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Amplification and cloning of human interferon gamma gene in mammalian expression vector and its expression study

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Abstract

The human interferon gamma gene was amplified by reverse transcriptase polymerase chain reaction with M-MuLV reverse transcriptase enzyme from the total RNA of human peripheral blood lymphocytes. Taq DNA polymerase enzyme was used to amplify the gene as it adds 'A' overhang in the PCR product. This 'A' tailed product was cloned in TA cloning vector pTarget and transformed in *E. coli* DH5 α cells. The recombinant plasmid containing the human interferon gamma gene insert in right orientation was selected after characterization using restriction enzyme analysis, directional PCR and nucleotide sequencing. The recombinant plasmid pTarget.hu IFN-g was observed to express IFN-g protein in vero cell as detected by fluorescent antibody and immunoperoxidase tests. The modeling of the IFN-g protein using DNASTAR and SWISS-MODEL softwares revealed the structure and epitopes of the protein.

Keywords: Interferon gamma, cloning, human, PCR, accession no. AM903379

Introduction

Interferon was characterized as an antiviral entity by (Isaacs et al., 1957). Two major classes of acid-stable (type I) interferons have been recognized in man: leukocyte interferon, released by stimulated leukocytes, and fibroblast interferon, produced by stimulated fibroblasts. They differ not only immunologically but also in their target cell specificities,

although both induce a virus-resistant state in human cells. The 2 are encoded by separate mRNAs. Human interferons have been classified into 3 groups: alpha, beta, and gamma.

Both alpha- and beta-IFNs, previously designated type I, are acid-stable, but they differ immunologically and in regard to some biologic and physiochemical properties. The IFNs produced by virus-stimulated leukocytes (leukocyte IFNs) are predominantly of the alpha type. Those produced by lymphoblastoid cells are about 90% alpha and 10% beta. Induced fibroblasts produce mainly or exclusively the beta type. The alpha- and beta-IFNs differ widely in amino acid sequence. Nucleotide sequencing of cDNA for leukocyte and fibroblast IFNs confirms the differences while showing some homologies (Streuli et. al., 1980). The gamma or immune IFNs, which are produced by T lymphocytes in response to mitogens or to antigens to which they are sensitized, are acid-labile and serologically distinct from alpha- and beta-IFNs. (Celada et. al., 1985) demonstrated and partially characterized the interferon-gamma receptor on macrophages. Interferon-gamma has an important role in activating macrophages in host defenses.

Materials and methods

Isolation of RNA

The peripheral blood mononuclear cells were isolated from the blood of healthy human using histopaque (Sigma, USA) as per manufacturer's instructions (Boyum, 1976). The total RNA was isolated from lymphocytes using TRIZOL reagent.

RT-PCR

The oligonucleotide primers were designed as forward primer 5'-TCT CTC GGA AAC GAT GGA AT -3' and reverse primer 5'- CCA TTA CTG GGA TGC TCT TC-3' using the published sequence (Genbank accession no. M29383). RT-PCR reaction was performed to obtain cDNA using 10µl of total RNA with 25 pmol random hexamer primer, 200mM dNTPs, and 40U of M-MuLV reverse transcriptase enzyme (Fermentas, USA) in 1X reaction buffer in total volume of 20 µl. The amplification step was performed at 37°C for 1 hour and

72°C for 10 min. The PCR was performed using 6µl of cDNA along with forward and reverse primers (25 pmol each), 200 µM dNTPs, 1.5 mM MgCl₂ and 3U of Taq DNA polymerase (Fermentas, USA) in 1X reaction buffer in total volume of 50 µl. The cyclic conditions were initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 54°C for 50 sec and primer extension at 72°C for 50 sec (Sambrook and Russell, 2001) with final extension time of 7 min to facilitate the addition of 'A' nucleotide at 3' ends of amplified products.

