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Gene Synthesis by a LCR-Based Approach: High-Level Production of Leptin-L54 Using Synthetic Gene in *Escherichia coli*

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Synthetic genes are very useful in genetic and protein engineering. Here we propose a general method for construction of synthetic genes. Short oligonucleotides are joined through ligase chain reaction (LCR) in high stringency conditions to make "unit fragments" which are then fused to form a full-length gene sequence by polymerase chain reaction. The procedure is simple and accurate and does not place constraints on sequence and length. In this report, a recombinant leptin gene was synthesized according to the codon preference of *Escherichia coli.* Besides, a substitution of the only Met at position 54 for Leu and an addition of a Met at the N-terminus were introduced in the synthetic gene. The gene was cloned in the pQE-31 expression vector and was expressed in *E. coli.* A large amount of recombinant leptin containing 6× His tag was produced and purified by Ni-NTA affinity column. Finally, intact leptin-L54 was released after removing the tag by CNBr cleavage at the Met residue.

Key Words: **oligonucleotide; synthetic gene; LCR; CNBr cleavage.**

Advances in the technology of oligodeoxyribonucleotide (ODN) synthesis and purification have led to opportunities for *de novo* gene synthesis. Synthetic genes are very useful when natural DNA clones are unavailable or when genes are expressed in a heterologous host, which may require optimization of codon usage. The synthetic gene approach may also be considered when mutations or restriction sites are to be introduced or when a novel gene sequence needs to be created for the expression of a man-made protein.

There are two major approaches to the construction of a synthetic gene. The first approach involves the synthesis of ODNs comprising the entire sequence. ODNs are annealed in a piecemeal fashion followed by joining with a T4 DNA ligase (1-6) which usually reacts at 37. The DNA fragments are then cloned into a plasmid vector either directly or after amplification by polymerase chain reaction (PCR). However, this approach is sensitive to secondary structures of the ODNs and adverse interactions between them often occur. The second approach is a PCR-based method (7-11) in which long ODNs belonging to the two strands of the gene sequence concerned are annealed by short overlaps. The longer ODNs are then extended by a thermal-stable polymerase using overlapped regions as primers. A full-length gene is generated after the PCR amplification step. This method is not affected by the secondary structure of the ODNs. However, synthesis of long ODNs requires hundreds of cycles of detritylation, activation, coupling, capping and oxidation reaction. The inborn mutation rate per nucleotide of long ODNs is thus significantly higher than that of short ODNs. Apparently, the success of the PCR-based approach is dependent on the derivation of a correct gene.

This paper described the synthesis of a leptin-L54 gene by a LCR-based method which circumvents drawbacks associated with the existing methods. Besides, substitution and/or addition of a methionine make CNBr cleavage feasible for removing tag peptide.

MATERIALS AND METHODS

Assembly of "unit fragment by LCR." Double-stranded fragments A and B were assembled separately by two sets of ODNs (A1–A9 and B1–B9) as shown in Fig. 1. A5 and B5 were 38mers. A9 and B9 were 20mers. The remainders were 58mers. The amino-acid codons were switched to those preferred by *Escherichia coli* and the only Met residue at position 54 of leptin was substituted by Leu. (The sequences of these ODNs are available on request.) All the ODNs except A1, A9, B1 and B9 were 5′-phosphorylated. The ODNs were custom-made and gel-purified by Oligos Etc. (Wilsonville, OR). Fifty microliters of reaction mixture contained 2.2 μM of each ODN (A1–A9 or B1–B9), 8 units Pfu DNA ligase (Statagene La Jolla, CA) and

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1× reaction buffer provided with the enzyme. LCR was conducted as follows: 95 °C 1 min; 55 °C 1.5 min, 70 °C 1.5 min, 95 °C 30sec for 15 cycles; 55 °C 2 min; 70 °C 2 min.

