

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.

MJIRI, Vol. 12, No. 3, 285-290, 1998

Keywords: Human growth hormone, RT-PCR.

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

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 cell line (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 (BglIII, HindIII, BamHI, EcoRI) 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

pHSA2000GH¹⁰ plasmid containing genomic sequence of hGH was used for this study (Fig. 1A). A 2.2 kb BamHI-EcoRI 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 BamHI and EcoRI 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 BamHI, 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 μ L of RPMI 1640 containing 24 μ g (12 μ L of 2 mg/mL) lipofectamin¹³ (Gibco BRL, U.K.). The mixture was incubated at room temperature for 50 min and after the addition of 800 μ L 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 μ g/ μ L neomycin.¹⁴ After 3 weeks from the start of the experiment, sporadic colonies resistant to neomycin appeared. The

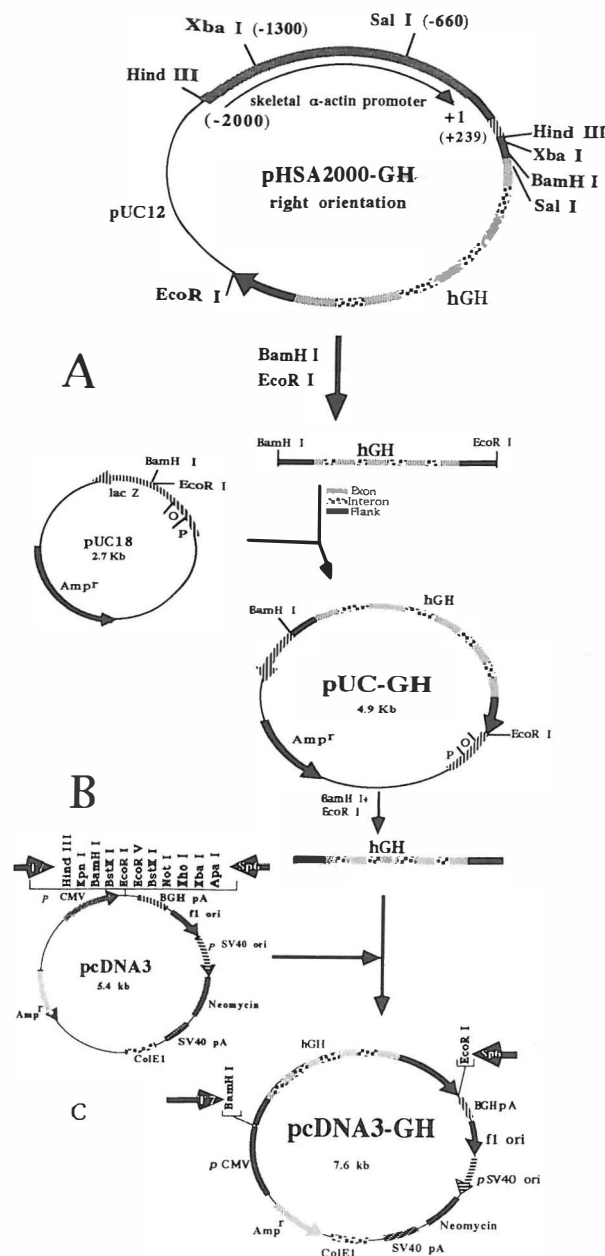


Fig. 1. 2.2 kb BamHI-EcoRI fragment (hGH gene) was cleaved from PHSA2000GH plasmid (A) and cloned in BamHI-EcoRI digested PUC-18 and PUC-GH was constructed for identification of hGH gene (B). The same fragment was cut from PUC-GH with BamHI-EcoRI and ligated to PCDNA3 plasmid at the same sites to obtain 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

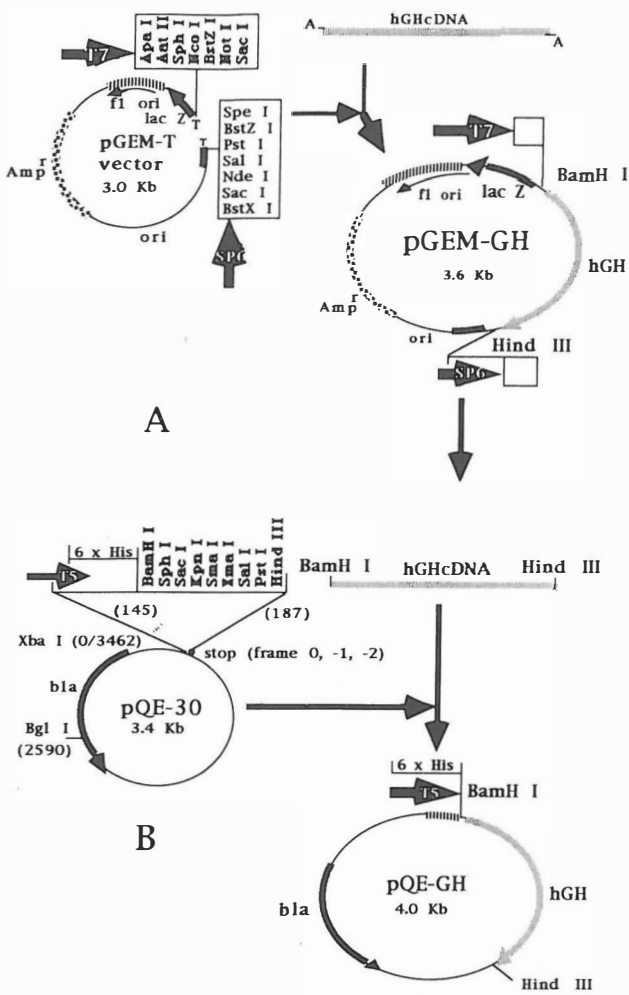


Fig. 2. Plasmid pGEM-T as a T-vector was subcloned with RT-PCR product, 0.6 kb hGHcDNA, and pGEM-GH plasmids were constructed (A). The hGHcDNA fragment was excised from the pGEM-GH plasmid with BamH1-HindIII and cloned in PQE-30 at the corresponding sites. The resulting plasmid was designated PQE-GH(B).

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.

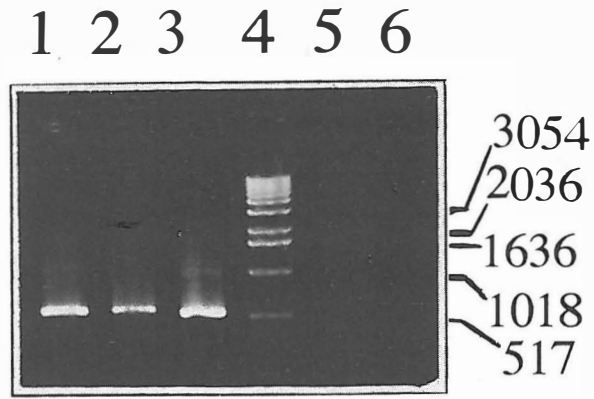


Fig. 3. RT-PCR analysis of hGH cDNA: Lane 1,2 and 3, 3T3 hGH cDNA samples 1-3, respectively; lane 4, molecular weight markers; lane 5, 3T3-Neo and lane 6, NIH-3T3.

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 spectrophotometrically¹⁵ 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 MgCl₂, 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 MgCl₂, 10 mM dNTP mix,

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