Research Article

Synthetic Human Erythropertin Gene-Construction, Cloning and Sequencing

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ABSTRACT

Human erythropoietin (huEpo) is a glycoprotein, with 34.4KD molecular weight, which was subsequently identified as the humoral regulator of red blood cell production. In understanding the mode of erythropoietin action is of obvious importance. It would not only serve as a useful model for studying the differentiation of development of mammalian erythroid cells, it is commonly used to treat renal of non renal anemia. Recombinant human erythropoietin (rhuEpo) and endogenous human erythropoietin (huEpo) are similar with respect to their biological and chemical properties. In this report we describe the construction of synthetic huEpo gene to produce rhuEpo. The synthetic huEpo gene were constructed by overlapping oligonucleotides assembly and amplified by polymerase chain reaction. The oligonucleotides sets showing huEpo gene sequence and two restriction enzymes were amplified using pfu DNA polymerase to produce the expected DNA products with sizes of 500 bp 600 bp. The PCR products were ligated into PGEM–T Plasmid Vector to facilitate DNA sequencing process. DNA sequence analysis showed correctly assembled oligonucleotide sets, representing the huEpo gene sequence with minor base mutations. The human Epo gene synthesized was cloned in pblue script with Nde I and EcoRI restriction sites. Ligation of the NdeI and EcoRI restrict digested vector and epo PCR product was carried out followed by transformation of the ligation mixture with DH5α competent cells which yielded two positive transformants. The recombinants were screened for gene integration by PCR with gene specific primer (500 bp). Hence, oligonucleotides assembly of PCR amplification provides a convenient and speedy method for the synthesis of rhuepo.

Keywords: HuEpo- Human erythropoietin, rhuEpo – Recombinant Human, PCR – Polymerase chain reaction.

INTRODUCTION

Human erythropoietin (huEpo) is a glycoprotein with important physiological functions, such as erythropoiesis, angiogenesis and wound healing. Erythropoietin acts directly on certain RBC progenitors and precursors in the bone marrow and controls the proliferation, differentiation and maturation of RBCs *in vitro* and *in vivo*[1-3]. In response to decrease in tissue oxygenation, Epo synthesis increases in the kidney. The secreted hormone binds to specific receptor on the surface of red blood cell precursor in the bone marrow leading to their survival, proliferation and differentiation and finally increases in hematocrit.[4,5] Human erythropoietin (huEpo) is highly effective at correcting renal anemia and non renal anemia associated with cancer. HIV infection, Myelodyplastic syndromes, prematurity autologus blood donation, bone marrow transplantation, restoring energy levels and increasing patient well being and of quality of life.[6, 7]

The large quantities of the hormone required to satisfy the clinical demand are currently met by recombinant expression in mammalian cells, namely Chinese hamster ovary (CHO) cells and baby hamster kidney cells (BHK).[8-11] currently, rhuEpo produced in CHO cells line is extensively used in the therapy to cure several anemia[12]. Oligonucleotide and polymerase chain reaction (PCR) provide the alternative in cloning characterization and expression of the gene of interest. The availability of gene sequence from Gen Bank provides the convenience to synthetically construct any particular gene. This eliminates the need to have the original gene source; the tissue sample, the cell sample or the organism itself, as well as, the necessary processing of the gene sources. Methods of DNA sequences assembly from oligonucleotides such as DNA ligase assembly, POKI gene synthesis method, self-priming PCR method and DNA shuffling are quite laborious, troublesome and time consuming[13] in this study. The synthetic huEpo was synthesized using PCR protocols which involve amplification of single step assembly of overlapping oligonucleotides and the synthetic huEpo gene was successfully constructed in a fast and convenient process.

MATERIALS AND METHODS

Desalted oligonucleotides ranging from 39 mer to mer. Restriction enzymes NdeI and EcoRI. ThermostableTaq DNA polymerase and pfu proof reading polymerase DNA ligation kit.

Oligonucleotide Design

The Oligonucleotide sets were designed according to *pichia pastoris* codons preference. There are many computer programs available for Oligonucleotide design, such as DNA works[14] which automates the process of oligonucleotide design for synthetic gene construction. As an input, the program requires an amino acid sequence of the target protein as well as any desired flanking sequences (for directional cloning). It then creates a set of oligonucleotide sequences (composing of gene of interest) that have been optimized to match the codon bias of the chosen host for expression and highly

homogenous melting temperatures of all overlapping oligonucleotide sections. Once synthesized these oligonucleotides combined and assembled in a two step PCR protocol to form the synthetic gene. (Fig.1)

MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLE
AKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQA
VEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGL
RSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKLFRVYSNF
LRGKLKLYTGEACRTGDR

Figure 1: Amino Acid Sequence of EPO Gene.

Overlapping complimentary oligonucleotide sets were used to produce huEpo gene. A single step gene assembly allowed the introduction of two restriction enzymes NdeI and EcoRI at 5' and 3' ends of huEpo gene for directional cloning. The computer program, DNA works [14] was used to design the optimal oligonucleotides sets that would enhance the hybridization efficiencies between the complimentary oligonucleotide sets; making sure the G+C contents remain in the range of 33% to 42% and the melting temperature within 60% and 77%.

