Biotechnology International, Vol 1,No.3 , 2008

ISSN 0974-1453

Online www.bti.org.in

Published by Biotechnology Society

Biotechnology International, Vol 1,No.3 , 2008

Amplification and Cloning of human interleukin-4 gene in mammalian expression vector

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Abstract

The human interleukin-4 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* DH5α cells. The recombinant plasmid containing the human interleukin-4 gene insert in right orientation was selected after characterization using restriction enzyme analysis, directional PCR and nucleotide sequencing. The modeling of the IL-4 protein using DNASTAR and SWISS-MODEL softwares revealed the structure and epitopes of the protein.

Keywords: Interleukin-4, cloning, human, PCR, accession no. AM937235

Introduction

IL4 is an 18-kD glycoprotein. IL4 is a cytokine produced by T cells that regulates proliferation and differentiation of a variety of cells. Interleukin-4 (IL4) plays a major role in immunoglobulin E (IgE) production. Its signal is conferred to effector cells through binding to the alpha chain of the IL4 receptor (IL4RA). Osteoporosis is a common complication in

patients with the hyper-IgE syndrome , a heritable immunodeficiency in which B lymphocytes function as if exposed to excess IL4 (Leung and Geha, 1988). A number of studies have recently addressed the correlationship between diabetic pregnancy/macrosomia and differentiation of T-cells into Th1 and Th2 subsets. Diabetic pregnancy has been found to be associated with a decreased Th1 phenotype and IL-4 mRNA expression. Th2 response and its mediators: interleukin-4, interleukin-5, interleukin-10 and interleukin-13 inhibit the development of atherosclerosis. Atherosclerosis is therefore a chronic inflammatory disease, in most cases initiated by hypercholesterolemia. Treatment of mice chronically infected with H. felis with a somatostatin analog resolved the inflammation. (Zavros et. al., 2003) concluded that IL4 resolves inflammation in the stomach by stimulating the release of somatostatin from gastric D cells. In a study of Helicobacter infection and the immune response regulation of acid secretion, (Zavros et. al., 2003) demonstrated that treatment with the Th1 cytokine Ifng induced gastritis, increased gastrin , and decreased somatostatin in mice, recapitulating changes seen with Helicobacter infection. In contrast, the Th2 cytokine Il4 increased somatostatin levels and suppressed gastrin expression and secretion. The transcription factor NFATC2 controls myoblast fusion at a specific stage of myogenesis after the initial formation of a myotube and is necessary for further cell growth. By examining genes regulated by NFATC2 in muscle, (Horsley et. al., 2003) identified the cytokine IL4 as a molecular signal that controls myoblast fusion with myotubes.

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'- CTA ATT GTC TCA CAT TGT CAC TG -3' and reverse primer 5'- ACT CAT AAA TTA AAA TAT TCA GCT C -3' using the published sequence (Genbank accession no. BC070123).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 5µl of cDNA along with forward and reverse primers (25 pmol each), 200 µM dNTPs, 1.5 mM $MgCl_2$ 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 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 54°C for 50 sec and primer extension at 72°C for 1 min (Sambrook and Russell, 2001) with final extension time of 6 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 IL-4 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 1.5% 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 interleukin-4 gene using the DNASTAR software.

Results and Discussion

The 520 bp human interleukin-4 gene was successfully amplified from the total RNA isolated from PBMCs (fig 1). The gene sequence was assigned accession number AM937235. The sequence homology studies using DNASTAR software revealed 96.3% homology at nucleotide level and 100% at amino acid level with the available human interleukin-4 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 interleukin-4 gene in the recombinant plasmid designated as pTargeT.hu.IL-4 was successfully detected by release of 550 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 520 bp PCR product in correct orientation while no product was obtained in wrong orientation (fig.4).

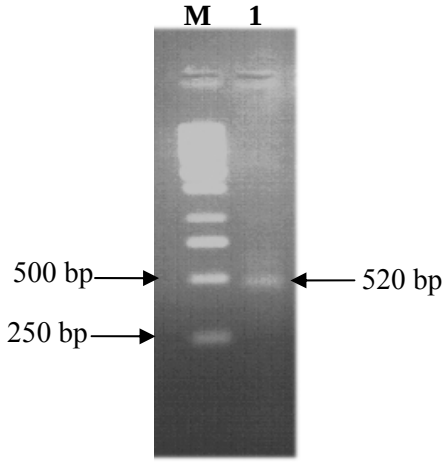

Fig. 1. PCR amplification of human interleukin-4 product size of 520bp.
Lane M: 1 kb DNA ladder,1: PCR product of human IL-4 gene 520bp

