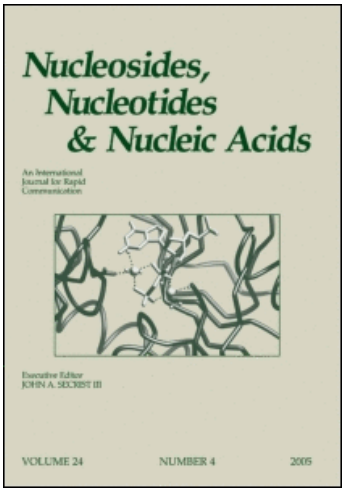

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Oligonucleotide Directed Mutagenesis of the E. coli hisS Gene: Introduction of an NcoI Restriction Site at the Initiating GTG and Cloning of the Mutated Gene to the Expression Vector pKK 233-2

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OLIGONUCLEOTIDE DIRECTED MUTAGENESIS OF THE E. coli hisS GENE: INTRODUCTION OF AN NcoI RESTRICTION SITE AT THE INITIATING GTG AND CLONING OF THE MUTATED GENE TO THE EXPRESSION VECTOR pKK 233-2

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Abstract: Site directed mutagenesis of the E.coli hisS gene with a double mismatch primer changed the initiation codon GTG to ATG and introduced an NcoI restriction site at the start codon. The promoter-deleted structural gene was cloned to the expression vector pKK 233-2.

The Escherichia coli hisS gene codes for the histidyl-tRNA synthetase which very accurately aminoacylates $tRNA^{His}$ in the course of protein biosynthesis (1). The enzyme is composed of two identical subunits (α_2) and has a molecular weight of 94.000 Daltons. The hisS locus was cloned and the nucleotide sequence of the coding region determined (2). The translated polypeptide sequence is comprised of 424 amino acid residues. The sequence alignment with other kown primary structures of aminoacyl-tRNA synthetase did not show any significant homologies.

We report here the site directed mutagenesis of the hisS gene and the cloning to the expression vector pKK233-2 (3) as a basis for the overproduction of the enzyme.

MATERIALS AND METHODS

The single stranded phage DNA M13pTU901, harboring a 1.7 kb BamHI/SalI insert of the hisS gene (4) served as template for the mutagenesis. Expression vector pKK233-3 was purchased from Pharmacia (Freiburg, F.R.G.). TG-1 and W3110 cells (5,6) served as hosts for the plasmids. Restriction enzymes NcoI, NciI and HindIII were purchased from New

England Biolabs (Beverly, MA, USA). DNA-polymeraseI (Klenow-fragment), T4 DNA ligase and exonuclease III were a gift of Dr. Eckstein (this department). The mutagenesis was performed according to the procedure developed by Eckstein and coworkers (7,8). The mismatch primer was synthesized on a DNA-synthesizer (SAM-ONE, Biosearch, New Brunswick, NJ, USA) using phosphite chemistry. The primer was 5'-phosporylated and annealed to the single strand DNA of pTU901 in equimolar amounts (3 pmol). In vitro polymerisation of the second strand was carried out in the presence of dCTPαS (0.4 mM). After ligation (30 U T4 DNA ligase) the resulting circular closed double stranded DNA (ds-DNA, RF IV) was separated from the single-stranded DNA (ss-DNA) by nitrocelluose filtration. The RF IV DNA (2 pmol) was incubated with NciI (5 U), the nicked (+)-strand digested by exonuclease III for 5 min and the resulting gap filled up by repolymerisation using dNTPs. All following steps were carried out using standard procedures (9). The ds-DNA was used directly for the transfection of competent TG-1 cells. Recombinant ds- and ss-phage DNA was isolated and analysed by digestion with NcoI and HindIII. The hisS fragment was isolated by preparative agarose gel electrophoresis and ligated to the NcoI/HindIII digested expression vector pKK233-2 in equimolar amounts (0.12 pmol). The recombined plasmid pGE87 was transformed to competent E. coli W3110 cells. pGE87 was isolated from W3110:pGE87, digested with NcoI and HindIII and the fragments analysed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

As a basis for the overexpression of the E. coli histidyl-tRNA synthetase we performed site directed mutagenesis of the hisS gene. The vector pTU901, a derivative of the ss-DNA phage vector M13mp9, which carries the (-)strand of the hisS gene, served as template for the mutagenesis. The rational for chosing pTU901 is, that it provides a NciI restriction site suitable for the nicking step step of the mutagenesis procedure about 738 base pairs upstream from the start codon of the gene.