Fusion of unit fragments by PCR. After LCR, reaction mixture of fragment A and B sharing a 23-bp overlap (see Fig 1) were pooled and phenol-extracted. Then, 10 μl of the pooled mixture was aliquoted in 100 μl fusion solution containing 0.2 mM dNTP, 2 units Vent DNA polymerase (New England Biolabs, Beverly, MA) and 1× reaction buffer provided with the enzyme. The fusion reaction was conducted as follows: 95 °C 1 min; 55 °C 1 min, 72 °C 50 sec, 95 °C 30 sec for 10 cycles; 55 °C 2 min; 72 °C 2 min. After that, 5 ml of the fusion product was mixed in 100 μl PCR solution containing 0.2 mM dNTP, 2 mM $MgSO_4$, 0.2 μg each of the 5g -and 3-a-end primers: TT**GGATCC**GGGTATGGTTCCGATCCAGAAAGTTCA and TTAGCATTCCGGAGAAACGT (Regions complementary to the synthetic leptin gene are underlined. The *Bam* H1 restriction site introduced is indicated in bolded-letters and codons for Gly-Met are boxed), 2 units of Vent DNA polymerase and 1× reaction buffer provided. The PCR was conducted as follows: 95 °C 2 min; 55 °C 30 sec, 72 °C 50 sec, 95 °C 30sec for 15 cycles; 50 °C 1 min; 72 °C 2 min.

Cloning of synthetic leptin gene. The PCR product was purified by phenol-chloroform extraction and alcohol precipitation. The DNA was digested by *Bam* H1 and then ligated to the pQE31 vector (Qiagen, Chatsworth, CA) which had been cut at the *Sma* I and *Bam* H1 sites. Recombinant clones were derived using standard plasmid cloning procedure. The nucleotide sequences of different clones were determined by an automatic sequencer.

Production of leptin-L54. The *E. coli* SG13009 transformed with the recombinant pQE-31 vector was grown in 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) and was induced by IPTG at log phase. After centrifugation, the cell pellet was suspended in buffer (25mM Tris.HCl pH 7.6, 50mM NaCl, DNase I (150 m0g/ml)) and the bacterial cells were broken with glass beads. The inclusion bodies which were in insoluble fraction were dissolved in denaturing buffer containing 6M of guanidine HCl, 20 mM Tris.HCl (pH7.5) and 4 mM bd -mercaptoethanol. After centrifugation, the supernatant was passed through a Ni-NTA column. The column was then washed sequentially with washing buffers (6M guanidine HCl, 50 mM sodium acetate) of pH 6.5, 6 and 5.5. The recombinant leptin was eluted in washing buffer of pH 5. The protein was precipitated out by dialyzing the eluent in 1× PBS. To cleave the 6× His tag moiety, the recombinant leptin was treated with 50% formic acid containing 0.1% CNBr at room temperature for 6h. The protein was precipitated out as described above followed by dissolved in denaturing buffer. The solution was passed through Ni-NTA column to remove the 6× His tag moiety. The effluent was dialyzed in renaturing buffer (15 mM Tris.HCl pH 8.4, 10% DMSO, 2 mM cysteine) for 24h and then in buffer of 10mM Tris.HCl pH 8.4.

