

Synthesis and Expression of Modified bFGF Gene in *Escherichia coli* Cells

Fariba S. Mozafari* and Valery V. Bakayev

Department of Biochemistry, Pasteur Institute of Iran, Tehran 13164, Iran.

ABSTRACT

A new strategy for construction of synthetic gene encoding human basic fibroblast growth factor comprising DNA annealing-ligation and augmentation by polymerase chain reaction was introduced. The sequence of the gene and corresponding amino acid chain were modified in order to increase stability of the protein. First, 300 bp and 160 bp fragments of the gene were assembled from 18 oligonucleotides and ligated separately. Then, the shorter fragment was completed by using PCR and combined with the longer one in a proper orientation in pUC 19. One extra nucleotide that had been found in the gene after DNA sequencing and resulted in frame shift, was rectified through the use of PCR directed mutagenesis. Finally, 5'-terminal region of the gene was augmented by means of PCR in order to restore the N-terminal part of the protein and to introduce the NdeI recognition site. The gene was subcloned into the inducible pET-3a expression vector under control of T7 promoter and expressed in *Escherichia coli*. The identity of the recombinant protein and level of expression were detected by using Western blot analysis and immunoassay. The proposed method has provided a useful strategy for synthesizing modified proteins that might be applied for protein engineering.

Keywords: gene synthesis, fibroblast growth factor, polymerase chain reaction, gene expression, recombinant protein

INTRODUCTION

Since the pioneer work of Khorana and his colleagues, widespread application of synthetic genes has led to the great improvement of the chemistry for oligonucleotide (oligo) synthesis [1]. However, methods for assembling gene were the same as originally proposed. So far, more than 100 synthetic genes were assembled according to strategy based on ligation of short (15-30 bases) overlapping oligos into large blocks. The blocks were either ligated to form the gene [2, 3] or cloned separately, then combined and cloned again [4, 5].

As an alternative approach, a synthesis of oligos sharing a complementary sequence at their 3'-ends had been proposed [1]. By this method, after annealing of the oligos, DNA was completed by DNA polymerase. Although this technique was more economic, it had been used rarely for the construction of genes [6-9].

Genes coding for cell growth factors were among the first candidates for synthesis. Human basic fibroblast growth factor (bFGF) is a heparin-binding non-glycosylated single chain protein with a basic

isoelectric point (pI 9.6). It is a member of a family that includes at least fourteen structurally related polypeptides and corresponded genes probably derived from a common ancestral gene [10]. bFGF is potent modulator of cell proliferation, motility, differentiation and survival. It plays an important role *in vivo* in normal physiological processes such as embryonic development, angiogenesis, nervous system differentiation and wound repair [11]. Although recombinant bFGF has become available, it is desirable to compare features and biological activity of normal and modified factors that enables to tackle questions concerning its physiological activity. The latter is important for bFGF to be used in preclinical studies on angiogenesis in ischemia and after transplantation, on wound healing and as an essential component of serum-free culture medium.

In this report, a new strategy was applied to the construction of modified human bFGF gene. We have chosen a direct approach of synthesizing longer oligos (more than 50 bases) and assembling the whole gene from two blocks. The gene cloned in this way directed the synthesis of bFGF that showed spe-

*Corresponding author.

cific antibodies binding activity when expressed in *Escherichia coli*.

MATERIALS AND METHODS

Synthesis, Purification and Ligation of Oligonucleotides. Oligos were prepared by automated phosphoramidite chemistry using an Applied Biosystem (UK) or Novosibirsk (Russia) synthesizers. Cleavage and deprotection were carried out by standard methods. Crude oligos were purified by reversed phase HPLC and verified by a 8% polyacrylamide gel electrophoresis (PAGE). For ligation one to another, 5'-ends of oligos (1.0 μ M of each) were phosphorylated by T4 polynucleotide kinase in a buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% PEG 8000) at 37°C for 1 hour. Then, the enzyme was inactivated by heating at 90°C for 5 min. Equimolar amount of each of complementary oligos were mixed, heated at 90°C for 4 min, quickly transferred to 60°C and cooled to room temperature. Adjacent pairs were combined, and after adding T4 DNA ligase (1.0 U) were incubated at 28°C for 1 hour. We used this temperature in the reaction to avoid unwanted pairing [12].