Oligonucleotide Directed Mutagenesis

The procedure of Eckstein and coworkers (7,8) was used to generate a NcoI restriction site at the start codon of the hisS gene. A 18mer mismatch-primer of the sequence GAG AAT AAC CAT GGC AAA with two nucleotides noncomplementary to the start region of the hisS gene at positions 9 (C-T) and 11 (A-C) was synthesized by automatic solid phase synthesis.

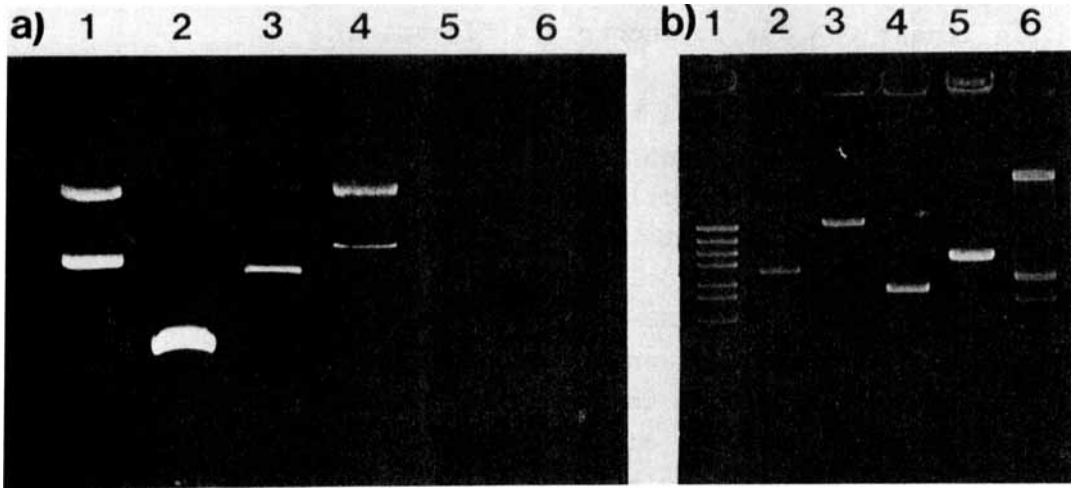

FIGURE 1: 1% agarose gel analysis of a) the steps of the site directed mutagenesis (lane 1: ds-RFII and RFIV DNA of pTU901; lane 2: single stranded DNA of pTU901; lane 3: pTU901 RFIV DNA after mismatch primer insertion; lane 4: pTU901 after nicking; lane 5: gapped pTU901 after exonuclease digestion; lane 6 pTU901 after repolymerisation) and b) of the plasmids constructed (lane 1 and 6: MW standards λBstEII and λEcoRI/HindIII; lane 2: pTU901; lane 3: NcoI linearised mutated pTU901; lane 4: NcoI linearised pKK233-2; lane 5: NcoI linearised pGE87.

The primer was 5'-phosphorylated and annealed to the ss-DNA of pTU901 (FIG. 1a, lane 1 ds-DNA of pTU901 (RFII and RFIV), lane 2 ss-DNA). In the polymersisation of the second strand dCTPαS was used beside the three other dNTPs (7,8). After ligation the RF IV DNA (FIG. 1a, lane 3) was separated from the ss-DNA. Upon incubation with NciI the RFIV DNA was only nicked in the template (+)strand because the phosphorothioate linkages of the newly synthesized (-) strand of the ds-DNA efficiently protects the DNA from linearisation by NciI although not completely (FIG. 1a,lane 4).

After nicking roughly 750 base pairs were removed from the (+)strand by exonuclease III to pass the mutation locus (FIG. 1a, lane 5). Repolymerasation of the ss-gap and

ligation led to RFIV DNA (FIG. 1a, lane 6, the upper band is linear DNA due to incomplete digestion).

Cloning of the Mutated hisS into Expression Vector pKK233-2

The double stranded DNA with the desired mismatches in both strands was directly transformed to competent TG-1 cells. Restriction site analysis (NcoI) of plasmids isolated from ten independent colonies yielded two plasmids with the desired mutations (FIG. 1b, lane 3). The mutations were further confirmed by Sanger sequencing (data not shown). The promoter deleted 1.55 kb NcoI/HindIII hisS fragment was subcloned to pKK233-2 digested with the same enzymes (FIG. 1b, lane 4). The translation initiation codon ATG of hisS is now located within the unique NcoI restriction site of pKK233-2 (FIG. 1b, lane 5) at an appropiate distance from the ribosome binding site.

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