The Role of Bacterial Vectors in the Expression of Human IFN-γ Gene

Short Communication

Moeen Rezakhanlou, A.,[1] Mahboudi*[2], F., Maghsoudi, N.[3]*and Habibi, G.R.[4]

1. Immunology and Hematology Dept., School of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran

2. Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

3. Biotechnology Research Center, Malek Ashtar Industrial University, Tehran, Iran

4. Protozoology Dept., Razi Vaccine & Serum Research Institute, Tehran, Iran

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Summary

The Interferon gamma (IFN-γ) gene 1 was isolated from phytohemagglutinin stimulated peripheral blood mononuclear cells of a healthy individual blood donor using RT-PCR technique. The gene was cloned under the control of the different promoters' expression vectors such as pET32a (Novagen), pQE30 (QIAGEN), pSKA-IBA (Strep-Tag), pRSET (Invitrogen), and then expressed in *E.coli*. The transcription of IFN-γ mRNA was determined by northern blot analysis. The level of expression of human IFN-γ in pRSET vector under the control of T7 promoter was determined by laser-based densitometry of SDS-PAGE and found more than 26% of total bacterial protein. The expression was confirmed by western blotting. The expression of IFN-γ under the control of promoters of pET32a, pQE30 and pSKA-IBA plasmids was detected by SDS-GAGE and western blotting.

Keywords: interferon-γ, expression, *E. coli*, northern blotting

Introduction

Human interferon-γ (hIFN-γ), a homodimer glycoprotein regulates human immune system to protect the body from malignant and infectious diseases. This cytokine produced by activated $CD4^+$ and $CD8^+$ T cells, antigen presenting cells and natural

* Author for correspondence. E-mail:mahboudi@institute.pasteur.ac.ir

killer cells (Fruncht *et al* 2001). A mature monomer of IFN-γ is a glycoprotein with 143 amino acids (Rinderknech *et al* 1984). The molecular weights of the different IFN-γ complexes range between 16.5-48Kda (Miyata *et al* 1986, Justesen & Berg 1986). IFN-γ has been cloned and expressed in yeast (Schaber *et al* 1986), insect cells (Sareneva *et al* 1996), Chinese hamster ovary cells (Scahills *et al* 1983, Xie *et al* 1997) and in the murine genome (James *et al* 1996).

hIFN-γ cDNA has been cloned in *E.coli* earlier, though the level of expression not mentioned (Gray *et al* 1982). Some investigators have reported high expression of the hIFN-γ cDNA in *E.coli* but they used synthetic, not native, gene (Wang *et al* 1995). The aim of this study was to isolate native cDNA IFN-γ gene, to clone it in various expression vectors, to produce natural IFN-γ protein in *E.coli* and also to determine the best vector for the expression.

Materials and Methods

Cultivation and stimulation of PBMCs to express IFN-γ. Peripheral blood mononuclear cells (PBMCs) were isolated from 5ml of heparinized peripheral blood taken from an apparently healthy donor using Histopaque (Sigma-Aldrich Chemicie, Germany) washed three times with RPMI 1640 (Sigma) supplemented with 100IU/ml penicillin, 100μg/ml streptomycin and 17% fetal calf serum (JRH, Biosciences). The 5×10^6cells/ml were stimulated by 5μg/ml phytohemagglutinin (PHA) (Sigma) and incubated 2h at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of total RNA. Total RNA was isolated from 5×10^6PBMCs using RNAZole™B RNA isolation reagent according to the manufacturer instructions (Biotex International Inc., Friedswood, TX).

cDNA synthesis. To prepare complementary DNA (cDNA), 1μg of total RNA extracted was reverse-transcribed by incubation at 37°C for 60min in a 20μl reaction mixture containing 1XRT buffer (Boehringer Mannheim), 10U RNase inhibitor,

500μM dNTP, 160pM Oligo $(dT)^{15}$primer and 20U Murine Moloney Leukemia Virus RT (Boehringer Mannheim).

Amplification of IFN-γ cDNA. Two primers were synthesized according to the IFN-γ sequence present in the Gene Bank (Accession: C 0111) and *BamH*I sites were added to each end (underlined).