Additionally primer-dimer formations were minimized and hairpin loops formations were avoided. All these would create a favorable environment for the overlapping complimentary oligonucleotide sets to correctly assemble the huEpo gene.

Gene assembly and Amplification:

The assembly of the synthetic gene from component oligonucleotides were performed according to the protocol [14] previously described. Equal volumes of the overlapping complimentary oligonucleotides were mixed together in a tube. The mixture was further diluted to give a final concentration of 0.2ng/ µl for each of the oligonucleotide. The mixture was then added into a reaction solution (20mm for tris Hcl (pH - 8.8), 10 mM for KCl, 10mM for $(NH_4)^2So_4$, 6mM for $MgSo_4$, 0.1% v/v) for triton x-100, 0.1mg/ml for Bovine serum albumin, 0.2mM for each dNTP of 2.5U for Pfu polymerase. This would further dilute the oligonucleotides 5-fold. The PCR protocol for gene assembly began with one 5 min denaturation step for 95°C during which the polymerase was added to avoid any possible mispriming. This step was followed by 25 cycles of a denaturation temp 95°C for 30s,a variable annealing temp (60°C to 77°C) for 30s and an extension temperature of 72°C for 1.5 min. The last step in this protocol was an incubation cycle at 72°C for 10 min. For gene amplification 1.25µl of gene assembly mixture was added into a 50 µl PCR reaction (1x pfu buffer, 1.5mM $MgSo_4$, 2mM dNTPs mix, 5% DMSO, 5µm each of the outermost 5'end and 3'end primers (oligonucleotides) 1.25 Unit of pfu DNA polymerase) . This would further dilute the gene assembly mixture 40-fold. Then the mixture was subjected to 20 cycles of amplification and the reaction was set up as in the assembly PCR program. The PCR product was desalted using a PCR purification kit. [15]

Cloning and sequencing[14, 15]

The synthetic gene fragments, purified by gel extraction were digested with restriction endonucleases NdeI and EcoRI and ligated into the vector PAED4[16] using 1µl T4 DNA ligase. The ligation mixture was incubated overnight at 4°C and the ligation products were transformed into DHα *E.coli* cells and selected for on LB plates with 50 µg/ml ampicillin, 0.5 mM IPTG and X-gal (50 mg/ml). Colony PCR was performed with modifications [16] on white colonies to detect the presence of huEpo gene. A white colony was picked and mixed into 25 µl PCR mixture (2.5 µl 10x Taq buffers, 2.0 Mm $Mgcl_2$, 400µm dNTPs mix, 0.4µm each of the outermost 5'and 3' end primers and 0.5 unit of Taq DNA polymerase). The mixture was subjected to a standard PCR protocol. Plasmids from the white colonies were extracted, purified and DNA sequencing was done.

RESULTS

Gene synthesis

The procedure for synthesizing genes is relatively fact of stream lined, with the most laborious step being the solubilization of lyophilized oligonuelotides. Since single strand ends of complementary DNA fragments are filled in during the gene assembly process, gene amplification reaction gave rise to a dominant single band of the correct size. (500bp) fig.2.

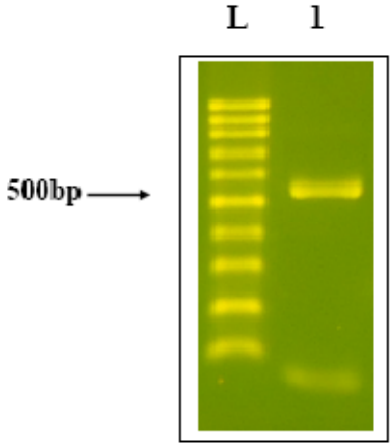

Figure 2: PCR product loaded on 2%. Agarose gel against 100bp DNA Ladder

The use of overlapping oligonucleotides necessitated the design of the oligonucleotides used for the synthesis of huEpo gene great attention to detail, owing to the requirement for a large number to be mixed in one PCR. For these reasons the designed oligonucleotides would be screened and matched in order to meet several criteria as followed.[17] (i) Elimination of palindromic sequences (ii) minimization of tandem of inverted repeats (<10bp in length) (iii) optimization of the short region overlap between each primer and (iv) allowing subsequent use of the primers for DNA sequencing. The overlapping oligonucleotides were designed to give huEpo gene construct and two restriction sites NdeI and EcoRI were

introduced at both ends of the constructed huEpo gene to allow the directional cloning of the gene into *Pichia pastoris* expression vector (pPICZ∝-A). In this study the annealing temperatures for the oligonucleotides were within the range of 55°C to 75°C to facilitate optimal PCR.

The gene assembly reaction involved the construction of the full length huEpo gene from a stoichimetic mixture of overlapping oligonucleotides. The assembly process took the advantages of complementary overlapping regions between the sense and anti-sense strands. The presence of DNA polymerase and dNTPs in the PCR cycles sealed up the gaps between the two strands and aliquot of this gene assembly reaction was then used as a template for the gene amplification process, together with the outermost 5' and 3' – end primers. Analysis of the amplification process products on 2% agarose gel revealed the presence of the expected DNA bands at 500 bp (Fig-2). The DNA bands were excised from the agarose gel and purified using a standard purification kit.

