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Cloning and Characterization of Directly Amplified Antiviral Gene Interferon Alpha-2b (HuIFNα-2b) from Human Leukocytes Chromosomal DNA

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Interferons are cytokines that confer resistance to viral infection and inhibit cellular proliferation. The interferon alpha gene from human blood samples was amplified, cloned and expressed in *E. coli* (BL21). Leukocyte chromosomal DNA was used as a source of template DNA. Using specific primers, the gene for HuIFNα-2b was amplified and inserted into the *E. coli* vector, pET21b, by ligation of the *Hind*III and *Bam*HI linkers of the vector and insert. The insert was further analyzed by PCR, DNA restriction mapping and sequencing, and expressed in a suitable *E. coli* strain. The production of this important cellular protein in the laboratory has significant applications in production of the recombinant pharmaceutical proteins.

Key words: Interferon alpha, Human, Gene cloning, PCR

INTRODUCTION

Interferons are vertebrates cytokines, whose main property is to elicit an antiviral state in many cell types, which also exhibit antiproliferative and immunomodulation properties (De-NIU *et al.*, 1995). At least 24 subtypes of human interferon alpha (HuIFNα) have been identified, with molecular weights ranging from 19 to 26 kD (Pestka, *et al.*, 1987; Sen *et al.*, 1992; Capon, *et al.*, 1985). IFNα is a type I IFN, produced by monocytes/macrophages, lymphoblastoid cells, fibroblasts and a number of different cell types, such as Namalwa and KG1, following the induction by viruses, nucleic acids and low-molecular weight (*n*-butyrate, 5-bromodeoxy uridine) and glucocorticoid hormones. (Kurane, *et al.*, 1987; Lepe-Zuniga, *et al.*, 1989). These types of interferon are involved in virus resistance on target cells, inhibition of cell proliferation, induction of cytokines and regulation of expression of MHC class I antigens (Pestka, *et al.*, 1987; Sen *et al.*, 1992; Aman *et al.*, 1996). Recombinant human IFNα-2 is a single, non-glycosylated, polypeptide chain, containing 165 amino acids (Callard and Gearing, 1994). HuIFNα genes are clustered on chromosome 9p22. It is not known whether all these genes are expressed following stimulation of the cells. HuIFNα genes do not contain intron sequences found in many other eukaryotic genes. In contrast to interferon gamma they are stable at pH 2 (Pulido *et al.*, 1986). Due to the large demand for interferons, the idea of producing these products has attracted many biotechnologists. Human leukocyte interferon cDNA was first cloned in *E. coli* using the plasmid, pBR322, as a vector. Expression of the IFN gene in *E. coli* with different promoters gave different levels of IFN production. For instance, the use of β-lactamase, *lac-uv5* and *trp* synthetase promoters resulted in the production of 5×10^3; 1.8×10^8, and 1.8×10^7 U of the product per gram of dried cells per liter of culture medium, respectively (Baron and Narrola, 1990).

HuIFNα is mainly used as a standard therapy for hairy cell leukemia, metastasizing renal carcinomas and AIDS associated angiogenic tumors of mixed cellularity, known as Kaposi sarcomas. It is also active against a number of other tumors and viral infections, including chronic hepatitis C. However, in less than 15 to 20 percent of patients the serum HCV RNA levels are undetectable after 48 weeks of treatment (Saracco *et al.*, 1993; Hoofnagle and Di Bisceglie 1997). It has been shown that in these patients a combination therapy, using HuIFNα and ribavirin, was

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more effective than treatment with interferon alone (McHutchison *et al.*, 1998). This study concerns the cloning and characterization of the HuIFNα-2b gene, amplified by direct PCR, from human leukocytes chromosomal DNA.

MATERIALS AND METHODS

Microorganisms

Escherichia coli strains, BL21-DE3 (from Novagen) and DH5α (from laboratory stocks), were maintained on sterile LB agar slants. The strains were grown at 37°C. The strains were stored long term at -20°C in 20% glycerol and nutrient broth.

Plasmid

The plasmid used was pET21b (Novagen), which possesses ampicillin (*amp*) resistance.

Chemicals and enzymes

The chemicals and enzymes used in this study were of analytical grade, and from either Sigma, Fluka, Fermentas or Cinagen (Iran). X-gal (5-bromo-4-chloro-3 indolyl β-D-galactopyranoside, Nothumberia Biologicals Limited) was dissolved in dimethylformamide, then to 20 mg·mL^{-1} in distilled water; filter sterilized and stored at -20°C.

