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Chemical Synthesis and Cloning of the Human Growth Hormone Releasing Factor Gene

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Human growth hormone releasing factor (hGRF) gene has been synthesized and cloned. The sequence of the synthetic hGRF gene, consisting of preferred codons for expression in *E. coli*, was designed with the aid of computer programs. Six segments with lengths ranging from 39 to 51 nucleotides were synthesized by solid-phase phosphoramidite method. The entire gene of 141 base pairs was constructed by enzymatic ligation of all synthetic segments and then cloned into plasmid pUC- 19. The positive colonies were confirmed by the screening of ampicillin resistance, inactive β-galactosidase, and analyzing by use of restriction enzymes and dot-blot hybridization. The cloned gene was sequenced by M13 dideoxynucleotide chain termination method and proven correct.

KEY WORDS hGRF; DNA synthesis; gene cloning

Human growth hormone releasing factor (hGRF) was first isolated from human pancreatic islet tumors that caused acromegaly by Guillemin et al.(1) and Rivier et al.(2) in 1982, independently, and then from human hypothalamic tissues (3). The two original hGRF are identical in primary structure and physiochemical properties. Both of them have the same sequence of 44 amino acid residues. Considerable research has revealed that hGRF is a biologically active peptide hormone, which especially induces synthesis and secretion of growth hormone in the pituitary (4).

hGRF has been successfully used in the treatment of pituitary dwarfism (5,6). Furthermore, it may be used in the diagnosis and therapy of other diseases caused by a deficiency of growth hormone. On the other hand, since hGRF has less species specificity, it also has potential applications in livestock production as a growth factor (7). However, it is difficult to isolate and purify the natural hGRF because of its limited amounts in hypothalamus. Several kinds of peptide molecules with a natural hGRF amino acid sequence, or with various amino acid substitutions, were chemically synthesized and they were biologically active (5,8). The procedures for the chemical

synthesis to produce the peptides of hGRF are, however, quite laborious and expensive. Recombinant DNA techniques have been used to prepare hGRF and its analogs (8,9) and other polypeptides with little molecular weight in recent years (10, 11). Here we describe the chemical synthesis and cloning of hGRF gene.

MATERIALS

Plasmid DNA and bacteria

Plasmid pUC-19 and pAT-153 were from Sino-American Biotechnology Co. (SABC). *E. coli* JM83 and TG-1 were kindly provided by Dr. Guo Lihe in Shanghai Institute of Cell Biology and Dr. Yang Shenli in the Shanghai Institute of Materia Medica, The Chinese Academy of Sciences, respectively.

Reagents for DNA synthesis

All reagents used in the solid-phase phosphoramidite method were provided by the Shanghai Institute of Cell Biology, The Chinese Academy of Sciences.

DNA synthesizer

The ABI-380A DNA synthesizer was from American Applied Biosystems Inc.

Enzymes and other reagents

T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase I(Klenow fragment) and all other restriction enzymes were purchased from SABC. γ-^{32}P-ATP and α-^{32}P -dGTP (10 mCi/ml, 3000 Ci/m mol) were products of Amersham Co., England. dNTP, ddNTP and isopropyl β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and M13 17-mer oligonucleotide primer were from Bio-Rad Company. Buffers for the restriction enzymes, LB media and other buffers used in the experiments were prepared as described in Ref.(12).

METHODS AND RESULTS

Gene design

The sequence of the synthetic hGRF gene was mainly designed according to the sequence of amino acids of the natural hGRF(1), and only a few amino acids were changed. A methionine was added to the N-terminal of the hGRF, the Met^{27} was substituted by a leucine and the Leu^{44} by a methionine. The designed structural gene of the hGRF consists of the preferred condons and was favorably used in *E. coli* (13). BamHI and EcoRI restriction sites were inserted at the 5' and 3' ends of the structural gene,respectively. Both sticky ends would facilitate direct cloning of the gene into the plasmid pUC-19 digested by BamHI and EcoRI.

The preliminary designed gene sequence was checked and analyzed by computer, locating the restriction sites and inverted or non-inverted sequence. According to the design principles, a few condons were modified. Some restriction sites, unfavorable to the next cloning procedure, and self-complementary sequences consisting of more than six nucleotides were eliminated, and also a single XhoI site was placed into the structure gene.

