Journal of Biochemistry and Molecular Biology, Vol. 34, No. 6, November 2001, pp. 502-508

JOURNAL OF
Biochemistry
and
Molecular Biology

A Rapid and Simple Method for Construction and Expression of a Synthetic Human Growth Hormone Gene in *Escherichia coli*

Sittiruk Roytrakul[†,‡], Lily Eurwilaichitr[†,‡,*], Chittiwat Suprasongsin[§] and Sakol Panyim[‡]

[†]National Center for Genetic Engineering and Biotechnology (BIOTEC), Rama 6 Rd, Bangkok 10400, Thailand

[‡]BIOTEC Training Center for Genetic Engineering and Biotechnology at Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhonpathom 73170, Thailand

[§]Ramathibodi Hospital, Rama 6, Bangkok 10400, Thailand

Received 8 May 2001, Accepted 31 July 2001

A cDNA, encoding the human growth hormone (hGH), was synthesized based on the known 191 amino acid sequence. Its codon usage was optimized for a high level expression in *Escherichia coli*. Unique restriction sites were incorporated throughout the gene to facilitate mutagenesis in further studies. To minimize an initiation translation problem, a 624-bp cassette that contained a ribosome binding site and a start codon were fused to the hGH-coding sequence that was flanked between the *Eco*RI and *Hind*III sites. The whole fragment was synthesized by an overlapped extension of eight long synthetic oligonucleotides. The four-short duplexes of DNA, which were first formed by annealing and filling-in with a Klenow fragment, were assembled to form a complete hGH gene. The hGH was cloned and expressed successfully using a pET17b plasmid that contained the T7 promoter. Recombinant hGH yielded as much as 20% of the total cellular proteins. However, the majority of the protein was in the form of insoluble inclusion bodies. N-terminal amino acid sequencing also showed that the hGH produced in *E. coli* contained formyl-methionine. This study provides a useful model for synthesis of the gene of interest and production of recombinant proteins in *E. coli*.

Keywords: Codon usage, *E. coli*, Human growth hormone, Synthetic gene, T7 promoter

Introduction

The human growth hormone (hGH) is a pituitary-derived polypeptide with a wide range of biological functions, including protein synthesis, cell proliferation, and metabolism (Kystyo and Isaksson, 1977). The hormone consists of 191 amino acid residues, which fold into a four-helix bundle structure with two disulfide bridges (De Vos *et al.*, 1992). Due to the variety of biological activities, the growth hormone has therapeutic applications in the treatment of dwarfism, bone fractures, skin burns, and bleeding ulcers. Since hGH is a non-glycosylated protein, prokaryotic expression systems, such as *Escherichia coli*, are preferred in the production of recombinant hGH (Goeddel *et al.*, 1979; Chang *et al.*, 1987; Kato *et al.*, 1987).

Synthetic genes are becoming increasingly popular for the production of proteins (Engels and Uhlmann, 1989). Desirable features of a synthetic gene include the use of codons that are optimal for expression in the desired host (Makoff *et al.*,1989; Hernan *et al.*, 1992), the silent removal of DNA sequences that effect transcription or translation (Romanos *et al.*, 1991), and the introduction of unique restriction sites (Climie and Santi, 1990). Several methods exist for the assembly of synthetic constructs (Ikehara *et al.*, 1984; Engels and Uhlmann 1989). hGH was the first that was chemically synthesized by sequentially ligating 78 gene fragments, totaling 726 bases (Ikehara *et al.*, 1984). Here, the design, construction, and assembly of the PCR product from a few oligonucleotides, based on the amino acid sequence of the human growth hormone, are described. We used a method that begins with the chemical synthesis of overlapping oligonucleotides from alternating strands of the gene (Dillon and Rosen, 1990). A primer extension of these overlapping oligonucleotides was then performed. The method does not rely on DNA ligase, but instead employs DNA polymerase to build increasingly longer DNA fragments during the assembly process. This was followed by a PCR amplification that produced a full-length sequence, which was subsequently trimmed with restriction endonucleases and cloned into suitable vectors. The synthetic hGH facilitates a more rapid generation of large numbers of site-directed mutagenesis by

*To whom correspondence should be addressed.
Tel: 66(2)4419003-7 (x1266); Fax: 66(2)4419906
E-mail: lily@biotec.or.th

the simple replacement of small restriction fragments with synthetic DNA duplexes that carry the desired mutations. This approach is made possible by the addition of more than 30 unique restriction sites within the hGH coding sequence. Expression of the synthetic hGH and the optimized procedure for the culture system in *E. coli* are also presented.

