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RT-PCR MEDIATED CLONING OF HUMAN GROWTH HORMONE GENE AND ITS EXPRESSION IN *E. coli*

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ABSTRACT

Human growth hormone (hGH) genomic sequence containing 5 exons and 4 introns was cloned in pcDNA-3 and the constructed plasmid was subsequently used for transfection of NIH-3T3 cell line using lipofection technique. Expression of hGH in stably transfected cells was assayed using ELISA. Total RNA was extracted from transfected cells and hGH cDNA was amplified by RT-PCR using specific primers. The product thus obtained was cloned in pGEM-T vector and the presence of the growth hormone coding region was verified by restriction enzyme analysis and Southern blotting. The hGH cDNA fragment was cloned in pQE-30 and the expression of hGH gene in *E. coli* was assayed using ELISA and immunoblotting. In this experiment 20.9 µg/mL purified rhGH was obtained.

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INTRODUCTION

The human growth hormone (hGH) gene consists of 5 exons and 4 introns and is located on the long arm of chromosome 17.[1] The gene is fully characterized and sequenced and encodes the classic pituitary growth hormone in the form of a prohormone which is proteolytically cleaved into the active form.[2] Differential processing of the hGH gene prohormone product leads mostly to two forms of hGH with a molecular weight of 20 and 22 K. The 20K form contains 176 amino acids and the 22 K form consists of 191 amino acids with two disulfide bonds.[3] The human growth hormone gene in its native form also contains a leader sequence encoding for a 26 amino acid peptide.

Growth hormone stimulates bone growth, but its biological role is not restricted to the stimulation of longitudinal bone growth alone. It is also believed that among various metabolic effects, GH might act as an immunomodulatory substance.[4] Complete growth hormone deficiency occurs in about one in 4000 individuals. Congenital and acquired defects of hypothalamic or pituitary origin can lead to growth hormone deficiency which is characterized by short stature, decreased skeletal muscle mass with an increase in body fat and a pathological growth rate. Treatment of growth hormone deficiency consists of daily injections of growth hormone.[5]

In view of the need for pure hormone, several strategies have been employed to obtain recombinant human growth hormone (rhGH) using both eukaryotic and prokaryotic hosts. For this purpose, several plasmids such as pUC-18, pcDNA3 and PQE-30 were used. Since human growth hormone is a non-glycosylated protein, *E. coli* is the most widely used host for the production of rhGH.[6-9] In the present study, cloning and expression of hGH in *E. coli* was attempted.

MATERIALS AND METHODS

Plasmids, bacteria and cell line

Plasmid containing genomic human growth hormone gene (pHSA2000GH) was a gift from Dr. George E.O. Muscat (Center for Molecular and Cellular Biology, University of Queensland, Australia). pUC18 was purchased

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from Pharmacia Biotech (Sweden), pcDNA-3 and pQE-30 were kindly provided by Dr. F. Kashanchi, Molecular Virology Lab., NIH, USA, and pGEM-T was purchased from Promega (U.K.). *E. coli* strains HB101 and JM109 were used as hosts for cloning of the constructed plasmids and obtained from Gibco BRL (U.K.). NIH-3T3 cellline (a murine fibroblast cell line) was a gift from Dr. Michel Goosens (Department of Biochemistry and Genetics, University of Paris XII, France).

Enzymes and primers

All restriction enzymes (BgIII, HindIII, BamHl, EcoRl) used were from Gibco BRL (U.K.) and PCR primers with the following sequences were synthesized in the Biotechnology Department, Pasteur Institute of Iran (Tehran, Iran). Forward primer was 5′-GGG CGG ATC CTT CCC AAC CAT TC-3′ and Reverse primer 5′-ACC CAA GCT TCT AGA AGC CAC AGC-3′.

Plasmid construction

pHSA2000GH[10] plasmid containing genomic sequence of hGH was used for this study (Fig. 1A). A 2.2 kb BamH1-EcoR1 fragment of hGH was excised from pHS A2000GH and inserted at the same sites in pUC18 and pUC-GH was thus obtained (Fig. 1B). This plasmid was used for hGH gene sequencing using universal primers. hGH gene as a 2.2 kb fragment was cleaved from pUC-GH with BamH1 and EcoR1 and recloned in pcDNA3 plasmid in corresponding sites and pcDNA3-GH was constructed (Fig. 1C).

pGEM-GH was obtained by cloning the RT-PCR product, a 0.6 kb fragment of hGH cDNA in pGEM-T vector (Fig. 2A). This plasmid was cut with BamH1, HindIII and 0.6 kb hGH cDNA was cleaved. This fragment was cloned in PQE-30, and PQE-GH containing hGH cDNA was constructed (Fig. 2B).

