

Molecular Cloning and Expression of Human Gamma Interferon (IFN- γ) Full cDNA in Chinese Hamster Ovary (CHO) Cells

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ABSTRACT

Background: IFN- γ is mostly secreted by activated CD4⁺, CD8⁺ T cells and NK cells. This cytokine has immunomodulatory, anti-cancer and anti-microbial effects and is important for prophylaxis, diagnosis and treatment of chronic infections and cancers. **Objective:** The purpose of this study was to clone the full cDNA of human IFN- γ and express it in CHO cell line. **Methods:** Lymphocytes from a healthy individual were isolated and activated by phytohaemagglutinin (PHA) in vitro. After 4 hours, total RNA extracted and first cDNA strand was synthesized. cDNA was amplified with primers containing EcoRI and NotI sites. The amplified fragment and the pCDNA3.1 vector were cut by EcoRI and NotI and ligated. The construct (pCDNA3.1-IFN- γ) was transferred into *E.coli* (DH5 α strain) using CaCl₂ method and selected by plating on a medium containing ampicillin. The construct sequence was confirmed by PCR and sequence analysis. Construct expression was achieved by performing a calcium phosphate-mediated transfection into CHO cells and followed by selection of stable drug (G418) resistant clones by limiting dilution assay (LDA). The IFN- γ production by transfected CHO cells was measured using ELISA technique. **Results and Conclusion:** Out of 33 grown transformed bacterial colonies, only 6 had the entire sequences of the inserted fragment and one of them was used for the transfection experiment. Out of 768 wells, 5 clones produced more than 100 ng/ml/10⁶ cells of IFN- γ . Among the 5 clones, one with the maximum production of INF- γ (143 ng/ml/10⁶ cells) was selected and used for propagation.

Keywords: IFN-g, CHO, cDNA

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INTRODUCTION

Interferons (IFNs) are currently divided into three groups: IFN- α , IFN- β and IFN- γ which are known as leukocyte, fibroblast, and immune interferons, respectively. IFN- γ is secreted by lymphocytes (CD4⁺Th0, CD4⁺Th1, CD8⁺Tc), memory cells (CD45RO⁺), NK cells (CD16⁺, CD56⁺), dendritic cells (CD23⁺, CD35⁺) and B lymphocytes (CD22⁺, CD23⁺). The single IFN- γ gene is located on chromosome 12. This gene includes four exons and three introns and low polymorphisms in this region have been identified (1-3).

IFN- γ , also called type-II interferon, is a homodimeric glycoprotein consisting of two 21 to 24 Kd subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains one identical 18 KD polypeptide encoded by the same gene. IFN- γ is a multifunctional cytokine which involves in activation of mononuclear phagocytes, up-regulation of MHC molecule expression, differentiation of T-lymphocytes to Th1 subset and activation of neutrophils (4-6).

IFN- γ is used therapeutically, prophylactically and mixed and may be injected subcutaneously, intracutaneously, intramuscularly, intravenously, intranasally or even locally. Therapeutically it is used for the treatment of chronic infectious diseases like *L. Donovanii*, *M. Leprae* and *T. gondii*. Its application as prophylactic agent is to defend against opportunistic infections in AIDS, used for the prophylaxis of infective complications in chronic granulomatous disease and T-cell immune deficiency syndrome. The mixed form of IFN- γ injection is mainly used in the treatment of oncological diseases (2,5).

Chinese hamster ovary (CHO) cells have been a popular mammalian host for the commercial production of the therapeutically important proteins, and IFN- γ produced from these cells is one of the most important biopharmaceuticals that has a considerably large market in the world.

MATERIALS AND METHODS

Lymphocyte Isolation and Stimulation. Briefly, 10 ml sterile blood was obtained from a healthy donor and mixed with 1 ml 10% EDTA and diluted with 22 ml Hank's solution. Peripheral blood lymphocytes (PBLs) were isolated by Ficol and washed twice with Hank's solution. 5×10^6 lymphocytes were cultured as monolayer culture in 10 ml RPMI1640 medium, supplemented with 15% heat inactivated FBS, 100 μ g/ml of streptomycin and 100 U/ml of penicillin. PBLs were stimulated to produce IFN- γ by adding 10 μ g/ml of phytohaemagglutinin (PHA) to the medium. The cells were collected 4h after stimulation and used for total RNA extraction (6,8-11).