Gene Assembly and Cloning. The bFGF DNA sequence was modified so that *E. coli* preferred usage codons were chosen. It was segmented into 18 oligos, mostly more than 50 nucleotides (nt) each with an overlap between adjacent oligos up to 15 bases. Sectional construction of the gene from two separate blocks (AB and C) was performed by combination of annealing-ligation and augmentation polymerase chain reaction (auPCR) as follows. The oligos comprising the gene were phosphorylated with the exception of the 5'-terminal oligos of each block. We preferred to ligate the oligos stepwise to avoid rearrangements since T4 DNA ligase is known to catalyze ligation at mismatch overlaps and small gaps [3]. Complementary oligos were annealed to each other in pairs, the pairs were mixed and ligated as above to produce segments. Finally, segments of each block were collected in a separate tube and incubated with additional portion of T4 DNA ligase at 15°C for 1 hour.

AuPCR with thermostable Vent DNA polymerase was done for filling gaps between oligos and extending parts of the C block. One of the structural oligos (O131) and HindIII oligo (5'-T

ATACGAAGCTTATTAGGATTTAG-3'), derived from 3'-terminal part of the gene and containing HindIII recognition site, were used for priming DNA synthesis. The mixture contained 1 μ M of each primer, 10 ng template DNA, 2 μ l 10x buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, pH 8.0, 20 mM Mg_2SO_4 , 1% Triton X100), 10.5 μ l H_2O , 0.2 mM of each dNTP, and 2 u Vent DNA polymerase (New England Biolabs, UK). In view of different melting temperature for primers, we used special thermocycling regimen: 30 cycles of denaturation at 94°C (1 min), annealing at 50°C (30 sec) for the first 10 cycles, and at 60°C (30 sec) for the rest of 20 cycles followed by elongation at 72°C for 1 min. PCR was completed by step at 72°C for 5 min. The blocks after restriction enzyme treatment were separated on a 8% polyacrylamide gel and corresponding bands were purified from the gel, separately ligated to EcoRI + XbaI (AB block) or to XbaI + HindIII (C block) fragments of pUC19 and used for transformation. HB101 *E. coli* transformants grown on LB-agar (100 μ g/ml ampicillin) were identified by restriction enzyme analysis of plasmids.

Characterization of Clones. DNA of bFGF inserts of plasmids prepared by equilibrium centrifugation in CsCl-EtBr gradients, was sequenced by a dideoxy chain termination method for double-stranded templates [13] using Automated or Macrophor DNA sequencing system (Pharmacia, Sweden).

Site-directed Mutagenesis. PCR mediated. Mutagenesis was used to rectify mutations or to generate modified sequence. Primers derived from the gene and endonuclease recognition sites were used to get the full-length gene by Vent DNA polymerase. Sequence of PCR product was verified when cloned into SmaI site of pUC 18. NdeI primer (5'-ATTACGCATATGCCAGCT CTGCCGGAAGACG-3') complementary to 5'-segment of the gene was used together with reverse HindIII primer in auPCR to introduce sequence coding for N-terminal amino acids and the NdeI site.

Production, Purification and Immunodetection of Recombinant bFGF. The verified gene was sub-cloned into the pET-3a expression vector (NdeI-BamHI) under the control of a bacteriophage T7 promoter (Novagen, USA). BL21-DE3-PlysS *E. coli*

cells were transformed with recombinant p3FGFM and grown at 37°C in LB medium (50 µg/ml ampicillin). When A_{550} reached 0.6, culture was supplemented with isopropyl-β-D-thiogalactopyranoside (a final concentration of 0.4 mM) and further incubated for 2.5 hour. Cells were collected by centrifugation and resuspended in a buffer (10% sucrose, 0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA) containing freshly prepared phenylmethylsulfonyl fluoride (PMSF, 1 mM) and hen egg white lysozyme (1 mg/ml). The cells were lysed by incubation at 4°C for 30 min and brief sonication (5 times, pulse of 30 sec). After centrifugation at 10,000 ×g for 50 min, the yield of soluble bFGF was confirmed by immunoassay with anti-bFGF antibodies as recommended by supplier (R&D, UK). A quantitative sandwich enzyme immunoassay technique was also employed to measure the amount of target protein using standard curve prepared for diluted commercial bFGF.