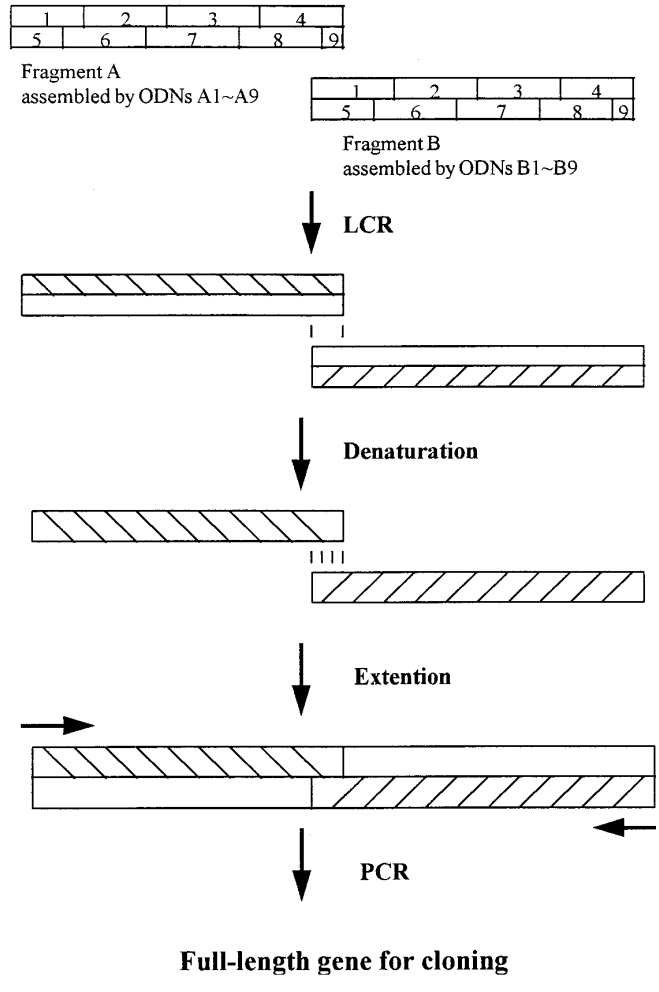

FIG. 1. A scheme for leptin gene synthesis. Fragments A and B were separately assembled by LCR using two sets of ODNs (A1–A9 and B1–B9). After LCR, fragments A and B with 23-bp overlap were fused by cycles of denaturation, mutual priming, and extension reaction. Finally, the full-length synthetic gene was amplified by PCR using 5′-and 3′-end primers.

RESULTS AND DISCUSSION

Leptin, a 16-kDa plasma protein which is secreted by adipose tissue (12), has recently been shown to control food intake and body fat in mice (13,14). Using the proposed method, we synthesized mouse recombinant leptin gene for expression in *E. coli.* To optimize the translation efficiency, codons were switched to those preferred by *E. coli.* The only Met residue at position 54 of mouse leptin (12) was substituted by Leu, which has similar steric conformation to Met. The sequence of a 441-bp synthetic gene sequence was first dissected into two unit fragments A and B with a 23-bp overlap between two fragments (see Fig. 1). Each fragment was assembled by a set of short ODNs covering both strands of the sequence (Fig. 1). Each set of ODNs was annealed and joined separately by ligase chain reaction (LCR) using a heat-stable ligase (see "Materials and Methods"). Short ODNs have lower inborn mutation rate. However, they create more ends to be joined. A high efficient ligation method, LCR, can thus overcome this problem. Since annealing and ligation were performed at higher temperature (high stringency), any secondary structures in the ODNs were diminished and ODN pairs containing mismatches were rendered unfavorable for pairing and ligation. Larger amounts of intact fragments A and B were generated through repeated cycles of ligation reaction (Fig. 2, lanes 2 and 3). After LCR, aliquots of the reaction mixtures of fragments A

and B were pooled. The two fragments containing 23-bp overlap were fused through an extension reaction (Fig. 1) and finally, the full-length leptin gene was PCR-amplified (24). The PCR product (Fig. 2, lane 4), which showed a single band with expected length, was ready for cloning plasmid.

We had introduced a *Bam*H I cutting site and Gly-Met codons in the PCR 5′-primer. The PCR product was inserted into pQE-31 expression vector. The recombinant leptin-L54 expressed by this vector is expected to have an amino-acid sequence configuration as shown in Fig. 3A. The leptin genes isolated from four independent clones were sequenced. It was found that three of them had the correct sequence, and the fourth contained a single nucleotide substitution.