Materials and Methods

All restriction endonucleases and DNA modifying enzymes were from Boehringer Mannheim, Promega, or New England Biolabs and were used according to the manufacturers protocols. All other chemicals were obtained from BDH or Sigma.

The bacterial strains used in this study were: DH5α (F-f80*dlac*ZDM15D(*lac*ZYA-argF) U169 *en*dA1 *rec*A1 *hs*dR17 (r K-m K+) *deo*R *thi*-1 *sup*E44 l-*gyr*A96 *rel*A1), purchased from Life Technology Inc., Gaithersburg, MD., BL21 (DE3) (F'*hsd*S_B(r_B^-, M_B^-) ompT gal(λcIts857) *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene1), BL21(DE3) pLysS (F'*hsd*S_B(r_B^-, M_B^-) ompT gal(λcIts857) *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene1 pLysS plasmid), and pET17b (*bla* gene, T7 promoter and terminator, pBR322 origin) were from Novagen Madison, WI. pUC18 (containing the *bla* gene, *lacZ* gene, and M13mp19) was obtained from New England Biolabs.

E. coli DH5a was used for construction of all of the plasmids. The BL21(DE3) and BL21(DE3)pLysS strains were used in the expression that was driven by the T7 promoter.

All of the DNA manipulations and electrophoresis procedures were carried out according to Maniatis *et al.* (1982).

hGH gene design and assembly The sequence of the synthetic hGH cassette is shown in Fig. 1. The synthetic hGH, used in this study, was designed based on the published sequence of the synthetic hGH (Ohtsuka *et al.*,1983). Our synthetic hGH was different from that in the database. Ours consisted of codons preferred by *E. coli*, and more than thirty unique restriction sites were introduced throughout the synthetic fragment. A 5' untranslated region, containing a purine-rich Shine-Dalgarno (SD) sequence for ribosome binding upstream from a translation start codon in order to ensure efficient translation initiation, was also included in the fragment. Stop codons for three different reading frames were placed upstream of a hGH start codon to prevent a possible fusion protein. Unique *Eco*RI and *Hin*dIII sites were added

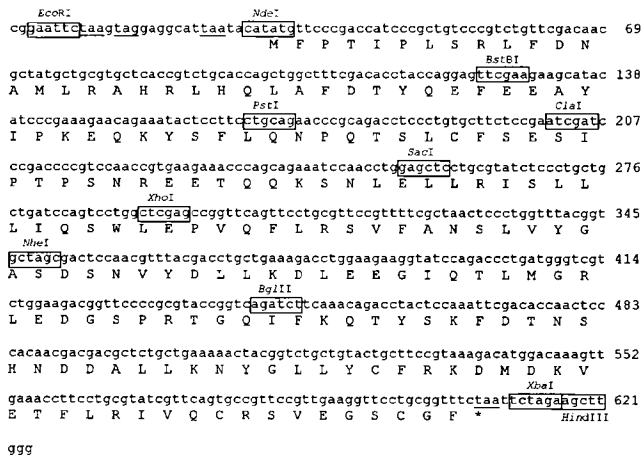

Fig. 1. The nucleotide sequence and its deduced amino acids of the synthetics human growth hormone (hGH) gene. Restriction sites unique to this hGH molecule are boxed. Stop codons are underlined.