Lipofection

NIH-3T3 cells[11,12] were cultured in 35 mm tissue culture plates until the monolayers were 50-80% confluent. Each plate was washed with serum-free RPMI 1640 medium and the liposome-DNA complex was prepared by gently mixing 100 µL of RPMI 1640 containing 24 µg (12 µL of 2 mg/mL) lipofectamin[13] (Gibco BRL, U.K.). The mixture was incubated at room temperature for 50 min and after the addition of 800 µL serum free RPMI 1640 to the DNA-liposome complex, the solution was added to each plate and incubated for 5 h. Following incubation, 1 mL RPMI 1640 medium containing 20% fetal calf serum (FCS) was added to the plates without removing the transfection mixture. Twenty-four hours later, the medium was replaced with fresh RPMI 1640 containing 10% FCS and 400 µg/µL neomycin.[14] After 3 weeks from the start of the experiment, sporadic colonies resistant to neomycin appeared. The

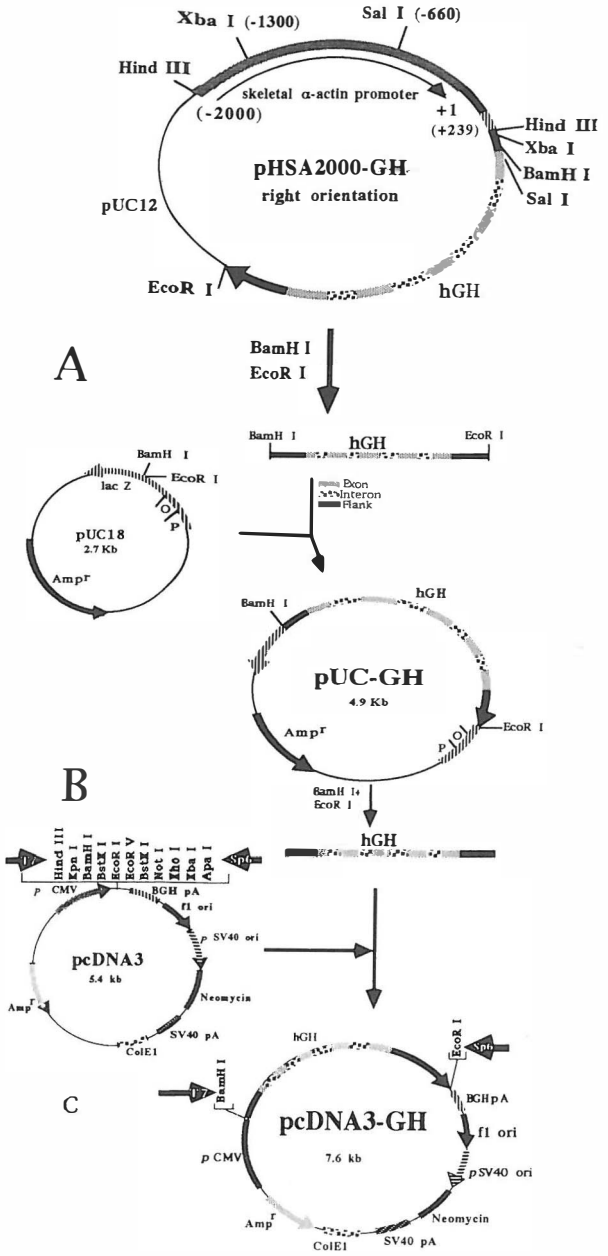

Fig. 1. 2.2kb BamH1-EcoR1 fragment (hGH gene) was cleaved from PHSA2000GH plasmid (A) and cloned in BamH1-EcoR1 digested PUC-18 and PUC-GH was constructed for identification of hGH gene (B). The same fragment was cut from PUC-GH with BamH1-EcoR1 and ligated to PCDNA3 plasmid at the same sites to obtain PCDNA3-GH(C). The plasmid pUC-GH creates a possibility for sequencing the hGH gene using universal primers.

experiment was extended for 8 weeks and every 2 days cells were refed. Then, hGH expression was assayed by ELISA.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the tissue culture plate cells 8 weeks following the start of transfection. For this