5′(GGCGGATCCATGCAGGACCCATATGTAAA)3′

5′(CAGGCAGGATCCGCAGGCAGGACAACCATT)3′. The primers were synthesized at Biotechnology Research Center (Pasteur Institute of Iran). Amplification of cDNA was performed in 20μl PCR reaction mixture containing 2μl cDNA, 2μl 10XPCR buffer [20mM Tris-HCl, pH8.8, 10mM KCl, 2mM MgSO4, 10mM $(NH_4)_2SO4$, 0.1%TritonX-100], 0.4μl dNTP (10mM) (Boehringer Mannheim), 0.5μl of each primer (50pM/μl), 0.5μl VentR DNA polymerase (1U/μl) [New England Bio Labs] under the following conditions: 94°C (1min) plus 35 cycles of 94°C (30sec), 65°C (1min), 72°C (1min) and a final extension cycle of 72°C (10min). A total volume of 400μl was amplified using this method.

Plasmid construction. The pure PCR product was cloned at the site of *Sma*I of pUC19 (Pharmacia) and then pUC19-IFN plasmid was made. This plasmid was transferred into *Top10F* [12]. The presence of the IFN-γ gene was confirmed by restriction enzymes analysis and sequencing (ALF system, Pharmacia). After sequence verification, the gene encoding hIFN-γ was sub-cloned into pET32a (Novagen), pQE30 (QIAGEN), pSKA-IBA (Strep-Tag) and pRSET (Invitrogen) expression vectors; and pET32a-IFN, pQE30-IFN, pSKA-IBA-IFN and pRSET-IFN plasmids obtained. These plasmids transformed into *E.coli* BL21-(DE3) (Studier *et al* 1990). The clones containing the appropriate IFN-γ gene were selected on Luria Bertain (LB) media, containing 100μg/ml ampicillin.

Northern blotting. *E.coli* cells containing different recombinant plasmids (pET32a-IFN, pQE30-IFN, pSKA-IBA-IFN) were induced by 1mM IPTG at exponential phase (absorbency=0.5). The appropriate amount of cells was harvested after 4h

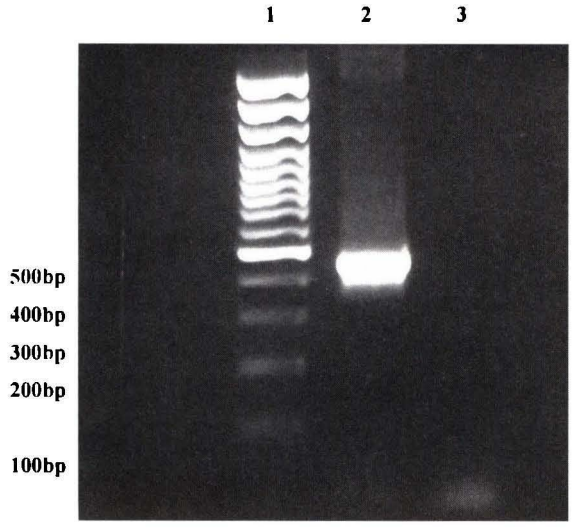

Figure 1. *Agarose gel electrophoresis of amplified nhIFN-γ gene by PCR. Lane 1, size marker; lane 2, amplified IFN-γ gene; lane 3, negative control*

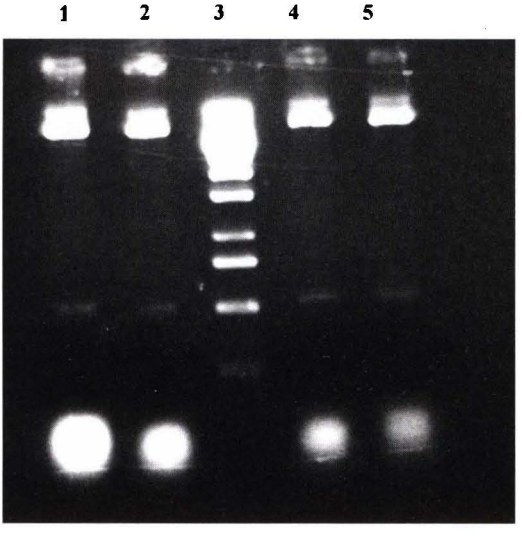

Figure2. *pUC19-IFN recombinant plasmids with nhIFN-γ gene digested with BamHI. Lane1,2,4 and 5: positive clones (~500bp), lane 3: molecular weight marker*

The hIFN-γ gene subcloned into pET32a, pQE30, pASK-IBA and pRSET as mentioned earlier, they were transferred into *E.coli* BL21-(DE3) for expression.