Total Cellular RNA Extraction. The cells were lysed with 4M guanidinthiocyanate. RNA was extracted with phenol-chloroform after addition of 0.2M sodium acetate and were precipitated twice by ethanol. The precipitate was dissolved in diethylpyrocarbonate (DEPC) treated water (6, 9-10).

RT-PCR. Extracted RNA was reverse transcribed to make the first cDNA strand. Briefly, 0.5 μ g of RNA was added to a tube containing 5mM MgCl₂, 1mM of each dNTPs, 50 mM KCl, 10mM Tris buffer(pH=8.3), 2.5 μ M Oligo dT (18), 20 units of RNase inhibitor and 50 units of M-Mulv-reverse transcriptase. The mixture was incubated at 45°C for 35 min, heated to 95 °C for 5 min and placed on ice until used

for PCR. The cDNA was amplified by PCR using the following oligonucleotides as primers: 5'-GGC TTA ATT CTC TCG GAA ACG-3' and 5'-AAA TTC AAA TAT TGC AGG CAG G-3'. The PCR was performed with an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 45sec at 94 °C, 60 sec at 58 °C, 60 sec at 72 °C, and the extension step of 5 min at 72 °C. Following the primary amplification, a second PCR was performed using two specific nested primers containing EcoRI and NotI restriction sites namely 5'-ACT TCT TTG GCT TAA TTC GAA TTC CTC GGA AAC ATG AAA-3' and 5'-ATT AGT GTT GCG GCC GCG GAT CCT TAC TGG GAT GCT CTT CGA CTT-3' (6,10).

Construction of Recombinant Plasmid (pcDNA3.1-IFN- γ or Construct). The resulting PCR product, the 558 base-pairs(bp) of human IFN- γ coding sequence containing restriction sites of EcoRI and NotI enzymes and expressing pcDNA3.1 vector (Invitrogen, USA) were digested by EcoRI and NotI simultaneously and purified by phenol/chloroform. IFN- γ expressing vector (pcDNA3.1-IFN- γ) was constructed by ligating of the entire human IFN- γ coding region (EcoRI & NotI fragment, 528bp) and the pcDNA3.1(10).

Transformation of E.coli (DH5 α) with the Construct. The resulting construct (pcDNA3.1-IFN- γ) was transferred into the competent *E.coli* (DH5 α strain) using CaCl₂ method and selected by plating on a medium containing ampicillin (10).

Screening of Transformed Bacterial Colonies by PCR. To confirm the existence of IFN- γ cDNA in the construct, PCR was performed for all colonies which were grown in the presence of ampicillin using a pair of primers amplifying 273bp sequences: 5'-AAT GCA GGT CAT TCA GAT G-3' and 5'- AAC TGA CTT GAA TGT CCA A-3'. Briefly, small amount of each colony was added to separate tubes containing all of the PCR reagents except the enzyme (Taq) and heated for 10 min. The Taq was then added to each tube and PCR was performed (10).

Sequence Analysis of the Construct. The complete nucleotide sequence analysis of the inserted construct in 6 grown colonies was performed in MWG-AG (Germany).

Construction of CHO Stable Cell Line Producing IFN- γ . CHO cells (Pasteur institute, Iran) cultured in DMEM medium for 24h were treated with 25 μ g construct suspended in 1 ml of TE buffer containing 0.25M CaCl₂, and an equal volume of HEPES buffer, and incubated in 5% CO₂ at 37°C, for 6 h. At the end of incubation period, the medium of cells was replaced with fresh medium. After 48 h, the level of IFN- γ in the conditioned media was evaluated by ELISA. Stable expression of the IFN- γ was achieved using the medium supplemented with 400 μ g/ml of G418 (Life Technologies, Germany) for selection. The cells were splitted in 8 \times 96-well plates by LDA. After approximately two weeks, drug-resistant cells forming clones were picked up. Stable human IFN- γ expressing clones were evaluated by ELISA and western blot analysis after 24 hours when the cells reached confluence (10,12).