Design of the oligonucleotide primers

The oligonucleotide primers were designed based on a comparison of the published sequences for the interferon alpha-2 gene. The interferon alpha-2 DNA sequence was accessed using GenBank, NCBI. A *Bam*HI or *Hind*III restriction site was added at the 5' end of each primer, followed by three additional bases. The DNA sequences of the designed oligonucleotide primers contained: F1 (forward primer): 5'> CAGGGATCCTTGTGATCTGCCT-CAAACC>3' and F2 (reverse primer): 5'> CGCAAG-CTTTCATTCCTTACTTCTTAAAC>3'.

Cloning of the PCR product

The PCR reaction product was purified from 0.8% (w/v) agarose gel, precipitated, washed with ethanol and dissolved in distilled water. The PCR product was cloned into the plasmid vector mentioned above. Approximately 2 μg of the cleaned PCR product was doubly digested with *Bam*HI and *Hind*III overnight at 37°C. 5 μg of plasmid was also doubly digested with *Bam*HI and *Hind*III overnight at 37°C. The DNAs were purified and washed with ethanol. The resulting pellets were each dissolved in 20 μL of distilled water. DNA fragments with cohesive ends were ligated in a final volume of 20 μL ligation buffer. Up to 0.5 μg of digested vector DNA was incubated, overnight at 15°C, with a three fold molar excess of the fragment to be cloned, and one Weiss unit of T4 DNA ligase. The transformation was carried out by a standard published $CaCl_2$ method (Sambrook et al., 1989).

Analysis of the plasmid derivatives

Single colonies of the transformant cells carrying plasmid derivatives were grown in LB, containing the appropriate antibiotic, and a small scale plasmid preparation carried out (Sambrook *et al.*, 1989). The plasmid derivatives were doubly digested with *Bam*HI and *Hind*III, and the restriction fragments run on a 0.7% agarose gel. Two bands corresponding to a ≈ 510 bp fragment (insert), and another corresponding to the vector, were expected. The DNA sequences were verified by the dideoxy chain-termination method (Sanger *et al.*, 1977).

Recombinant HuIFNα expression

E. coli BL21 (DE3), transformed with the plasmid pETHA, was grown for ~3 h until the cultures gave an A_{600} of 0.7. The expression of rHuIFNα was induced by the addition of 225 μM isopropyl-β-thiogalactopyranoside (IPTG, Life Technologies, Inc.). After further incubation for 4 h, bacteria were harvested by centrifugation at 3000×*g* for 15 min at 4°C. The bacterial pellets were lysed with 200:1 8 M urea (Dian *et al.*, 2002). Proteins from the lysed bacterial pellets were resuspended in an equal volume of 2×Laemmli running buffer, boiled for 4 min and separated under denaturing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with 5% stacking and 12% resolving gels. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. Immunoblotting was performed with a primary anti-HuIFNα polyclonal antibody (PBL Biomedical laboratories, New Brunswick) and anti-rabbit secondary antibody conjugated with horse radish peroxidase (HRP). The HRP signal was generated by incubation with a chemiluminescence detection kit (Amersham) and exposure on X-ray film.

RESULTS

DNA extraction

Human leukocyte DNA was extracted from the blood samples of healthy individuals. The quality of the DNA extract was evaluated by three digestion reactions, with the restriction enzymes *Bam*HI, *Hae*III and *Hind*III (Fig. 1). At the same time, plasmid preparations were carried out, and the plasmid preparation concentrations measured.

PCR experiments

An amplicon of the expected size (510 bp) was observed in the reaction (Fig. 2), which was not seen in the controls. The fragment was analyzed using a *Pvu*II enzyme, which would cut the fragment into two fragments