The extracted proteins were also evaluated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, when transferred onto nitrocellulose membrane [14]. The membrane was soaked with buffer A (0.5% BSA in PBS, 0.05% Tween 20) at 37°C for 30 min and incubated overnight at 4°C with 1/4000 dilution of the rabbit anti-bFGF antiserum in buffer A. After three-times washings, membrane was incubated with 1/10,000 diluted horseradish peroxidase conjugated to goat anti-rabbit antibodies at room temperature for 2 hours followed by three washes. Proteins were visualized by using diaminobenzidine-HCl (Sigma, Switzerland) and H₂O₂ as a substrate.

The extract prepared from 1 liter of culture was applied to a Sephadex G-50 column equilibrated with 0.5 M NaCl in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA). A flow through fraction was applied to a heparin-Sepharose column equilibrated with 0.6 M NaCl in the same buffer. After sample application, the column was washed with ten volumes of the buffer and with 1.0 M NaCl in buffer B, until the A_{280} of the effluent reached base line value. The bFGF was eluted from heparin-Sepharose with the buffer B containing 2.0 M NaCl.

RESULTS

Design of the Gene. A new multipurpose strategy for construction of bFGF gene was designed. The main body of the gene was assembled in standard way using annealing-ligation method. The terminal

parts were formed using in the augmentation PCR. This was useful for needed modification of these regions of the gene in order to change amino acid structure or to introduce endonuclease recognition sites for subcloning into expression vector. The overall gene assembly scheme, cloning strategy and oligo design are presented in Fig. 1 and Fig. 2. The sequence of a human bFGF mRNA (EMBL, accession nr. M27968) was used as the basis for the synthetic gene. Three substitutions were incorporated in the gene to produce three restriction enzyme sites that might be convenient for assembly. Additionally, we chose cysteins in positions 69 (#209-211) and 87 (#263-265) to replace for serines, creating TCT codons instead of TGC for stabilizing protein. The modified structure of recombinant bFGF could prevent antecedent oxidation of the protein and dimer formation that might secure its homogeneity during purification [12, 15, 16]. Finally, the stop codon and HindIII site were added beyond the natural TAA termination triplet.

Assembly and Cloning of Gene Blocks. The gene was constructed from two blocks, a 300 bp (AB) and a 160 bp (C) fragment, assembled independently. As a first step, subsets of oligos were ligated to generate the blocks. Appropriate oligos were assembled by annealing and multiple fragment ligation to yield AB fragment. In the case of C fragment, the combination of annealing-ligation and auPCR was undertaken. Oligo 132 was obtained of 32 nt instead of 54 nt leaving a gap of 22 nt between oligos 131 and 132. A gap of 6 nt between oligos 133 and 134 was also present (Fig. 1). To fill in these gaps and to extend the strand, auPCR was performed with oligo 131 as a forward primer and HindIII oligo as a reverse primer.

Blocks AB and C were digested with EcoRI + XbaI and XbaI + HindIII endonucleases, correspondingly. Then, they were fractionated by 8% PAGE and the size-verified DNA bands were extracted, ligated to appropriately digested, dephosphorylated pUC 19 vectors, and used to transform competent cells.

Conjunction of AB and C Blocks of the Gene. Clones containing the predicted DNA inserts were identified for further steps by double digestion of the plasmid DNA with appropriate restriction enzymes. Then, fragment AB was excised from pAB300 with EcoRI + XbaI and subcloned into pAB152 in front of C fragment in a way to construct the full-length gene in pABC450 (Fig. 2).