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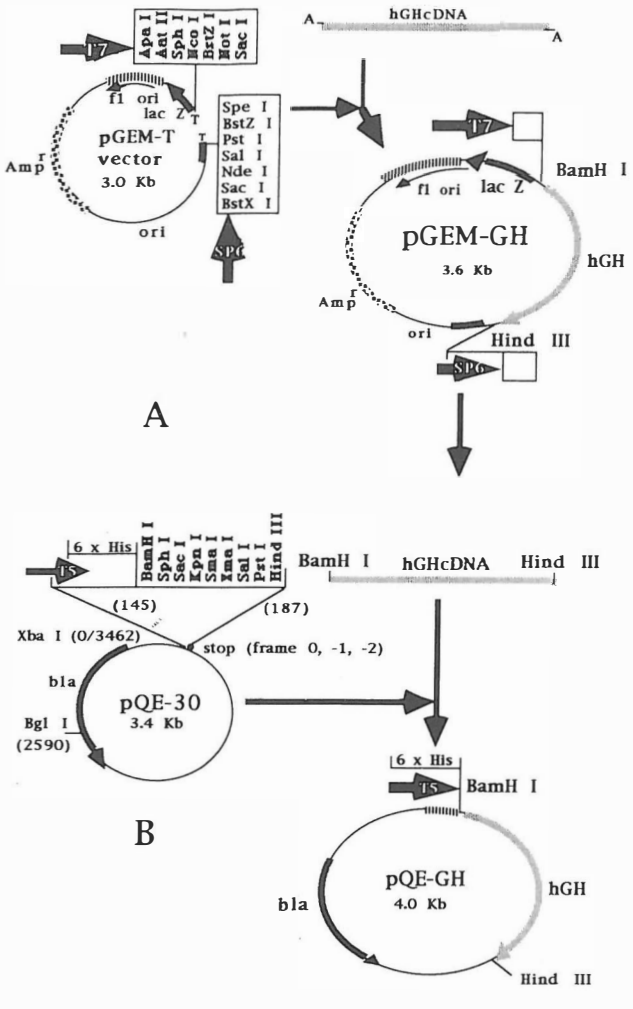

Fig. 2. Plasmid pGEM-T as a T-vector was subcloned with RT-PCR product, 0.6 kb hGHcDNA, and pGEM-GH plasmids were constructed (A). The hGHcDNA fragment was excised from the pGEM-GH plasmid with BamH1-HindIII and cloned in PQE-30 at the corresponding sites. The resulting plasmid was designated PQE-GH(B).

Table I. ELISA test to detect hGH in 3T3-GH.

Sample	hGH concentration ng/mL
1	108
2	120
3	104
4	-
5	-

Sample numbers 1 to 3, 3T3-GH; No. 4, 3T3-Neo; No. 5, NIH-3T3.

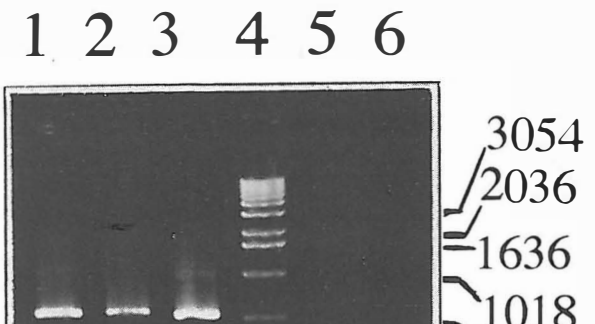

Fig. 3. RT-PCR analysis of hGH cDNA: Lane 1,2 and 3, 3T3 hGH cDNA samples 1-3, respectively; lane 4, molecular weight markers; lane 5, 3T3-Neo and lane 6, NIH-3T3.

purpose, cells were lysed directly by adding 1 mL Trizol (Gibco BRL) and incubated for 5 min at room temperature, then 0.2 mL chloroform was added to the mixture which was then centrifuged at 12000 × g for 15 min at 4°C. RNA was precipitated from the aqueous phase by the addition of 0.5 mL isopropanol and 10 min incubation at room temperature. The sample was then centrifuged at 12000 × g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed once with 75% ethanol. The pellet was air washed once with 75% ethanol. The pellet was air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically[15] and used for RT-PCR.

RT-PCR

Synthesis of first strand hGH cDNA using gene specific primers (GSP)

The superscript preamplification system (Gibco BRL) was used to convert total RNA to first strand cDNA.[16,17] This step is necessary to obtain cDNA from total RNA that was extracted from 3T3-GH. The RNA/primer mixture was prepared by addition of 2 µM gene specific primer and DEPC-treated water to 5 µg total RNA in an autoclaved 0.5 mL microfuge tube. Samples were incubated at 70°C for 10 min and on ice for at least 1 min. To each sample a mixture containing 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix and 0.1 M DDT was added, mixed gently and centrifuged briefly. Samples were incubated at 42°C for 5 min, after which 200U reverse transcriptase was added and incubation at 42°C was continued for a further 50 min. Reaction was terminated by incubation at 70°C for 15 min and chilling the samples on ice. After a brief centrifugation RNase H was added to each sample and incubated at 37°C for 20 min. The first strand cDNA thus obtained was amplified directly using PCR. A PCR mixture containing 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mix,