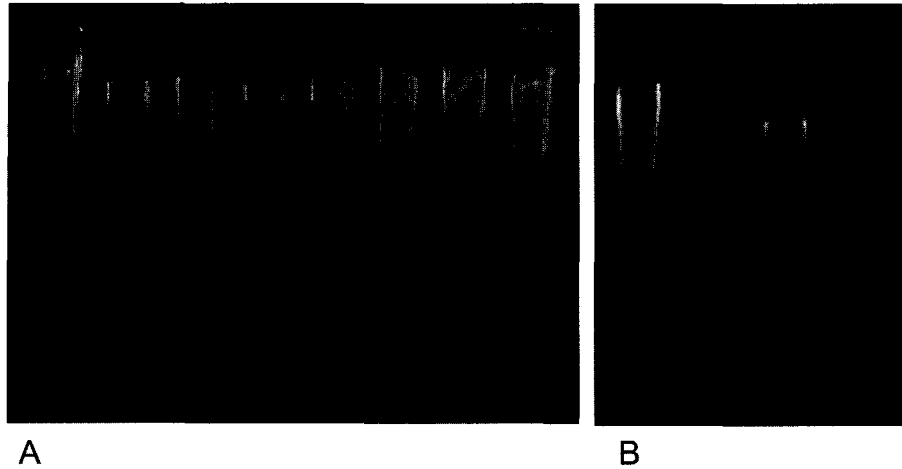

Fig. 1. (A) The human leucocyte DNA preparations and (B) digestion of the DNA with three restriction enzymes; *Bam*HI, *Hae*III and *Hind*III (first line is control DNA).

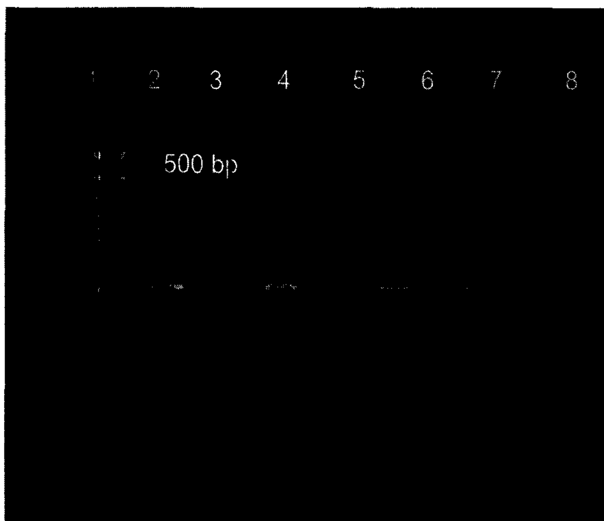

Fig. 2. The PCR reaction (Lines 7 and 8 are controls, without primers and without DNA template, respectively).

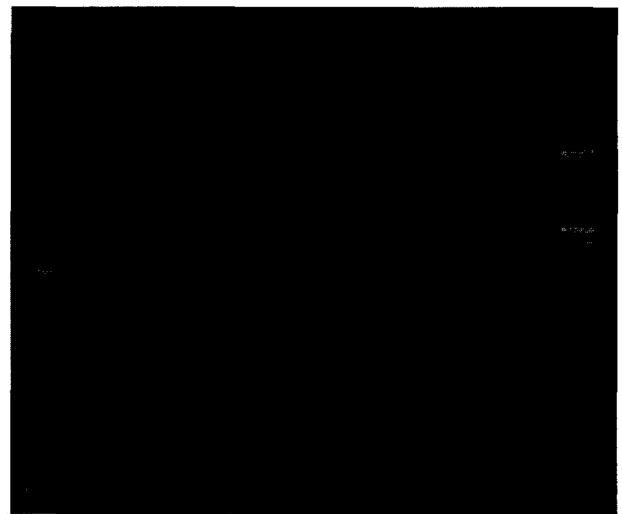

Fig. 3. Analysis of transformants; Restriction mapping of the recombinant vectors.

of 233 and 273 bps.

Cloning and characterization of the insert

The amplified fragment was then cloned into different vectors, as mentioned above. The resulting plasmids could be used for sequencing and further characterization of the insert. Following ligations, transformation experiments were carried out, and the transformants screened for the presence of the insert (Fig. 3).

Using oligonucleotide primers for the T7 promoter and terminator, sequencing reactions were carried out. The sequence of the cloned region was determined and deposited in GenBank (accession number AY532915). The sequence data was then submitted to the programmes FASTA and BLAST, for pairwise domain matching of the nucleotide sequence and the deduced gene products to the sequences on the databases (Altshul *et al.*, 1990; Pearson 1990). Highest DNA homology scores were observed between the inserts and HuIFNα-2 (gi|11414526|emb|AL353732.14|), with 460/462 (99%) identical nucleotides. The recombinant vector pETHA (Fig. 4) was then transformed into *E. coli* strain BL21-DE3 for further analysis.