To compromise between the yield, purity and the length of the gene fragments synthesized, the full gene was divided into six fragments with a size ranging from 39-51 nucleotides, ensuring in

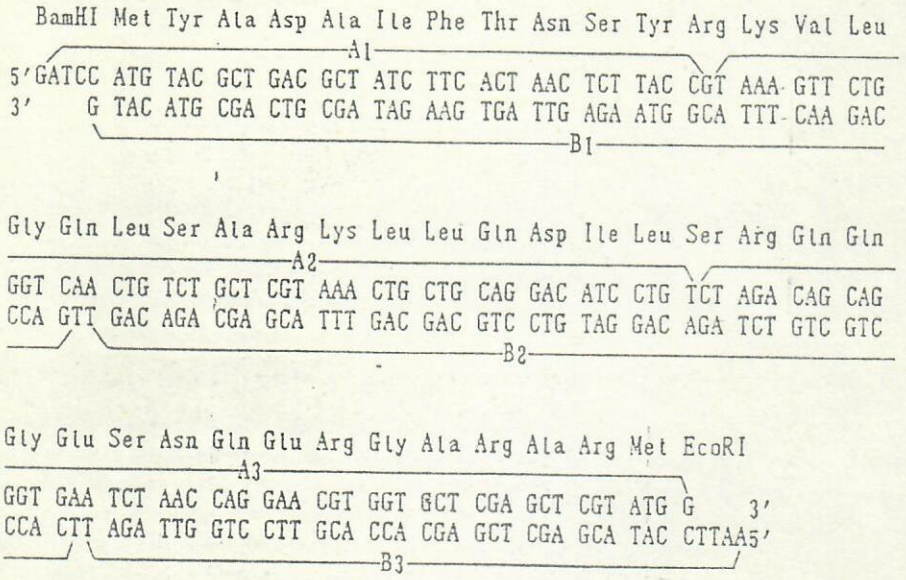

Fig.1
Designed sequence of the hGRF gene and its corresponding amino acids.
The gene was divided into 6 oligonucleotides for chemical synthesis.

each case an overlap of at least 15 nucleotides between the two complememtary fragments (Fig.1). The six fragments were synthesized separately.

Chemical synthesis of the oligonucleotides

Six oligonucleotides (A1-A3, B1-B3) were synthesized by the soli-dphase phosphoramidite method in an ABI-380A DNA Synthesizer. Controlled pore glass (CPG) was used as a solid support.

After the synthesis was completed, all protecting groups were removed by the concentrated ammonia at 55°C for 15-20h. The oligonucleotides were purified by PAGE under denaturing codition (8 *M* urea).

100 pmol/*l* of each purified fragment were labelled at 5'-and with γ-^{32}p -ATP and analyzed by 8 *M* urea-10% PAGE, followed by autoradiography to verify the purity and sizes of all the fragments (Fig. 2).

Gene construction

The synthetic fragments A2, A3, B1 and B2 were phosphorylated respectively at the 5'-end with T4 polynucleotide kinase (4u) in 20 μl reaction mixture containing 400 pmol oligonucleotides, 50mM Tris. HCl pH7.6, 10 m*M* $MgCl^2$, 10 m*M* spermidine, 10m*M* dithiothreitol (DTT) and 0.5m*M* ATP, incubated at 37°C for 1 h. The enzyme was inactivated by incubation at 100°C for 5 min. The four 5'-phosphorylated fragments and equimolar fragments of A1 and B3 were mixed and denatured at 100 C for 5 min followed by cooling slowly to room temperature. The entire gene was constructed in a ligation system containing 10 u T4 DNA ligase, 50 m*M* Tris HCl pH 7.6, 10m*M* $MgCl_2$, 10m*M* DTT, 0.5m*M* ATP, and reacted overnight at 4°C. The ligated product with 141 base pairs was

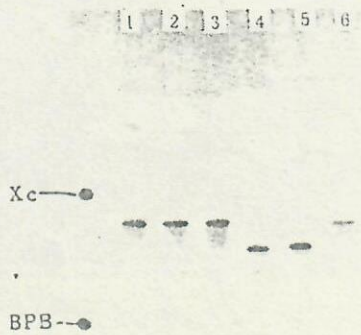

Fig.2
Analysis of the purified oligonucleotides (5'-end labeled by α- P^{32}) by 8*M* urea-10% PAGE.
1. A2, 50-mer; 2. B2,51-mer; 3. B1, 51-mer; 4. B3, 39-mer; 5. A1, 40-mer; 6. A3, 51-mer.
Xe. Xylene cyanol; Bb. Bromophenal blue.