The 17-kDa recombinant leptin was produced upon IPTG-induction of the transformed *E. coli* SG13009. The recombinant leptin reached 30% of total cellular proteins, and formed inclusion bodies (Fig. 3B, lanes 2–4). Recombinant leptin was purified by passing a Ni-NTA affinity column (Fig. 3B lane 5). The 16-kDa intact leptin-L54 was released after CNBr cleavage at Met residue (Fig. 3B lane 6). A Gly introduced next to the Met just leaves no steric hindrance for the reaction. The removal of 6× His tag was verified by western blot using antibody against 6× His tag moiety (Fig. 3C). We had also done amino-acid sequencing for leptin-L54 protein. Result indicated the cleavage site and N-terminal sequence (legible from amino acid 1–24) were correct. The large amount of nearly pure protein are applicable in different purposes. It can be used as an antigen for raising anti-leptin antibody. The relation between the structure and function of leptin can be studied by amino-acid changes of the synthetic gene.

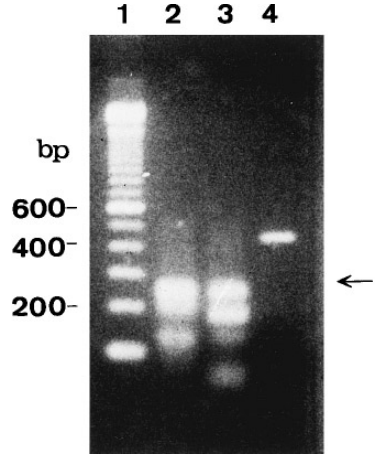

FIG. 2. Analysis of DNA fragments synthesized. Fragment A (lane 2) and fragment B (lane 3), which were generated by LCR, are indicated by an arrow. The full-length leptin gene produced by fusion of fragments A and B after PCR is shown in lane 4. Lane 1 is 100-bp DNA ladder (GibcoBRL, Gaithersburg, MD).

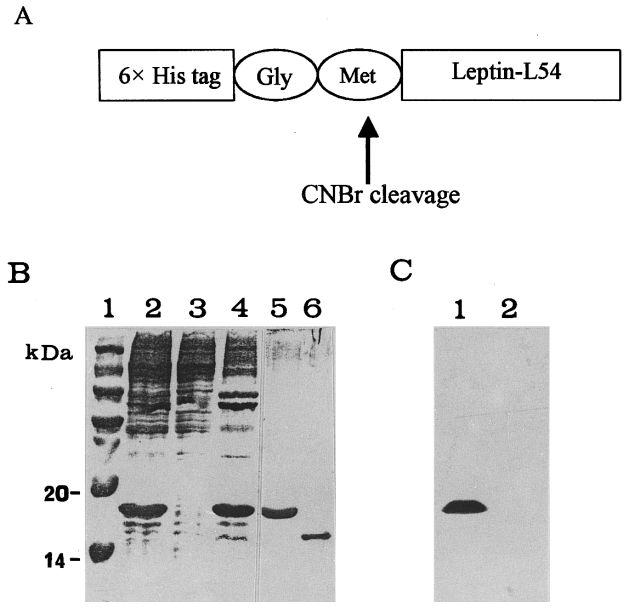

FIG. 3. (A) The amino-acid configuration of recombinant leptin produced and cleaving site for CNBr. (B) Molecular weight of markers (lane 1); total cellular proteins of transformed *E. coli* SG13009 induced by IPTG (lane 2); cells were broken by glass beads and fractionated by centrifugation: supernatant (lane 3), pellet (lane 4); recombinant leptin purified by Ni-NTA column (lane 5); leptin-L54 released by CNBr cleavage (lane 6). (C) Western-blot detection of 6× His tag moiety by antibody against the tag peptide (Qiagen, Chatsworth, CA): uncut recombinant leptin (lane 1); leptin-L54 (lane 2).

The biological activity of renatured leptin-L45 had also been verified. C57BL/6J (ob/ob) mice, deficient in leptin, were injected with leptin-L54 (5 m g per day) intraperitoneally for 2 weeks. It was found a 17% decrease in food intake concomitant with a 25% decrease in body-weight gain for those treated mice (data not shown).

In summary, the LCR-based gene synthesis is simple and accurate and is independent of the nature of the nucleotide sequences. For construction of longer synthetic genes, the procedure may be carried out by dissecting the gene into several overlapped unit fragments and following the same approach.

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