Screening of huepo clones and DNA sequencing

Transformed white colonies were screened for the presence of huepo gene by colony PCR and resolved on 2% agarose gel (fig-2). DNA bands, ~ 500 bp, were detected by ethidium bromide staining that represent huepo gene respectively plasmids from the transformed white colonies were extracted, purified and DNA sequencing was done (Fig-4). The sequencing process was carried out using the outermost 5' and 3' end primers and the DNA sequencing results show colonies carrying the correctly assembled huEpo gene. (fig-3).

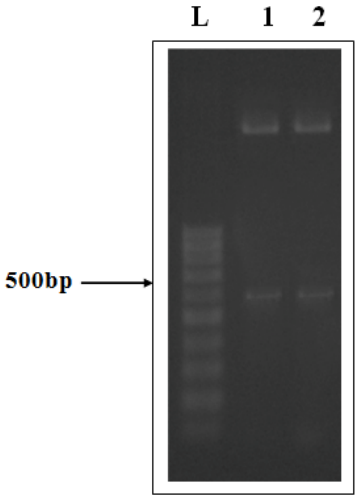

Figure 3: Restriction digestion of the probable clones (p Blue Script) with Nde I and ECORI, loaded on a 2% Agarose.

Lane Description:

1 – pBlueScript clone 1 digested with Nde I & Eco RI

2 – pBlueScript clone 2 digested with Nde I & Eco RI

L – 100bp DNA Ladder

>CLONE 1

TACTTCAGCATGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGGGGTGCACGAAT
GTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCTGTCGCTCCCTCTGGGCCTCCCAGTCCT
GGGCGCCCCACCACGCCTCATCTGTGACAGCCGAGTCCTGGAGAGGTACCTCTTGGAGG
CCAAGGAGGCCGAGAATATCACGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAAT
ATCACTGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAGGATGGAGGTCGGGCA
GCAGGCCGTAGAAGTCTGGCAGGGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCC
AGGCCCTGTTGGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCATGTGGATAAAG
CCGTCAGTGGCCTTCGCAGCCTCACCACTCTCCTTCGGGCTCTGGGAGCCCAGAAGGAAG
CCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCACTCCGAACAATCACTGCTGACACTTT
CCGCAAACTCTTCCGAGTCTACTCCAATTTCCTCCGGGGAAAGCTGAAGCTGTACACAGG
GGAGGCCTGCAGGACAGGGGACAGACACCATCACCATCACCACTAGGAATTCGAGCTCC
GTCGACA

- Restriction enzymes highlighted in yellow
- Human erythropoietin gene highlighted in blue

Figure 4: DNA sequence of Human Erythropoietin gene.

DISCUSSION

A large scale, high-through put means of generating numerous proteins depends dramatically on robust, reliable and routine methods of cloning genes and expressing protein. An approach utilizing synthetic genes presents an attractive option. The strategy described in this report, utilizing the appropriate computer software, minimizes the effort involved in designing oligonucleotides used for PCR-based gene synthesis. In many cases, the time required between identification of a protein sequence and product expression can be short, protein expression strongly depended on post-translational events. Thus, although the synthetic gene is optimized for expression, the yield of any particular protein may vary considerably.

The procedure is PCR-based single step gene assembly which comprises two main steps that are gene assembly and gene amplification. In the gene assembly step, proof reading, DNA polymerase is used to build long DNA fragment from a pool of overlapping complimentary oligonucleotides. This is followed by gene amplification step where the long DNA fragment would now serve as the template for the amplification process, using the same outermost 5' end 3' end primers.

In this work, the synthetic huEpo gene was designed according to codon preference of *Pichia pastoris,* methylotrophic yeast that has the potential to produce high protein. The assembly step of huEpo gene was impressive such that very low percent of base mutations were detected after gene amplification step, despite random oligonucleotides being pooled together in a single reaction. The use of proof reading pfu DNA polymerase certainly contributed to this phenomenon by ensuring both processivity and fidelity of the enzyme for gene assembly and amplification.

Using longer oligonucleotides has certain advantages, including the simplified gene design and a smaller number of oligonucleotides required for gene assembly reaction [18] utilization of longer oligonucleotides contribute to low stability and specificity, tendency to form secondary structure and the higher cost to synthesis longer oligonucleotides. Thus using shorter oligonucleotides

helps to avoid secondary structure formation and lowers the number of errors introduced during the assembly process. Since the frequency of PCR derived errors increases with the increasing number of amplification cycles [18]the gene amplification process was limited to twenty cycles. It would be almost impossible to eliminate the errors in random assembly reaction. However mutations in the synthetic gene could be reduced significantly by optimizations.

To conclude we have successfully constructed and synthesized huepo gene using the PCR-based single-step gene assembly and amplification. The strategy described in this report, utilizing the appropriate computer software, minimizes the effort involved in designing oligonucleotides used for PCR-based gene synthesis

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