Samples of the constructed plasmid were pre

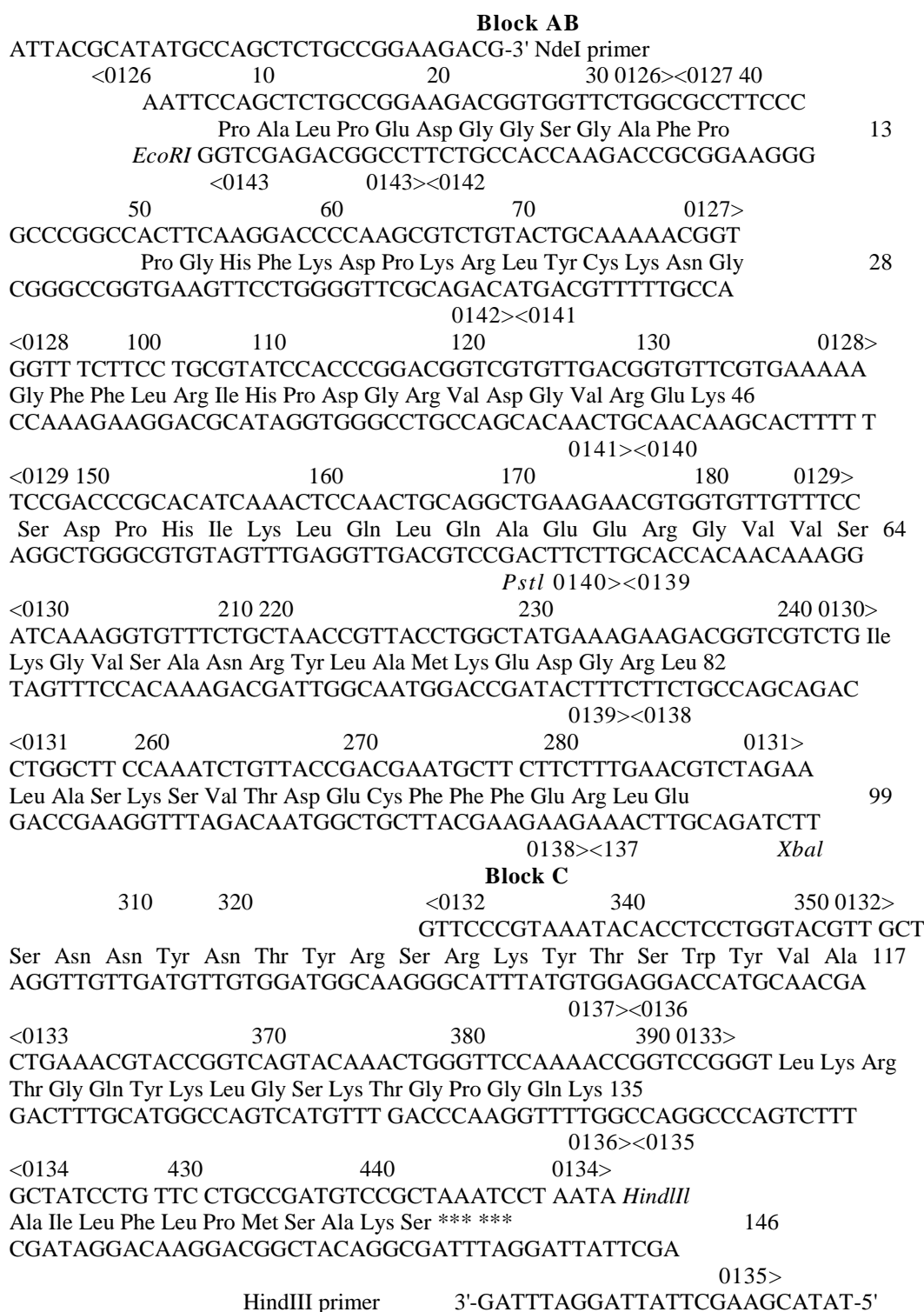


Fig. 1. Scheme of gene assembly and oligo design. Individual oligos are delineated by brackets above and below the sequence. The four restriction enzyme sites were added as a result of translationally silent nt substitutions. The sequences between *EcoRI* and *XbaI* sites (1-297 nt) and between *XbaI* and *HindIII* sites (298-459 nt) represent AB and C blocks correspondingly.

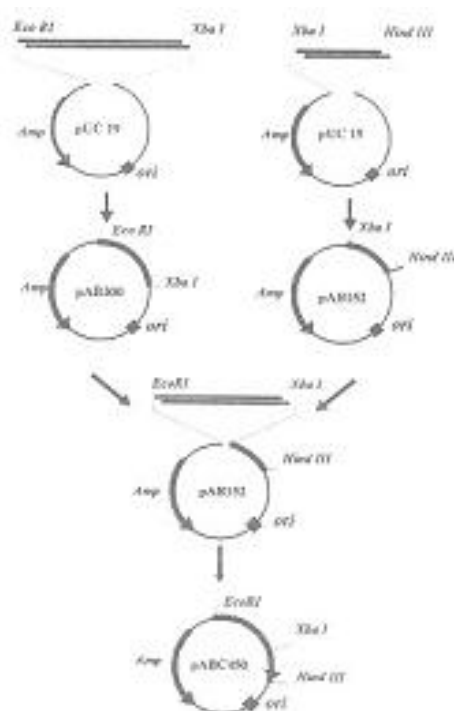


Fig. 2. Cloning of bFGF gene into pABC450. The designed oligos were annealing-ligated into two sections and cloned into pUC19 as shown schematically. The gel isolated AB section of pAB300 was assembled into EcoRI/XbaI cut of pAB152 yielding the complete bFGF gene.