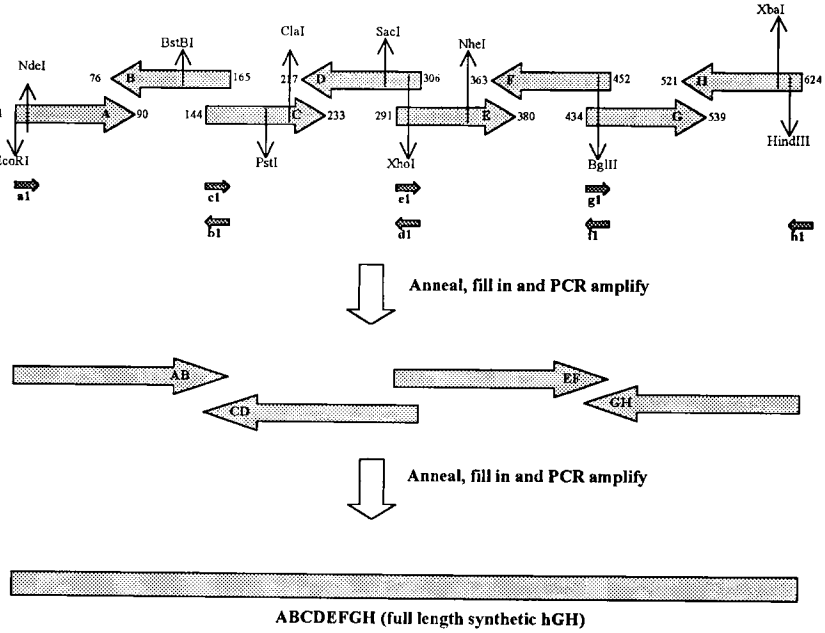

Fig. 2. Schematic representation of the gene synthesis process. Each overlapping segment (duplex) was annealed, extended, and amplified by PCR using the specific primer pairs as shown. Finally, the four short duplexes were assembled, extended to give a full-length hGH gene, and finally amplified by PCR using a1 and h1 as the flanking primers.

Table 1. A list of the oligonucleotides used in this study

No	Name	Nucleotide sequence	Length (bp)	Tm (°C)
1	Fragment A	5'-ggaattctaagtaggaggcattaatacatatgttcccgaccatcccgctgtcccgtctgttcgacaacg ctatgctgcgtgctcaccgt-3'	90	-
2	Fragment B	3'-gacgcacgagtggcagacgtggtcgaccgaaagctgtggatggtcctcaagcttcttcgtatgtag ggctttcttgtctttatgaggaag-5'	90	-
3	Fragment C	5'-gaaagaacagaaatactccttcctgcagaacccgcagacctccctgtgcttctccgaatcgatccc gaccccgtccaaccgtgagaaac-3'	90	-
4	Fragment D	3'-aggttggcacttctttgggtcgtctttaggttggacctcgaggacgcatagagggacgacgactagg tcaggaccgagctcggcaagtc-5'	90	-
5	Fragment E	5'-gctcgagccggttcagttcctgcgttccgttttcgctaactccctggtttacggtgctagcgactccaa cgtttacgacctgcgaaaga-3'	90	-
6	Fragment F	3'-aatgctggacgactttctggaccttcttccataggtctgggactacccagcagaccttctgccaagg ggcgcatggccagtctgaagtt-5'	90	-
7	Fragment G	5'-gtaccggtcagatcttcaaacagacctactccaaattcgacaccaactcccacaacgacgacgct ctgctgaaaaactacggttgctgtactgcttccgtaaag-3'	105	-
8	Fragment H	3'-acatgacgaaggcatttctgtacctgtttcactttggaaggacgcatagcaagtcacggcaaggcaa cttccaaggacgccaaagattaagatcttcgaaccc-5'	104	-
9	Primer a1	5'-aattctaagtaggaggc-3'	19	51.63
10	Primer b1	3'-cttgtctttatgaggaag-5'	20	47.69
11	Primer c1	5'-gaaagaacagaaatactcct-3'	24	56.47
12	Primer d1	3'-cgagctcggcaagtc-5'	16	59.10
13	Primer e1	5'-gctcgagccggttca-3'	16	59.10
14	Primer f1	3'-cgcatggccagtctgaagtt-5'	20	57.01
15	Primer g1	5'-gtaccggtcagatcttca-3'	20	55.20
16	Primer h1	3'-aagattaagatcttcgaaccc-5'	22	54.87

at the 5' and 3' termini, respectively, of the coding strand. The nucleotide sequences were submitted to GenBank. The accession number is AF359458.