Gene cloning

The PCR product was purified using QIAEX II gel extraction kit (Qiagen, Germany) and then ligated into pTarget vector (Promega, USA) using T4 DNA ligase (Promega, USA) at 16°C overnight. The description of pTarget mammalian expression vector has been given by Rai *et al.* (2006). The ligated plasmid after transformation into *E. coli* DH5α competent cells produced blue and white colonies. The white colonies were selected, subcultured and recombinant plasmids were isolated using QIAGEN miniprep plasmid isolation kit (Qiagen, Germany). The presence of IFN gamma gene in the recombinant plasmid was checked by restriction digestion with EcoR1 (Fermentas, USA) and the orientation of the gene insert was checked by colony PCR using T7 promoter primer present flanking 5' end of gene in the vector and reverse primer of the gene. The digested products and PCR products were analyzed on 1.2% agarose gel along with DNA molecular weight marker. The sequencing was done using T7 promoter primer and the sequence obtained was submitted to the GENBANK. The sequence was also compared with other available sequence of human IFN gamma using the DNASTAR software.

Gene expression

For expression analysis, the recombinant plasmid pTarget.hu IFN-γ (Saxena et al, 2006) was isolated using Qiagen plasmid mini kit (Qiagen), transfection was carried out in Vero cells using polyfect transfection reagent (Qiagen) according to manufacturer's

instructions and the expression was characterized by indirect fluorescence antibody test (IFAT) and immunoperoxidase test

Indirect fluorescence antibody test

Vero cells in 24-well plate with approximately 80% confluent monolayer were transfected with plasmid pTarget.hu IFN- γ and the cells were incubated at 37°C and 5% CO₂. After 48 hours, the cells were fixed with 80% chilled acetone. Mock-transfected vero cells were also fixed as a control. To the fixed monolayer, 1:50 diluted mouse anti-human IFN- γ hyperimmune serum was added and incubated at room temperature for 1 h. After the incubation, the wells were washed twice with PBS and again incubated with goat anti-mouse FITC conjugated secondary antibody and incubated further for 1h at room temperature. Cell monolayer were extensively washed with PBS, mounted in 50% glycerol in PBS and examined under fluorescent microscope.

Immunoperoxidase test

The protocol was similar to IFAT except that, goat anti-mouse HRP as the secondary antibody conjugate was used. The color was developed with DAB (1mg/ml in PBS with 1 μ l/ml H₂O₂) at room temperature for 5 min.

Results and discussion

The 517 bp human interferon gamma gene was successfully amplified from the total RNA isolated from PBMC's (fig 1). The gene sequence was assigned accession number AM903379. The sequence homology studies using DNASTAR software revealed 99.6% homology at nucleotide level and 100% at amino acid level with the available human interferon gamma sequence (fig. 2a. & 2b.). The basic property of Taq DNA polymerase was used for TA cloning by addition of 'A' nucleotide to PCR product, since pTarget mammalian expression vector has T overhang at 5' end assisting the ligation process. The

presence of interferon gamma gene in the recombinant plasmid designated as pTarget.IFN.γ.hu was successfully detected by release of 600 bp gene insert on digestion with EcoRI enzyme (fig. 3). The orientation of gene was confirmed by PCR using T7 promoter primer and reverse primer of gene using recombinant plasmid DNA as template which yielded full length 517 bp PCR product in correct orientation while no product was obtained in wrong orientation (fig.4). pTarget.hu IFN-γ transfected Vero cells showed good immunofluorescence while control healthy cells did not show any fluorescence (Fig. 5 - 6).

Similarly the Vero cells transfected with the recombinant plasmid DNA showed enhanced staining reaction in immunoperoxidase test while control cells did not show any staining (Fig. 7 - 8).

The expression of the pTarget.huIFN-γ gene in vero cells showed that the mammalian expression vector pTarget is highly efficient in expressing the gene cloned in it. It further demonstrated that it would express in appropriate host when injected.

The modeling of human IFN gamma using SWISS-MODEL software online showed the structural details and epitopes(Fig9).