RESULTS

The full length cDNA, encoding the signal sequence and mature human interferon, was amplified by RT-PCR and nested PCR. To determine the integrity and molecular weight of amplified fragments, electrophoresis in agarose gel was performed (Figure1). Recombinant plasmid was made by digestion and ligation of the nested PCR fragments (558bp) and a mammalian expression vector (PcDNA3.1, 5428bp)

(Figure 2,3). To select and amplify the correct recombinant plasmid, the resulting construct was transferred into E.coli. The results of PCR screening and the sequencing of plasmid derived from grown bacterial colonies are shown in figure 4 and 5, respectively. To establish IFN- γ producing cell line, CHO cells were transfected with the construct and permanent IFN- γ expressing clones in the cell culture supernatant were selected by ELISA test. To confirm the electrophoresis pattern and immunological characterization of produced IFN- γ , SDS-PAGE and western blot were performed (Figure 6).

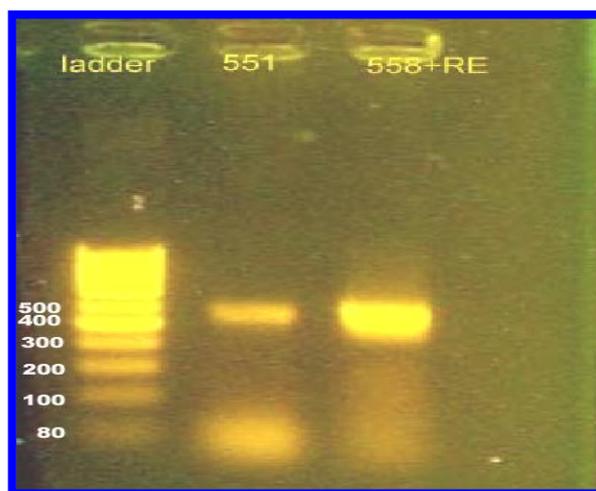


Figure 1. RT-PCR and nested PCR of IFN- γ full cDNA. The full length cDNA of human gamma interferon derived from PBLs was amplified by PCR and nested PCR followed by electrophoresis in agarose gel (1.7%). Lane1, molecular size marker; lane2, RT-PCR of full cDNA and lane 3 nested PCR with EcoRI & NotI sites.

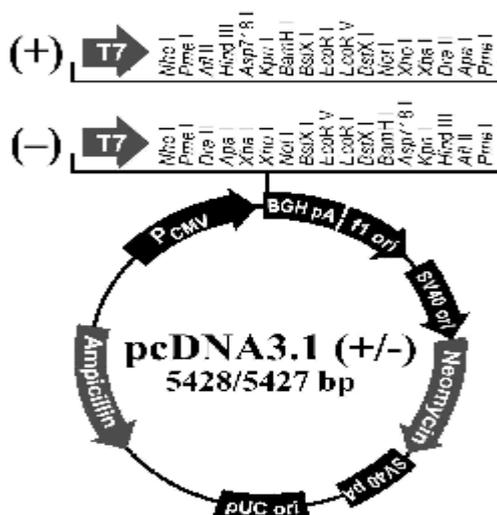


Figure 2. Construction of recombinant plasmid (pcDNA3.1), a mammalian expressing vector. The vector is under regulation of a cytomegalovirus (CMV) promoter which followed a cloning site for many restriction enzymes including EcoRI/NotI. In addition, it contains neomycin (G418) and ampicillin resistance gene for selecting eukaryotic and prokaryotic host cells, respectively. Also it has a bovine growth polyadenylation site (BGHpA).