SDS-PAGE analysis of the expressed natural hIFN-γ (nhIFN-γ) protein has been shown in figures 3. Confirmation of nhIFN-γ was done by western blotting (Figure 4). Laser densidometry analysis on SDS-PAGE showed over 26% expression of total bacterial proteins. The molecular weight of the expressed protein was 18KDa.

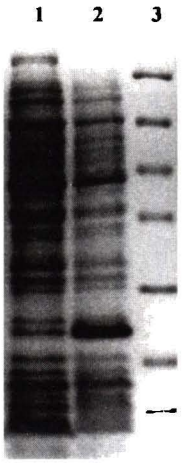

Figure 3. *SDS-PAGE (15%) analysis of expressed IFN-γ protein in BL21 (DE3). Lane 1: uninduced cells, lane 2: induced cells with 1mM IPTG in final concentration, lane 3: standard proteins*

Figure 4. *Western blotting analysis of expressed human IFN-γ in pET3a and pRSETA. Lane 1 is the expressed IFN-γ protein in pETa with synthetic gene. Lane 2 is expressed IFN-γ protein in pRSETA with natural Iranian gene*

The expression of hIFN-γ has been determined by the northern blotting because the IFN-γ protein has not been detected by SDS-PAGE and western blotting of pET32a-IFN, pQE30-IFN and pASK-IBA-IFN plasmids (Figure 5). Data of pQE30-IFN and pASK-IBA-IFN plasmids are not shown. However, the expression of IFN-γ protein has not been detected by SDS-PAGE and western blotting, the results of northern blotting showed that the gene of hIFN-γ was transcribed in cells with pET32a-IFN, pQE30-IFN and pASK-IBA-IFN plasmids.

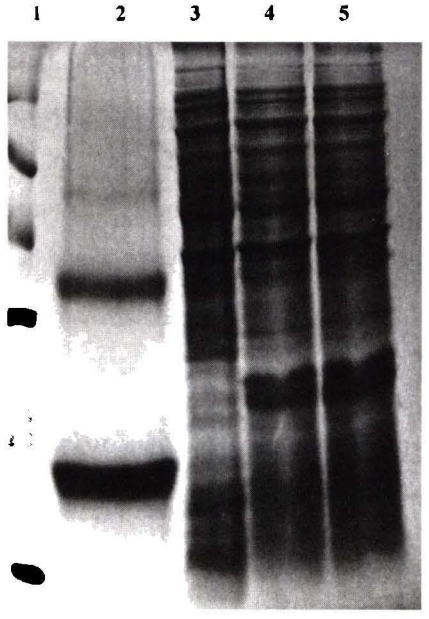

Figure 5. *SDS-PAGE analysis of expression of pET32a-IFN plasmid in E.coli. Lane1: Low molecular weight standard proteins. lane2: human IFN-γ monomer and dimmer, lane3: pET32a-IFN before induction, lanes 4, 5: pET32a-IFN after induction*

There are several factors that control the level of expression of protein in *E.coli* (Baney 1999). The secondary structure of mRNA is a main factor in protein expression (Tessier 1984) and the sequence of a vector that exists in mRNA is important for forming the secondary mRNA structure. We concluded that the secondary structure of mRNA of pRSET-IFN was suitable for translation, but

secondary structures of the mRNAs of pET32a-IFN, pQE30-IFN and pASK-IBA-IFN plasmids were not suitable for translation. The secondary structure of the mRNAs was analyzed by RNAdraw software (RNAdraw V1.1, Mazura Multimedia, Stockholm, Sweden).

Today, hIFN-γ expressed in *E.coli* is widely used as a therapeutic agent in various pathological conditions (Kruskal *et al* 1997) To the best of our knowledge; this is the first report of moderate expression (~26%) of nhIFN-γ gene in *E.coli.* Gene expression in prokaryotic systems is under the influence of such factors as promoter, upstream elements, mRNA stability, codon usage, and fusion proteins (Baney 1999). Based on this investigation, it seems that the sequence of upstream elements of a vector is a main factor in expression of a protein. The sequences of upstream elements influence the primary and secondary structures of mRNAs. Therefore, for the expression of a gene, the appropriate vector should be selected.

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