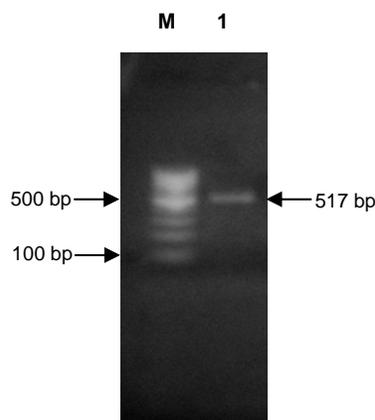


Fig. 1. PCR amplification of human IFN-γ product size of 517bp.
Lane M: 100 bpDNA ladder, 1: PCR product of human IFN-γ- 517bp.

Percent Divergence		Percent Similarity		
		1	2	
1		■	99.6	AM903379.seq
2	0.4	■		M29383.SEQ
		1	2	

Percent Divergence		Percent Similarity		
		1	2	
1		■	100.0	AM903379 protein.SEQ
2	0.0	■		M29383 protein.SEQ
		1	2	

Fig 2.a Alignment of hu IFN- γ gene. **Fig.2b.**Alignment of huIFN- γ amino acid sequence.

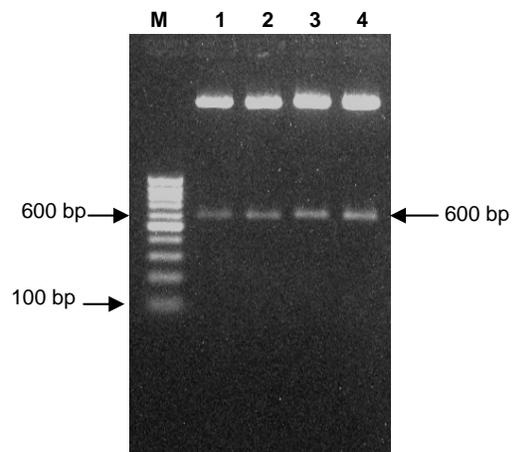


Fig 3: Confirmation of human IFN- γ gene in pTarget.hu IFN- γ . Lane M: 100 bp DNA ladder, 1–4: Release of human IFN- γ gene insert of 600 bp from pTarget.hu IFN- γ using EcoR1 enzyme.

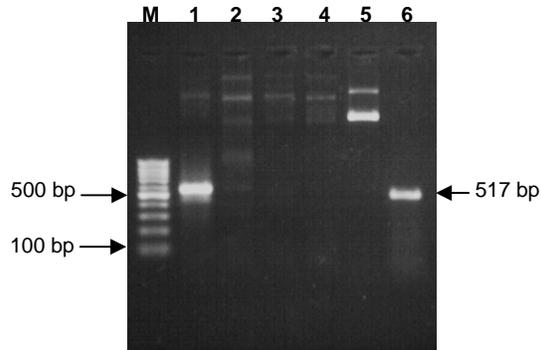


Fig 4: Confirmation of human IFN- γ gene in right orientation in pTargeT.hu IFN- γ .
Lane M: 100 bp DNA ladder, 1: PCR amplification of human IFN- γ using T-7 Primer as forward primer and gene specific reverse primer, 2-5: Non specific amplification, 6: PCR amplification of human IFN- γ using specific primers.