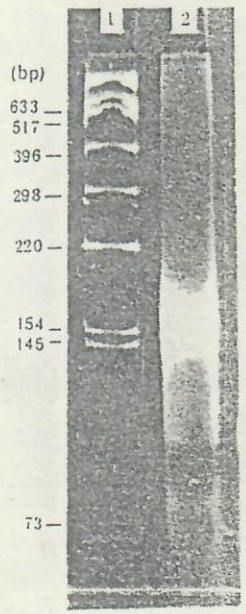

Fig.3
Purification of enzymatic ligation of the oligonucleotides by 8% PAGE.
1. Enzymatic ligation band;
2. pAT-153/ EcoRI-HinfI fragments as for molecular weight standards (values in bp);The gel was stained with ethidium bromide and examined under UV-illumination.

purified by 8% PAGE and sized by a molecular weight marker, pAT-153 HinfI-EcoRI digested fragments, and subsequently recovered according to the published procedures (14) (Fig.3).

Gene cloning

The constructed hGRF gene was cloned into plasmid pUC-19(Fig.4). The plasmid pUC-19 was digested with BamHI and EcoRI first, then the larger segments, prepared by the methods described in Ref.(12,15), and the constructed hGRF gene were mixed and ligated in the ligation system (same as described before) containing 10 u of T4 DNA ligase at 4°C overnight. The enzyme was inactivated after incubation at 65°C for 5 min. The ligation mixture was transformed 100 μl of competent *E. coli* JM83 cells (12). The transformants were selected on the plates which contained LB-agar medium plus 100 μg/ml of ampicillin and 40 μg/ml each of X-gal and IPTG. After incubation at 37°C overnight, the white colonies were selected and incubated in 5 ml LB containing 100 μg/ml ampicillin, and the recombinant plasmids were isolated by the alkaline method (12). The recombinant plasmids digested by BamHI and EcoRI were analyzed by 8% PAGE (Fig.5).

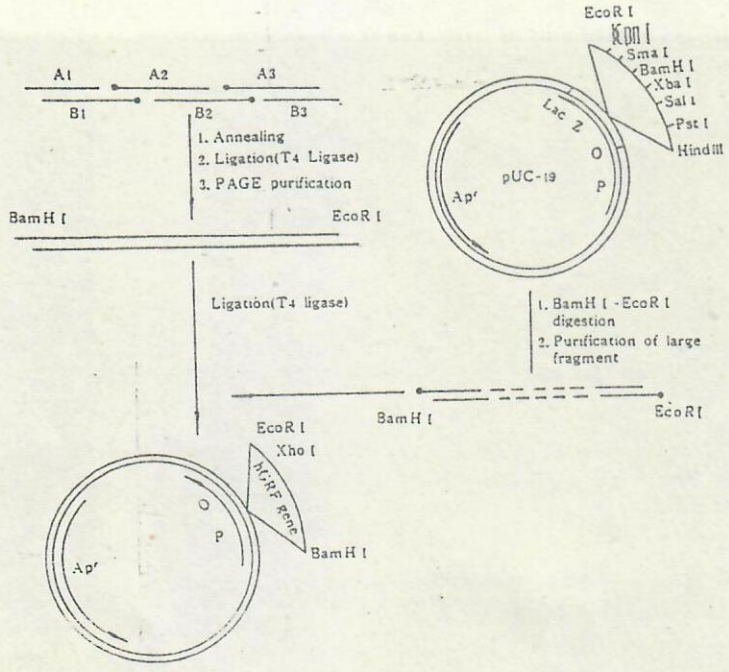

Fig.4
Construction and cloning strategy of synthetic hGRF gene (·—, 5'-phosphate).

The selected positive colonies containing recombinant plasmids were further analyzed by the following methods.

Analysis of the recombinant plasmid

Analysis by the restriction enzymes. There is an XhoI single site in the designed hGRF gene and KpnI single site in pUC-19. Recombinant plasmid and pUC-19 were digested by both XhoI and KpnI, respectively, and analyzed by 0.8% agarose gel electrophoresis. The results (Fig.6) revealed that the recombinant plasmids have indeed an XhoI site in the designed hGRF gene.