To construct the synthetic hGH gene, eight separate overlapping oligonucleotide fragments were designed (Fig. 2). The oligonucleotides that were used, their melting temperatures, and lengths are shown in Table 1. All of the oligonucleotides were ordered from GenSet Singapore Biotech. Pte Ltd. The gene was initially constructed as four segments (AB, CD, EF, GH) (Fig. 2). To fill in the overlapping oligonucleotides, DNA polymerization was carried out using the Klenow fragment of *E. coli* DNA polymerase. To anneal the overlapping oligonucleotide pairs, each of the overlapping oligonucleotides (100 ng/µl each) were mixed in pairs (i.e., AB; CD; EF; and GH) in the following conditions. A reaction mixture of 25 µl that contained 100 ng of each oligonucleotide, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 1.25 µM each dNTP, was heated to 94°C for 5 min and then allowed to cool slowly to 37°C in order to anneal and form duplexes. The extension reaction was initiated by the addition of 7 units of Klenow fragment, then further incubated at 37°C for 30 min. PCR amplification was performed to amplify the segments using segment specific primer pairs: a1b1, c1d1, e1f1, g1h1. For amplification, 30 cycles (94°C, 30 sec; 50°C, 1 min; 72°C, 1 min) were carried out using *Taq* Polymerase.

All of the gene segments (AB; CD; EF; GH) were heated to 94°C for 5 min and slowly cooled down to 37°C. The single-stranded gaps between the overlapped oligonucleotides (ABCDEFGH) were thus produced. The complete coding sequence for hGH was filled in by *Taq* Polymerase. The final construct was subjected to PCR amplification again using a1 and h1 as the flanking primers. The reaction mixture without polymerase was heated to 94°C for 5 min, then cooled down to 80°C. *Taq* polymerase was added, followed by 30 cycles of 94°C (1 min), 50°C (1 min), 72°C (1 min), and further incubated at 72°C for 10 min. The PCR product of the expected length (624 bp) was digested with *Eco*RI and *Hin*dIII. The digested band was purified by electrophoresis and eluted from a 2% (w/v) agarose gel using a Qiaquick kit (Qiagen). The *Eco*RI/*Hin*dIII digested 624-bp hGH gene was then ligated into pUC18 using a Rapid DNA ligation kit (Boehringer Mannheim). One-tenth of the volume of the ligation reaction mixture was used for transformation into the *E. coli* DH5α strain. Transformed cells were selected by plating and incubating on a medium that contained 100 µg/ml ampicillin. Transformants that contained the correctly constructed DNA fragments were identified by double digestion with *Eco*RI and *Hin*dIII. The hGH synthetic insert was verified by double-stranded plasmid sequencing with the PRISM™ Ready Reaction *Taq* DyeDeoxy Termination Cycler Sequencing kit (PE Applied Biosystems). The transformant carrying the right sequence was selected and named pUChGH231.

Construction of an *E. coli* expression plasmid The pEThGH231 was constructed to obtain a high-level expression of recombinant hGH under the T7 promoter. The first 30-bp sequences that contained the ribosome binding site and stop codons for three different reading frames were omitted. The *Nde*I /*Hin*dIII fragment of the synthetic gene was ligated to a pET17b plasmid that resulted in a pEThGH231 plasmid.

Gene expression *Escherichia coli* BL21(DE3) and BL21(DE3) pLysS cells were transformed with the pEThGH231, then selected at 37°C on LB-plates that contained 100 μg/ml ampicillin. For protein expressions, BL21(DE3) and BL21(DE3) pLysS cultures were grown at 25°C to approximately OD_{600} = 0.5-0.6 in either the LB broth (5 g/l NaCl, 10 g/l bactopeptone and 5g/l yeast extract) or the M9 medium (200 ml/l M9 salt (64 g/l $Na_2HPO_4 \cdot 7H_2O$, 15 g/l KH_2PO_4, 2.5 g/l NaCl and 5 g/l NH_4Cl), 2 ml/l of 1 M $MgSO_4$, 20% (w/v) glucose and 1 M $CaCl_2$). The *lac*UV5 promoter was induced by the addition of IPTG to a final concentration of 1 mM.

Automated DNA sequencing The ABI PRISM™ Sequencing Kit was used to prepare a sample for a sequencing analysis on the ABI PRISM 377 DNA Sequencer. Principles of this kit are based on using fluorescent-labeled terminator cycle sequencing (Smith *et al.*, 1982).