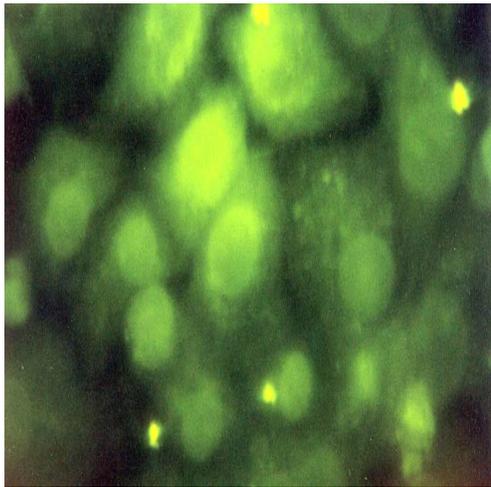


Fig5. Vero cells transfected with pTargeT.huIFNgamma showing fluorescence

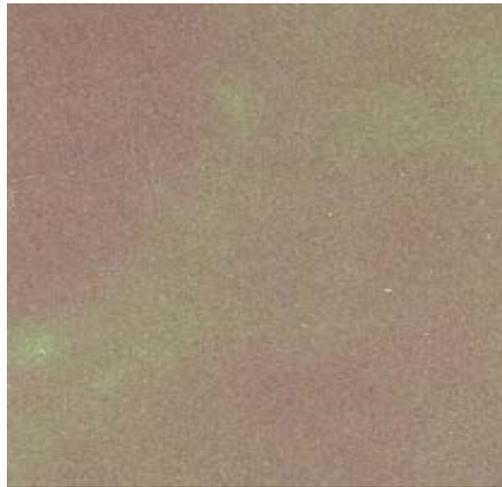


Fig6. Vero cells healthy.

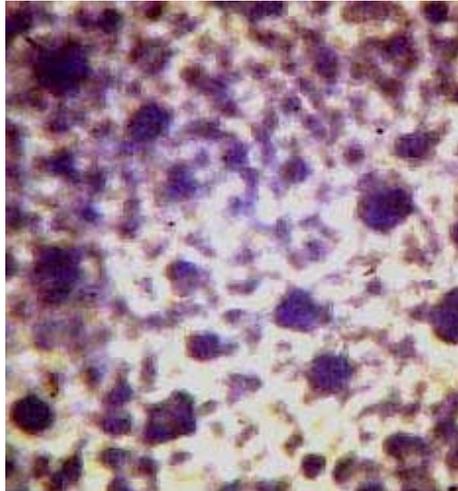


Fig7. Vero cells transfected with pTarget huIFN gamma showing IPT staining

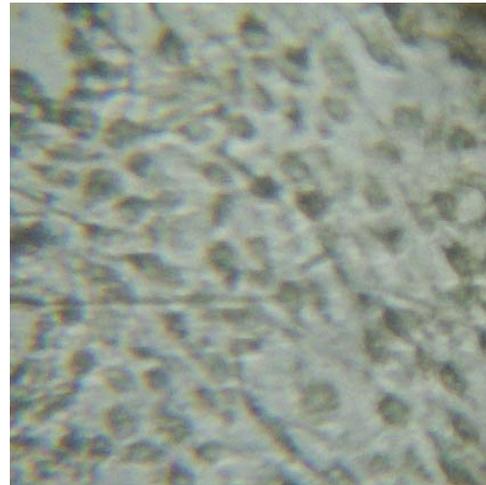
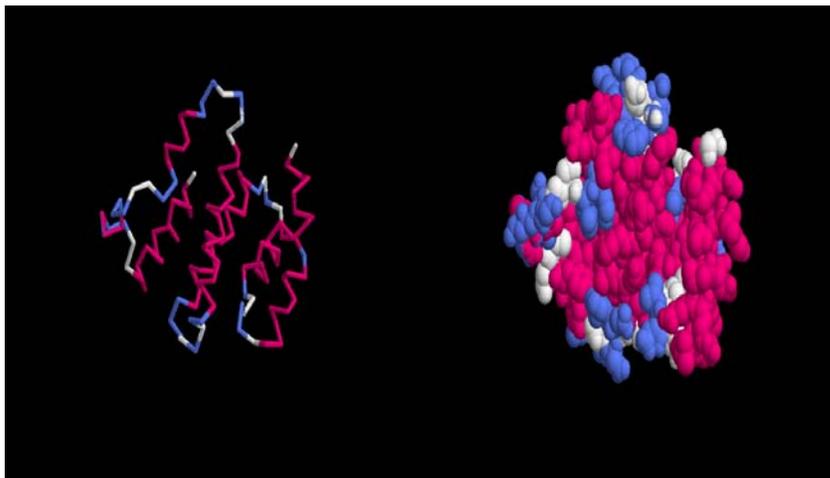


Fig8. Vero cells healthy.



A

B

Fig9. Human IFN gamma protein

Modeling A. color structure, Rasmol, B. Spacefill, Rasmopl

Acknowledgement

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