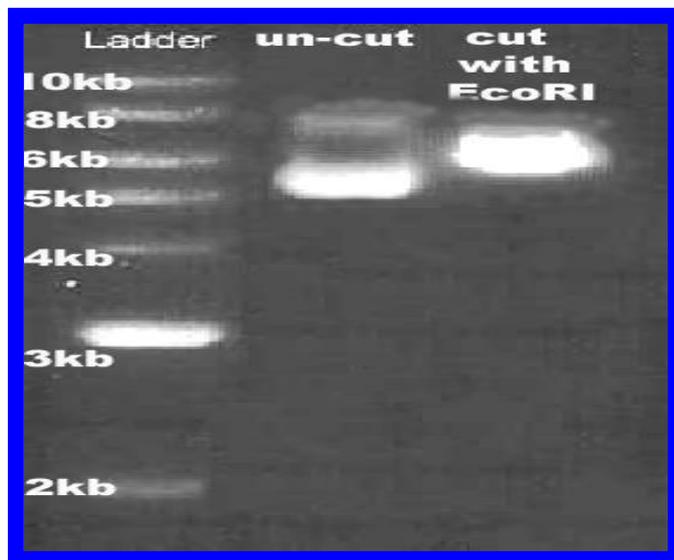


Figure 3. Agarose gel (1%) electrophoresis pattern of digested expressing vector pcDNA3.1. The expressing vector pcDNA3.1 which contains restriction sites for EcoRI & NotI in its cloning site was digested with these two enzymes and purified by phenol-chloroform. Lane1, molecular size marker; lane2, uncut vector (5428bp); and lane3, cut vector with EcoRI&NotI (5428bp).

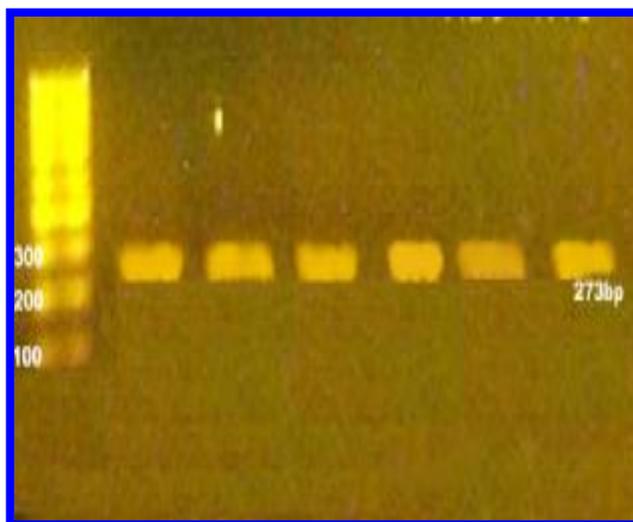


Figure 4. Screening of bacterial colonies for IFN- γ sequences by PCR. Agarose gel (1.7%) electrophoresis pattern of positive colonies for IFN- γ sequences. Lane1, molecular size marker; lanes 2,3,4,5,6 and 7 amplified fragments (273bp).

<XII211_col4_t7.fasta

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ATGAAATATACAAGTTATATCTTGGCTTTTCAGCTCTGCATCGTTTTGGGTTCTCTTGGC
TGTTACTGCCAGAACCCATATGTAAGAAGCAGAAAACCTTAAGAAATATTTAATGCA
GGTCATTGAGATGTAGCGGATAATGGAAGCTTTTTCTTAGGCATTTTGAAGAATTGGAAA
GAGGAGAGCGACAGAAAAATAATGCAGAGCCAAATTGTCTCTTTTACTTCAAACTTTTT
AAAACTTTAAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACAT
GAATGTCAAGTTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAA
TTATTCGGTAACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGAT
GGCTGAACTGTCGCCAGCAGCTAAAACAGGGAAACGAAAAGGAGTCAGATGCTGTTT
CGAGGTCGAAGAGCATCCCAGTAA
    
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Figure 5. Complete coding sequence of IFN- γ cDNA in the construct.