The PCR reaction was performed in a total volume of 20 μl using 8 μl of Terminator Premix (A-,T-,C- and G-dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCl pH 9.0, $MgCl_2$, thermal stable pyrophosphatase and Amplitaq DNA polymerase), 500 ng of a plasmid DNA template, 0.5 μM of a universal primer for pUC18, or a T_7 initiator and terminator primer for a T_7 promoter plasmid. The reaction mixture was performed in an automated thermal cycler for 25 cycles (Perkin Elmer Cetus Model 2400) with the following conditions: denaturing temperature at 96°C for 10 s, annealing temperature at 50°C for 5 s, and extension temperature at 60°C for 4 min. After completion of the PCR reaction, the PCR product was recovered by the phenol-chloroform extraction method. The dye-labeled-PCR product was analyzed on the ABI PRISM 377 DNA Sequencer.

Electrophoresis SDS/PAGE was carried out on 1-mm-thick 12% acrylamide slab gels according to Laemmli (1970). Standard molecular marker kits were purchased from BioRad and New England Biolabs.

Determination of protein concentration The amount of hGH was estimated from the electrophoresis band, comparing the total cellular protein from the integration of all bands using a scanning densitometer.

Fractionation of recombinant hGH protein A method was developed, based on published procedures, to separate the periplasmic proteins, soluble- and insoluble-intracellular proteins from the *E. coli* cells (French *et al.*, 1996). Cultured *E. coli* cells were collected by low speed centrifugation. The cell pellet was resuspended in a fractionation buffer that contained 500 μg/ml lysozyme, 200 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 20% (w/v) sucrose. The cell suspension was incubated for 15 min at room temperature before an equal volume of cold water was added. The suspension was incubated for a further 15 min before recovery of the periplasmic fraction by centrifugation. The cell pellet was then resuspended in a STET buffer (50 mM Tris-HCl, pH 8.0, 8% sucrose, 50 mM EDTA, 0.1% Triton X 100). After incubation at room temperature for 15 min, the cells were then disrupted with a sonicator and the clear cell lysate was centrifuged at 10,000 rpm for 15 min. The pellet was washed twice with a STET buffer and used as the insoluble fraction (inclusion bodies). The supernatant was used as the soluble fraction.

N-terminal peptide sequencing Immobilization of the protein for N-terminal sequencing was carried out following the method of Matsudiara (Matsudiara, 1987) with some modifications. The protein samples were resolved in SDS-PAGE and then transferred to a Problott™ membrane (PVDF membrane) by using a semi-dry blot apparatus (BioRad, USA). Electroblotting was carried out in a buffer that contained 10 mM of 3-(cyclohexylamine)-1-propanesufonic acid, pH 11.0, and 10% (v/v) methanol at a constant current of 100 V, 140 mA at 4°C for 1 h. The transblotted membrane was detected by staining with 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) methanol and 1% (v/v) acetic acid for 1 min. It was subsequently destained in 30% (v/v) methanol for 10 min. The membrane was briefly rinsed with de-ionized water, dried, and stored at −20°C until it was used. The protein bands of interest were excised from the membrane and further analyzed by N-terminal sequencing using an Applied Biosystem model ABI492 automated protein sequencer.

Results and Discussion

Synthesis of hGH and its expression in *E. coli*. In this study, a synthetic human growth hormone gene was designed based on a published sequence. This synthetic gene was constructed from eight oligonucleotides that ranged between 90-105 nucleotides in length, with complementary overlaps of 15-22 nucleotides. The gene was initially assembled in four short segments-the AB, CD, EF, and GH segments. The short duplexes were annealed, filled in, and amplified to produce the complete gene that encoded hGH using the PCR technique (Fig. 2). The PCR product was cloned into a pUC18 plasmid, pUChGH231. The correct nucleotide sequences of the synthetic hGH insert were verified by DNA sequencing. The expression vector, pEThGH231, was constructed by fusing the *NdeI*/*Hind*III fragment of the synthetic hGH from pUChGH231 to the pET17b vector.

High levels of expression were achieved with pEThGH231, in which the hGH gene was inserted immediately downstream of the T7 promoter. Different strains of *E. coli.* (e.g., BL21 (DE3) and BL21(DE3)pLysS) were transformed with the pEThGH231 plasmid. Upon induction of the cultures that contained pEThGH231, a protein that migrates at about 22 kDa on a SDS-PAGE gel was observed (Fig. 3).