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2. Karin M, Theill L, Castrillo JL: Tissue-specific expression of the GH gene and its control by GH factor-1. Recent Prog Horm Res 46: 43-57, 1990.
3. Muller EE, Cocchi D, Locatelli V: Advances in growth hormone and growth factor research. Berlin: Pythagora Press/Springer-Verlag, 1989.
4. Isaksson OGP, Eden S, Jansson JO: Mode of action of pituitary GH on target cells. Annu Rev Physiol 47: 483-499, 1985.
5. Jorgensen JOL: Human growth hormone replacement therapy: pharmacological and clinical aspects. Endocrinol Rev 12: 189-207, 1991.
6. Hsiung HM, Cantrell A, Luirink J, Oudega B, Veros AJ, Becker GW: Use of Bacteriocin release protein in *E. coli* for excretion of human growth hormone into the culture medium. Bio/Technology 7: 267-271, 1989.
7. Schoner RG, Ellis LF, Schoner BE: Isolation and purification of protein granules from *Escherichia coli* cells overproducing bovine growth hormone. Bio/Technology 3: 151-154, 1985.
8. Chang CN, Rey M, Bochner B, Heyneker H, Gray G: High level secretion of human growth hormone by *Escherichia coli*. Gene 55: 189-196, 1987.
9. Ghorpade A, Garg LC: Efficient processing and export of human growth hormone by heat labile enterotoxin chain B signal sequence. FEBS Lett 330: 61-65, 1993.
10. Dahler A, Wade RP, Muscot GEO, Waters MJ: Expression vectors encoding human growth hormone (hGH) controlled by human muscle-specific promoters: prospects for regulated production of hGH delivered by myoblast transfer or intravenous injection. Gene 145: 305-310, 1994.
11. Jaurez-Aguilar E, Castro-Munozeldo F: 22-kDa hGH isoforms show differential effects when assayed in 3T3-F442A/C4 adipocytes. Biochem Biophsy Res Comm 217: 28-35, 1995.
12. Uchida E, Shimokawa S, Takasu H, Ikehara M, Uesugi S, Tomita K, Tanaka A, Morikawa M, Nishikawa S, Hayakawa T: Activity of artificial mutant variant of human growth hormone changes in charged residues around 62-67. Biol Pharm Bull (Japan) 18: 797-801, 1995.
13. Brigham KL, Meyrick B, Christman B, Conary JT, King G, Berry LC Jr, Magnuson MA: Expression of human growth hormone fusion genes in cultured lung endothelial cells in the lung of mice. Am J Respir Cell Mol Biol 8: 209-213, 1993.
14. Muscat GEO, Gobius K, Emery J: Proliferin, a prolactin growth hormone-like peptide represses myogenic specific transcription by the suppression of an essential SRF-like DNA binding activity. Mol Endocrinol 5: 802-814, 1991.
15. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159, 1987.
16. Rappolee DA, Mark D, Banda MJ, Werb I: Wound macrophages express TGF-alpha and other growth factors *in vivo*: analysis by mRNA phenotype. Science 241: 708-712, 1988.
17. Akowitz A, Manuelidis L: A novel cDNA/PCR strategy for efficient cloning of small amounts of undefined RNA. Gene 81: 295-306, 1989.
18. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: a Laboratory Manual. 2nd edition, NewYork: Cold Spring Harbor Press, Chapter 9.31, 1989.
19. Salinovich O, Montelaro RC: Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 156: 341-347, 1986.
20. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman GJ, Smith JA, Struhl, K: Current Protocols in molecular Biology. New York: Greene Publishing Associates Inc. and John Wiley and Sons Inc., (Ch. 9-1 to 9-4), 1994.
21. Staggs DR, Burton DW, Deftos LJ: Importance of liposome complexing volume in transfection optimization. BioTechniques 21: 792-798, 1996.
22. Guise AD, West SM, Chaudhuri JB: Protein folding *in vivo* and renaturation of recombinant protein from inclusion bodies. Mol Biotechnol 6: 53-64, 1996.
23. Stamoyannou L, Karachaliou F, Neou P, Papataxiarchou K, Pistevos G, Bartsocas CS: Growth and growth hormone therapy in children with achondroplasia: a two-year experience. Am J Med Genet 72: 71-76, 1997.
24. Saggese G, Baroncelli GI, Bertelloni S, Cinquanta L, Di Nero G: Effects of long-term treatment with growth hormone on bone and mineral metabolism in children with growth hormone deficiency. J Pediatr 122: 37-45, 1993.

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