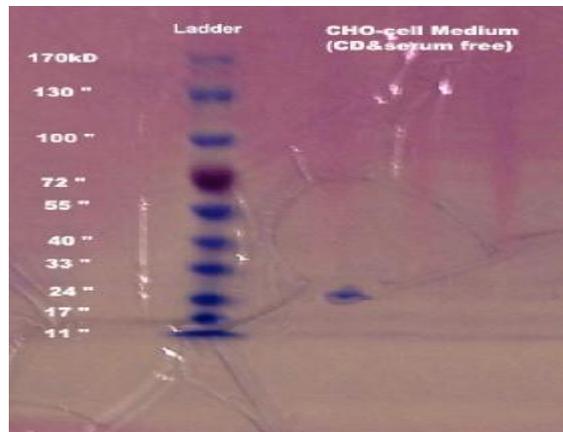


Figure 6. SDS-PAGE analysis of secreted IFN- γ by CHO cells. The medium of stable IFN- γ expressing CHO cells was centrifuged, concentrated and electrophoresed in 12% polyacrylamide gel in the presence of 1% SDS. The position of molecular weight marker is shown on the left and IFN- γ band (24KD) in the right. The size corresponds to that of natural IFN- γ secreted by human cells.

The product of nested PCR and expressing vector pcDNA3.1 (5428bp) were digested with EcoRI/NotI and ligated to make a construct (Figures 1-3) and was transferred into E.coli strain DH5 α . In the presence of ampicillin, 33 bacterial colonies appeared, while PCR screening showed only 6 colonies which had the entire inserted sequences (Figure 4).

CHO cell line was transfected with the construct and after two weeks, 47 G418-resistant clones were isolated from transfected cells. G418-resistant clones were transferred into 24-well microtitre plates. IFN- γ secreted in each plate was assayed by ELISA. Among 47 tested clones, 5 produced a significant level of IFN- γ when the cells had reached confluence (more than 100ng/ml/10⁶cells). The clone with the maximum production (143ng/ml/10⁶cells) was selected and propagated.

DISCUSSION

For the first time, Gray et al (1982) cloned and expressed human gamma interferon full length cDNA in E.coli. They produced a non glycosylated clone with an apparent molecular weight of 18 kd as evaluated by SDS-PAGE (13). However, the natural human interferon- γ has two forms with apparent molecular weights of 21kd and 24kd. Although their primary sequences were identical, they differed in their content of carbohydrates. The 24 kd form carries carbohydrates on both of its possible Asn-x-Ser/Thr glycosylation sites, whereas the 21kd form had only a single glycosylation site (4,14-16).

Apart from molecular size, the glycosylation can change the biochemical properties of the molecules such as antigenicity and solubility, as well as pharmacological and biological functions of the recombinant proteins. However, glycosylation seems not to be necessary for dimerization since recombinant E.coli-derived IFN- γ exhibits the expected apparent 36 kD dimer as analyzed by nondenaturing gel filtration (7,8, 13-16).

It has been shown that other recombinant proteins like human erythropoietin, human chorionic gonadotropin, coagulation factor VIII, follicle-stimulating hormone, human immunodeficiency virus envelope proteins and also require a proper glycosylation for their therapeutic and biological activities and half lives *in vivo*.

This requirement has been successfully resolved by production of recombinant proteins in CHO cells (17-24).

In addition, it has also been reported that recombinant human IFN- γ produced in CHO cells has two potential N-linked glycosylation sites at Asn28 and Asn100 (13,15,16). Based on the above, we chose CHO cells for the production of human IFN- γ which as indicated in the results, its relative molecular size revealed to be 24 kd indicating that it has been glycosylated on both glycosylation sites.

We found that the CHO cell line which was established upon introducing IFN- γ cDNA that was under the control of the CMV promoter, secreted IFN- γ into the culture medium up to 143ng/ml/10⁶cells. This is comparable to the amount produced by genetically engineered E.coli (13, 25-27) and by mouse cells in which IFN- γ cDNA had been introduced using bovine papilloma virus as a vector (7, 28). Therefore, the expression vector pcDNA3.1 may be used for further purification of other biologically active substances in a large scale production.

ACKNOWLEDGEMENTS

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