Because the expression level, or the amount of protein product, depends on the duration of the induction time, all cultures were collected at 3 and 24 h in order to determine the expression level and protein stability. Intracellular accumulation of 22-kDa hGH occurred at 3 h after induction and increased rapidly. The expression level of hGH, estimated from the densitometric scanning of a Coomassie-blue stained gel, was about 15-20% of the total cellular proteins in BL21(DE3) and BL21(DE3)pLysS cells (Fig. 3). This indicates that the T7 promoter-expression system was highly efficient. Recombinant hGH was stably expressed in the BL21(DE3) and BL21(DE3)pLysS cells at high levels, even after 24 h of induction. However, the transformant pEThGH231/BL21(DE3) that was grown in non-induced

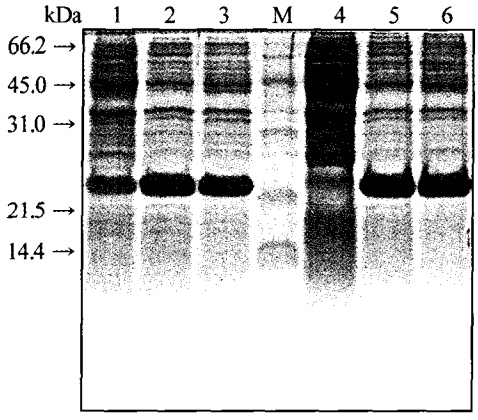

Fig. 3. SDS PAGE analysis of crude extract from either uninduced or induced *E. coli* cell harboring recombinant hGH (pEThGH231). Lane 1 was from the non-induced BL21(DE3)/pEThGH231 grown for 24 h. Lanes 2-3 were from BL21(DE3)/pEThGH231 induced for 3 and 24 h. Lane M was a protein molecular weight marker. Lane 4 was from non-induced BL21(DE3)pLysS/pEThGH231 grown for 24 h. Lanes 5-6 were from BL21(DE3)pLysS/pEThGH231 induced for 3 and 24 h.

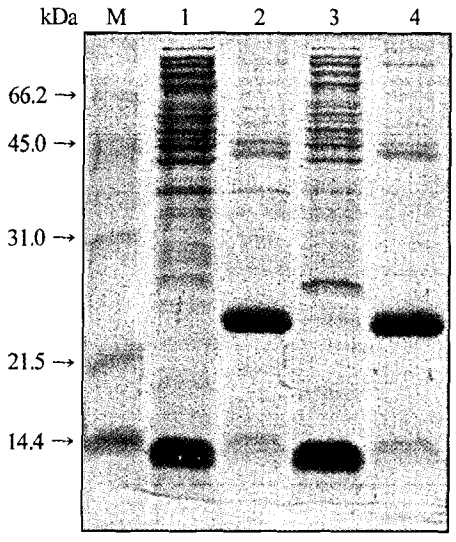

Fig. 4. SDS PAGE analysis of soluble and insoluble proteins extracted from the induced *E. coli* cells harboring recombinant hGH (pEThHG231). Lane M was a protein standard marker. Lanes 1-2 were soluble and insoluble fraction, respectively, from the 24 h-induced BL21(DE3)/pEThGH231 cell. Lanes 3-4 were soluble and insoluble fractions, respectively, from the 24 h-induced BL21(DE3)pLysS/pEThGH231 cell.

conditions (LB medium at 25°C) also produced as much hGH protein as that in the induced conditions. This suggests that the T7 promoter was leaky. It was, therefore, important to vary the induction conditions in order to obtain the optimal amount of hGH.

Physico-chemical properties of the expressed protein Following resolution on SDS-PAGE, the hGH protein was transferred to a PVDF membrane. The isolated protein band was subjected to Edman degradation in order to determine the amino-terminal sequence. The sequences of the first 6 amino acids at the N-terminus of this protein from either BL21(DE3) or BL21(DE3)pLysS cells were M-F-P-T-I-P. This is one disadvantage of direct expression of heterologous protein in *E. coli*, as it synthesized the protein that is initiated with formylmethionine (fMet). It is possible that overproduction of hGH in *E. coli* yielded a product that was incompletely deformylated, as was reported in other recombinant protein that were expressed in the bacterial host (Warren *et al.*, 1996). Several proteins were poor substrates for peptide deformylase (PDF), and exhibited incomplete deformylation, particularly when they were overproduced.