pared from the culture of selected transformants, digested by EcoRI + HindIII and loaded on 8% polyacrylamide gel along with DNA size markers to determine the size of the expected bFGF gene insert. The remaining parts were treated by XbaI + PstI to approve the restriction map of the gene (Fig. 3). Each of the positive clones displayed three DNA bands corresponding to parts of the gene: band of intermediate position (XbaI-HindIII fragment, 158 bp) with mobility close to that of 154-bp HindIII fragment of pBR322, upper band (EcoRI-PstI fragment, 169 bp), and lower band (PstI-XbaI fragment, 128 bp).

Sequence Verification and Site-directed Mutagenesis.

Sequence analysis of the bFGF gene clones showed only two nucleotide changes from an expected structure: extra cytosine in #50 resulted in a shift of reading frame and a substitution of A for C (at #197) inflicted the deduced amino acid sequence (Leu instead of Ile). This substitution was not supposed to greatly influence folding and activity of the protein because of similar features of both amino acids.

To remove the extra nucleotide, PCR mediated site-directed mutagenesis has been performed with

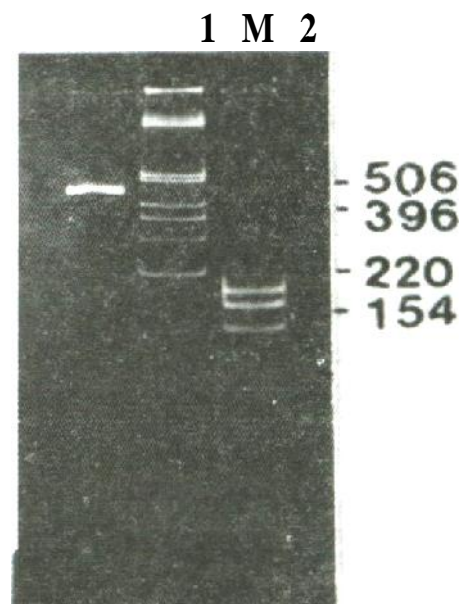


Fig. 3. 8% PAGE of cloned bFGF gene after digestion with restriction endonucleases. pABC450 DNA was digested with EcoRI + HindIII endonucleases (lane 1), then treated with XbaI and PstI (lane 2) in an appropriate buffer and loaded onto a polyacrylamide gel. M, molecular size marker (pBR322 DNA/HinfI fragments). Numbers next to the gel are length of size markers in base pairs.

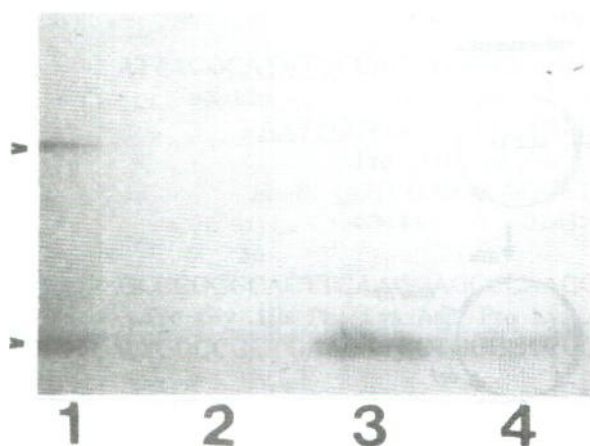


Fig. 4. Western immunoblotting of recombinant bFGF from supernatant fraction and pellet of the lysed cells. Standard bFGF (lane 1), a supernatant fraction prepared from the cells transformed with original pET3a (lane 2), the supernatant fraction (lane 3) and pellet (lane 4) prepared from the cells transformed with p3FGFM were dissolved in loading buffer and loaded onto SDS-PAGE. Then, bFGF immunoreactive bands were detected after electrophoretic transfer onto nitrocellulose membrane. Arrows indicate the position of the major and minor forms of the standard bFGF.