The expression of the cloned gene in the transformants was induced by the addition of isopropyl β-D-thiogalactoside (IPTG). Proteins from the bacterial lysates were subjected to electrophoresis on 12% slab SDS-PAGE and blotted onto nitrocellulose membrane. An anti-HuIFNα polyclonal antibody and anti-rabbit secondary antibody, conjugated with horse radish peroxidase, were used. A 20

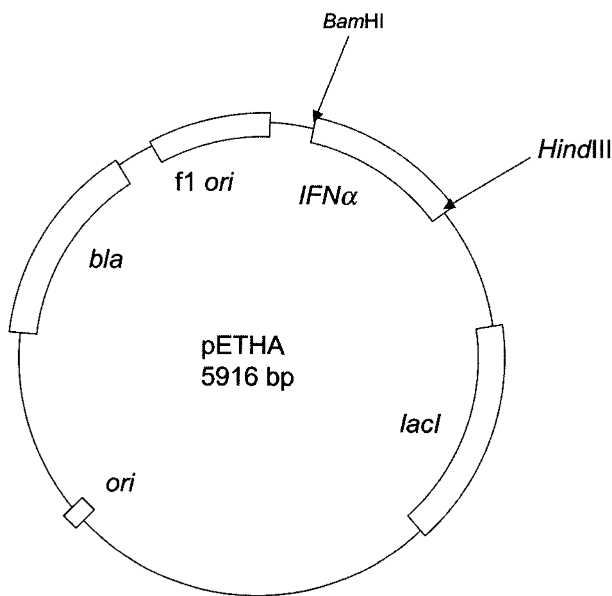

Fig. 4. Physical map of the recombinant plasmid pETHA. Features of the plasmid: ***bla***: the pBR322 β-lactamase coding sequence, ***ori***: pBR322 origin, ***lacI***: *lacI* coding sequence from *E. coli*, ***IFNa:*** the insert (HuIFNα-2b coding sequence)

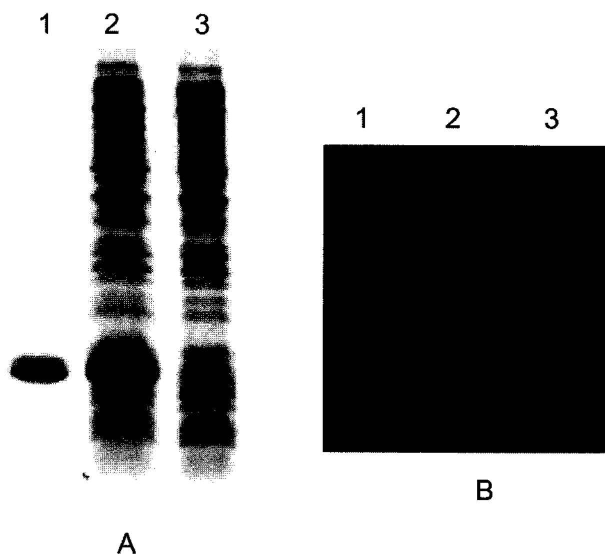

Fig. 5. SDS-PAGE (A) and Western immunoblot analysis (B) of rHuIFNα. Lanes from left to right 1. interferon-αA (sigma); 2. BL21-DE3 transformants, 4 h after induction; 3. BL21-DE3 transformants before induction

kD band, corresponding to the expected size for HuIFNα-2 protein, was observed (Fig. 5).

DISCUSSION

The cytokine, IFNα, plays a very important role in the treatment of various human diseases, including hairy cell leukemia, multiple sclerosis, laryngeal and genital papillomas, Kaposis sarcomas, acquired immunodeficiency syndrome and chronic viral hepatitis (Pfeffer, 1997). IFNα species are produced in response to a variety of inducers, such as viruses, double-stranded DNA, and T and B cell mitogens and antigens (Pestka, *et al.*, 1987; Sreevalsan 1995). Cloning of HuIFNα gene species has been carried out using a library of complementary DNA (cDNA) prepared from a template of partially purified mRNA isolated from human leukocytes (Pestka, 1997). In this study, the HuIFNα gene (without the signal peptide) was directly amplified from human leukocyte DNA and cloned into *E. coli*. The signal peptide is a short amino acid sequence, at the *N*-terminal of proteins, which help their secretion from mammalian cells. This signal is not necessary for the proteins expressed in *E. coli*.

The insert in the plasmid pETHA had 99% (460/462) identity to HuIFNα A (*homo sapiens* interferon alpha, GB: J00207) at the DNA sequence level. The expression of HuIFNα from the recombinant plasmid pETHA was confirmed by SDS-PAGE and Western blot analysis. Further studies are underway to characterize the antiviral and antiproliferative activities of the recombinant protein.

ABBREVIATIONS

PCR: polymerase chain reaction; AIDS: acquired immunodeficiency syndrome; HCV: hepatitis C virus.

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