The hGH that was obtained from this study contained methionine as the N-terminal amino acid. Most of the hGH protein accumulated as an insoluble form in the *E. coli* cells. It is possible that the intracellularly produced methionyl hGH might affect biological activities because of this additional residue, since incorrect folding was observed previously in a similar case of methionyl 22-kDa hGH. It was also reported that the methionyl residue at the N-terminus of the methionyl human growth hormone may play a role in antibody formation in patients that are treated with the hormone (Glasbrenner, 1986). However, it was shown previously (Kostyo *et al.*, 1985) that the methionyl-hGH has diabetogenic and insulin-like activities. In order to obtain the mature soluble hGH, an alternative approach may be to employ other host systems.

Effect of nutrient and temperature on solubility of recombinant hGH Fractionation of the cell extract revealed that the protein was localized mainly in the insoluble fraction (Fig. 4). Although being expressed in an inclusion body provides some advantages (including facile isolation of the protein in high purity and concentration), it results in a significant barrier to gene expression in the cytosol. These problems are further tasks of refolding the aggregated protein. It is uncertain whether the refolded protein retained its biological activity and the reduction in yield of the refolded and purified protein (Rudolph and Bennett, 1982)

Therefore, attempts were carried out to increase the proportion of soluble hGH relative to the insoluble form by reducing the level of protein expression. The expressed solubility changes may be due to nutrient starvation and induction at low temperatures, which leads to a lowering of specific growth rates, hence concomitant reduced rates of foreign protein production. It was reported that metabolic pools in *E. coli* are under strict physiological control (Tweeddale *et al.*, 1998). The protein synthesis rate and its solubility may respond to nutrient limitation. Here, changing either the media content (LB or M9), or harvesting after a longer post-induction period in 4°C (3 or 24 h) or both, were tested. The M9 medium, using glucose as a carbon source, was also tested. The result showed (Table 2) that the highest

Table 2. Expression of hGH in *E. coli* containing pEThGH231, when induced at 4×C in either a LB or M9 medium.

E. coli strains	Soluble hGH produced in LB (% total cellular protein)		Soluble hGH produced in M9 (% total cellular protein)	
	3 h post-induction	24 h post-induction	3 h post-induction	24 h post- induction
BL21(DE3)	6.4	9.4	6.1	6.6
BL21(DE3)pLysS	14.9	8.8	8.8	19.7

level of soluble hGH was obtained from the BL21(DE3) pLysS cells that were grown in a M9 medium (19.7% of total cellular protein). However, an Edman degradation analysis showed that the sequence of the N-terminal amino acids of the soluble hGH remained M-F-P-T-I-P. Therefore, downstream removal of the f-Met from the recombinant hGH is needed.

This study is a useful model for the synthesis and expression of genes of interest in *E. coli*. The expression of synthetic hGH (estimated by using densitometric scanning) that was obtained from our study was as high as 0.5 g/l. A previous method for synthesis of hGH involved the ligation of 78 gene fragments using DNA ligase with an expression level of approximately 168.7 mg/l (Ikehara *et al.*, 1984). Our procedures provide an easier and more rapid way to achieve production of the desired proteins with reliable results. More restriction sites can be easily added for a further site-directed mutagenesis study. It also allows codon usage to be designed in order to favor the host expression system. Therefore, this system is not only limited to *E. coli*, it can be successfully applied to eukaryotic host cells, such as *Pichia pastoris* (Eurwilaichitr L, Roytrakul S., Suprasongsin C, and Panyim S., submitted manuscript).

Acknowledgments We would like to thank Dr. Albert Ketterman and Dr. Kusol Pootanakit for their critical reading of this manuscript. We also thank Ms. Aruchalean Wattanachai for her technical assistance. This work was supported by the Thailand National Center for Genetic Engineering and Biotechnology (BIOTEC) at the National Science and Technology Development Agency (NSTDA).

References

Chang, N. C., Rey, M., Bocher, B., Heynecker, H. and Grey, G. (1987) High-level secretion of human growth hormone by *Escherichia coli*. *Gene* **55**, 189-196.

Climie, S. A. and Santi, D. V. (1990) Chemical synthesis of the thymidylate synthase gene. *Proc. Natl. Acad. Sci.* **87**, 633-637.

De Vos, A. M., Ultsch, M. and Kossiakoff, A. A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306-312.