oligo 5' -ATAGGATCCATATGCCGGAAGAC GGGTTCTGGCGCCTTCCCGCCCGGCCAC-3' used as forward primer in combination with HindIII oligo. Then, 5'-end of the gene was extended using auPCR performed with forward NdeI and reverse HindIII primers in order to introduce three amino acids into N-terminal part of the protein and NdeI site for subcloning under T7 promoter of the pET-3a expression vector. Final product was ligated to SmaI fragment of pUC 18 and used for cell transformation. Sequencing of the corrected DNA showed rectified structure of the gene.

Construction of Expression Vector. The cloned gene was used for expression in *E. coli* cells. bFGF gene insert was excised from pABC450R with NdeI + BamHI, ligated to NdeI + BamHI fragment of pET-3a, and used for transformation of *E. coli* strain BL21 (DE3). The transformants were grown by a fed-batch fermentation and used for preparation of soluble proteins. The protein with an affinity to anti-bFGF antibodies was found in the supernatant and pellet fractions. At this stage, induction of the protein expression led to the production of similar to 0.5 mg/liter.

When examined by Western blotting using an antiserum raised against human bFGF, a major polypeptide of approximately 17,000 MW, which

was similar in size to that of standard bFGF had been seen (Fig. 4). However, an additional band of lesser intensity at higher molecular weight was observed in standard bFGF which probably resulted from intermolecular interactions. There was no band in the control sample of the supernatant fraction prepared from the *E. coli* cells transformed with the original pET-3a.

Then, recombinant bFGF was purified from the supernatant fluid by heparin-Sepharose affinity chromatography as described in Materials and Methods and the eluted proteins were shown to have specific antibodies binding activity when analyzed by immunoblotting method.

DISCUSSION

Most of the gene assemblies in the recent literature employed short oligos. As a result, many more oligos and ligation steps were required. A major problem related to the use of long synthetic oligos for DNA synthesis was an expected increase in the frequency of synthesis-derived clonal mutations. Studies on the mutational effect of varying steps in synthesis and assembly procedures are very important. They are pointed to elucidate the source of mutation in DNA, and hopefully ways to reduce them further. It was previously shown that the mutation frequency in long synthetic fragments could be quite low (0.19% per nucleotide) [17]. In this study, 450 bp fragment containing human bFGF was assembled in a two-step procedure from a total of 18 oligos by using combination of annealing-ligation and auPCR. The presence of two mutations in 450-bp gene clone (total of 900 nt) found after the assembly, gives a mutation frequency comparable with that one observed. The considerable savings in time, labor and materials demonstrated in this report should encourage the wider usage of the long oligo synthesis for total gene construction.

Part of the gene was constructed using modification of PCR as a powerful method. auPCR was introduced not only to correct mutations but also for filling gaps and extending parts of the gene. The method proposed does not rely only on DNA ligase but in addition applies thermostable DNA polymerase to build increasingly longer DNA fragments. auPCR itself is well suited for several *in vitro* mutagenesis strategies and allows to examine both the role of N-terminal amino acids, as well as the molecular basis underlying protein function. This methodology could find applicability for those

seeking an efficient and rapid way to modify parts of a large gene or gene fragment(s).

Much remain to be learned about the function of bFGF *in vivo* and its possible application. The functionally important parts of the protein and mechanisms by which they are displaying the activity are not well known. The bFGF can bind to tyrosine kinase and non-tyrosine kinase transmembrane receptors [10,18]. In order to study more thoroughly the activities of bFGF, we have constructed a bacterial expression vector by inserting modified synthetic bFGF gene into the T7 RNA polymerase-based pET3a vector. The resulting construct is able to drive the expression of a high amount of immunoreactive bFGF of the correct size in *E. coli*. Previously, several structural analogues of human bFGF have been prepared by site-directed mutagenesis of a synthetic gene and analyzed for the effect of amino acid substitutions on dimer formation and protein stability [15,16]. The altered affinity of the bFGF derivatives have been found when amino acid structure was changed in the three putative heparin-binding domains [19]. The results of this study confirm that modified bFGF could be produced in a soluble state with domination of the monomeric form. Further experiments including bioactivity assay therefore seem relevant to elucidate the probable influence of modified amino acid composition.

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