucleotide changes outside the ORF, as shown in Fig. 1 (lowercase letters). These discrepancies might be due to differences in the strains used.

Having a genomic clone of deoxyhypusine synthase at hand allowed us to determine the copy number of the gene in yeast. The cloned gene (3.7 kb) with its 5′- and 3′-UTR enabled us to perform the complementation experiment by expression of the gene from its native promoter(s) in a null haploid yeast strain. In this study we have identified DYS1 as an intron-free single-copy gene in the yeast *S. cerevisiae* and provided compelling evidence that a functional copy of this gene is required for cell proliferation. To our knowledge, this is the first direct evidence showing the in vivo essentiality of deoxyhypusine synthase gene for cell viability and its copy number in any eukaryotic cell. These findings indicate that in yeast there are no alternative means for deoxyhypusination at least in the wild-type background. According to this study, protein expressed from the single-copy DYS1 gene appears to modify either one of the two precursors of eIF-5A expressed in aerobically or anaerobically grown cells with equal efficiency.

The availability of the recombinant enzyme and the ability to manipulate this gene using yeast genetics should provide powerful tools for future studies on the molecular mechanism of the biosynthesis of deoxyhypusine and the controlled expression of this essential gene.

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