Dillon, P. J. and Rosen C. A. (1990). A rapid method for the construction of synthetic genes using the polymerase chain reaction. *Biotechniques* **9**, 298.

Engels, J. W. and Uhlmann, E. (1989) Gene synthesis. Angrew. *Chem. Int. Ed. Engl.* **28**, 716-734.

French, C., Keshavarz-Moore, E. and Ward, J. M. (1996) Development of a simple method for the recovery of recombinant proteins from the *Escherichia coli* periplasm. *Enz. Microb. Tech.* **19**, 332-338.

Goeddel, D. V., Heynecker, H. L. and Hozumi, T. (1979) Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature* **281**, 544-548.

Glasbrenner, K. (1986) Technology spurt resolves growth hormone problem, ends shortage, *J. Ann. Med. Assoc.* **255**, 581-582.

Hernan, R. A., Hui, H. L., Andracki, M. E., Noble, R. W., Sligar, S. G., Walder, J. A. and Walder, R. Y. (1992) Human hemoglobin expression in *Escherichia coli*: importance of optimal codon usage. *Biochemistry* **31**, 8619-8628.

Ikehara, M., Ohtsuka, E., Tokunagam, T., Taniyama, Y., Iwai, S., Kitano, K., Miyamoto, S., Ohgi, T., Sakuragawa, Y., Fujiyama, K., Ikari, T., Kobayashi, M., Miyake, T., Shibahara, S., Ono, A., Ueda, T., Tanaka, T., Baba, H., Miki, T., Sakurai, A., Oishi, T., Chisaka, O. and Matsubara, K. (1984) Synthesis of a gene for human growth hormone and its expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81(19)**, 5956-60.

Kato, C., Kobayashi, T. and Kudo, T. (1987) Construction of an excretion vector and extracellular production of human growth hormone from *Escherichia coli*. *Gene* **54**, 197-202.

Kostyo, J. L., Cameron, C. M., Olson, K. C., Jones, A. J. S. and Pai, R. C. (1985) Biosynthetic 20-kilodalton methionyl-human growth hormone has diabetogenic and insulin-like activities. *Proc. Natl. Acad. Sci. USA* **82(12)**, 4250-4253.

Kystyo, J. L. and Isaksson, O. (1977) Growth hormone and the regulation of somatic growth. *Int. Rev. Physiol.* **13**, 255-274.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Makoff, A. J., Oxer, M. D., Romanos, M. A., Fairweather, N. F., and Ballantine, S. (1989) Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. *Nucleic Acid Res.* **17**, 10191-10202.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matsudiara P. (1987) Sequence from picomole quantities of protein electroblotted onto polyvinyllidone difluoride membrane, *J. Biol. Chem.* **267**, 10035-10038

Ohtsuka, E., Taniyama, Y. O., Iwai, S., Kitano, K., Miyamoto, S., Ohgi, T., Sakuragawa, Y., Fujiyama, K., Ikari, T., Kobayashi, M., Miyake, T., Shibahara, S., Ono, A., Ueda, T., Tanaka, T., Baba, H., Miki, T., Sakurai, A., Oishi, T., Chisaka, O. and Matsubara, K. (1983) The synthesis of human growth hormone gene. *Nucleic Acids Symp. Ser.* **12**, 79-82.

Romanos, M. A., Makoff, A. J., Fairweather, N. F., Beesley, K. M., Slater, D. E., Rayment, F. B., Payne, M. M. and Clare, J. J. (1991) Expression of tetanus toxin fragment C in yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucleic Acid Res.* **19**, 1461-1467.

Rudolph, R. and Lilie, H. (1996) *In vitro* folding of inclusion body proteins *FASEB J.* **10**, 49-56

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H. and Hood, L. E. (1986) Fluorescence detection in automated DNA sequence analysis. *Nature*. **321**, 674-679.

Tweeddale, H., Notley-McRobb, L. and Ferenci, T. (1998) Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool (metabolome) analysis. *J. Bacteriol.* **180**, 5109-5116.

Warren, W. C., Bentle, K. A., Schlitter, M. R., Schwane, A. C., ONeil, J. P. and Bogosian, G. (1996) Increased production of peptide deformylase eliminates retention of formylmethionine in bovine somatotropin overproduced in *Escherichia coli*. *Gene* **174**, 235-238.