Dot-blot hybridization. Fragments B1, B2, B3 labelled with α -^{32}P-ATP were used as probes and hybridized with the crude extracts of the recombinant plasmid No. 6,7,9,10, respectively. The dot-blot hybridization was carried out by the procedure modified from Ref.(16). The result (Fig.7) confirmed that the positive colonies contained the recombinant plasmid with the designed hGRF gene.

DNA sequencing. The cloned hGRF gene fragments from the recombinant plasmid extracts were subcloned into M13mp19 RF (replicative form) DNA and transformed to *E. coli* TG-1. By using the single strand DNA of the recombinant M13mp19 as a template, M13 17 mer oligonucleotide fragments as a primer and α-^{32}P-dGTPas the radioactive source, the cloned hGRF

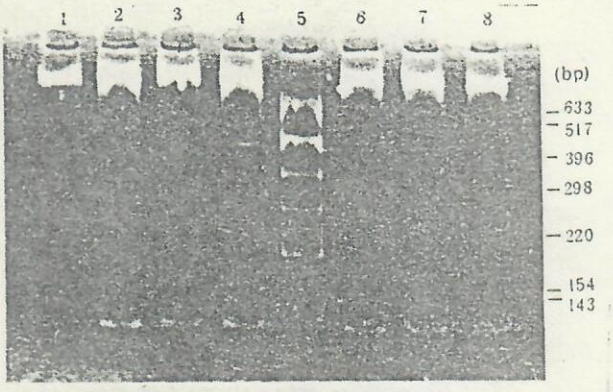

Fig.5
Examination of recombinant plasmids digested by BamHI and EcoRI.
1. Control plasmid pUC-19; 2,3,4,6,7,8. Recombinant plasmids No.10,9,8,7,6,3., respectiveiy;
5. Molecular weight standard, pAT-153/EcoRI-HinfI fragments.
Recombinant plasmids from the positive colonies were isolated by the alkaline method (12) and digested by restriction enzymes. The digested recombinant plasmids were analyzed by 8% PAGE. The gel was stained with EB and examined under UV-illumination.

Fig.6
0.8% agarose gel electrophoresis of the recombinant plasmid digested by restriction enzymes.
1,3. pUC-19 digested by KpnI and XhoI, respectiveiy;
2,4. Recombinant plasmid No.6 digested by KpnI and XhoI, respectively.

gene was sequenced by M13 dideoxynucleotide chain termination method(17). The DNA sequence of the synthetic hGRF gene was proven to be correct (Fig.8).

DISCUSSION

Our results have shown that computer program analysis is helpful to the design of a gene structure which could be beneficial for cloning, detection or even the isolation and purification of the gene. In our case, the unique Xho I site inserted is quite convenient for cloning and detection of the positive transformants, while the modification of Met^{27} —Leu, and Leu^{44} —Met could be useful to isolate the gene,if it is expressed as a fusion protein, by CNBr cleavage without damaging its biological activity.

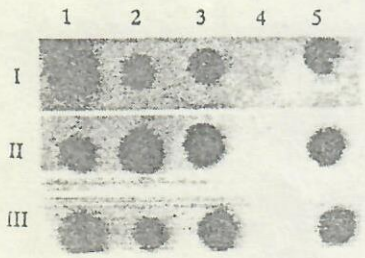

Fig.7
Autoradiography of dot blot hybridization of the recombinant plasmids with the probes labeled by α-^{32}P-ATP.
1,2,3,5. Recombinant plasmid No. 6,7,9,10, respectively;
4. Control plasmid pUC-19;
I,II,III, Probes P-B1, P-B2 and P-B3, respectively.

Fig.8
Autoradiography of DNA sequencing of the cloning hGRF gene, using the fragment 5'-GTAAAACGACGGCCAGT-3' as primer.
A. Electrophoresis for 2 h;
B. Electrophoresis for 3.5 h

In the construction of the full-length gene, the six oligonucleotides synthesized were annealled quite well without the interference of the ligation by repeated sequence or self-circulation which turned out 80% positive recombinant colonies as it was cloned in pUC-19.

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