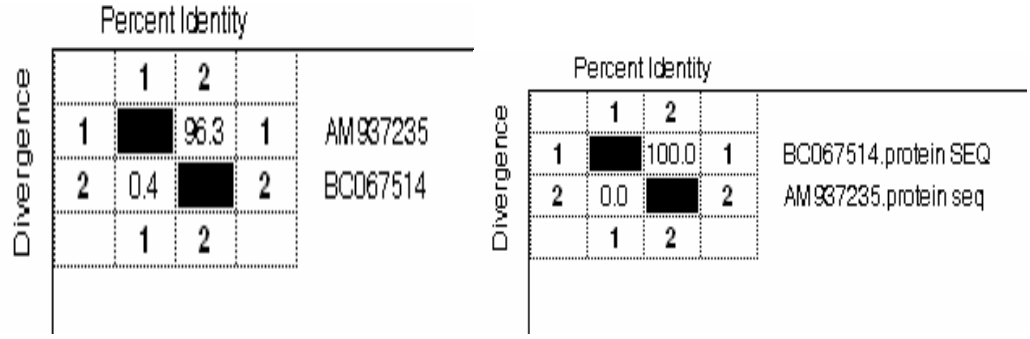

Fig 2.a Alignment of hu IL-4 gene. **Fig2b.** Alignment of hu IL-4 amino acid sequence.

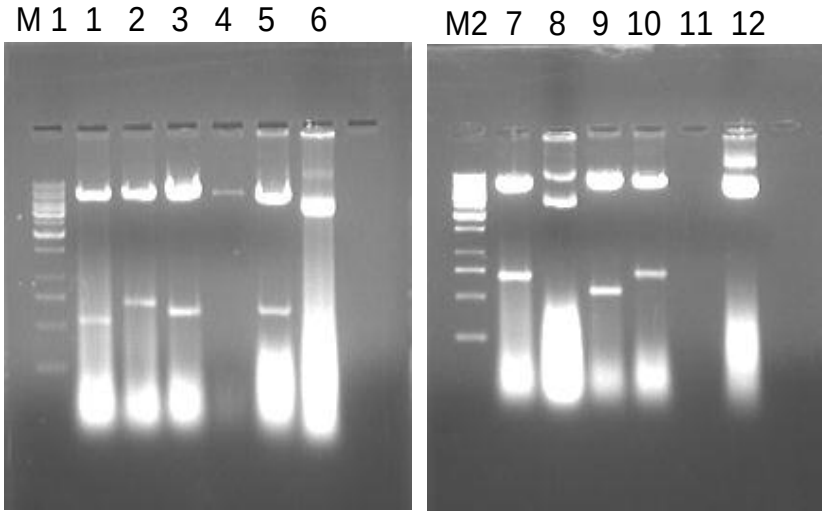

Fig 3 confirmation of IL4hu insert in r-plasmid

Lane M1, M2 : 1 kbDNA ladder, 6 and 12 : Uncut r- plasmid , 1 – 5 and 7-11 digested samples with EcoR1. Out of these 10 samples, Sample No.1, 2, 3, 5, 6, 8 and 9 have insert, as after digestion a fragment of aprox. 500bp is released.

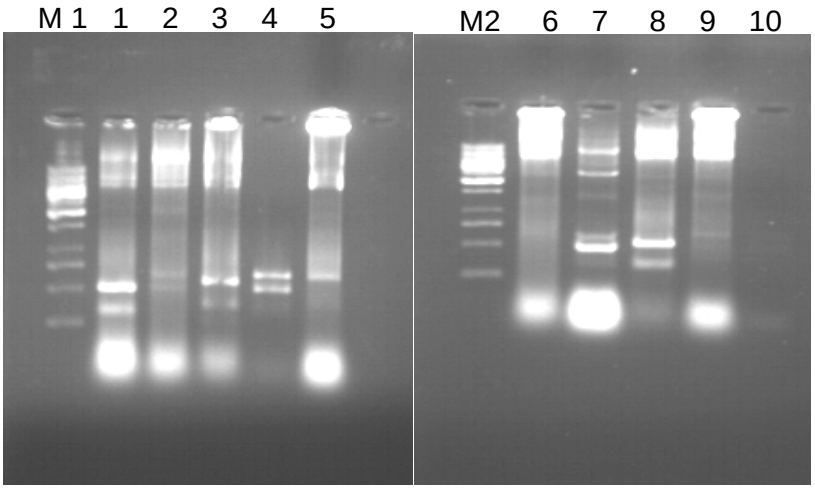

Fig 4 confirmation of IL4hu rplasmid in right orientation
Lane M1,M2 : 1 kbDNA ladder, Lane 1 – 10 PCR amplified taking T-7 primer as forward primer and gene specific reverse primer. Samples 1, 3, 4, 5, 7 and 8 are in right orientation, as an amplification of 500bp wasobtained.

Acknowledgement

The authors thank the Director, Indian veterinary research Institute, Izatnagar for providing facilities to carry out this work.

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