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A preferable approach to clone hLIF cDNA from the genomic DNA

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Abstract Complementary DNA (cDNA) is valuable for investigating protein structure and function in the research of life science, but it is difficult to obtain by traditional reverse transcription. In this study, we employed a novel strategy to clone the human leukemia inhibitory factor (hLIF) gene cDNA from genomic DNA directly isolated from the mucous membrane of mouth. The hLIF sequence can be acquired within a few hours by means of amplification of each exon and splicing using overlap-PCR. Thus, the new approach developed in this study is simple, time- and cost-effective, and it is not limited to particular gene expression levels of each tissue.

Keywords: cDNA cloning, hLIF, oral mucous membrane, overlap PCR

INTRODUCTION

In the molecular biology fields, cDNA cloning is one of the most frequently used technologies, and is the first and essential step in genetic manipulation. The traditional method to obtain the cDNA fragment is based on reverse transcriptase PCR amplification (RT-PCR). In short, the RNA is transcribed into cDNA which is used as a template for subsequent PCR with primers designed according to the target gene (Bertrand et al. 1994). However the cDNA is difficult to be acquired since gene expression could vary in each tissue influencing mRNA obtainment; mRNA preparation, which could be influenced by RNases, a very stable and difficult enzyme to remove and that degrades mRNA; and reverse transcription could fail (Eikmans et al. 2000; Bustin and Nolan, 2004). It's clear that cDNA cloning is exposed to some challenges.

As it is well known, the gene sequence contains exons and introns alternately, where the exons can reverse-transcribe into cDNA. So the overlap-extension PCR (Young and Dong, 2004) and successive PCR (Xiong et al. 2004) and assembly PCR (Hoover and Lubkowski, 2002; Xiong et al. 2006) can be used to synthesize the cDNA of the target gene. Assembly PCR was originally adopted in DNA shuffling for *in vitro* evolution of DNA molecules (Stemmer, 1994). With the improvement in genomic sequencing and databases, it has become easier to acquire information interrelated to any gene. However, a few studies have reported about the cloning of Bioactive cytokine directly from genomic DNA, since these cytokine genes have been observed in specific tissues, such as the hLIF gene that exist only in the blood and the embryo.

Here, we report a novel method to synthesize hLIF cDNA based on overlapping-PCR directly from the oral mucous membrane. Briefly, the exons of hLIF gene are PCR-amplified separately by using a pair of primers containing homologous sequence with two adjacent exons, which can be spliced together in their spontaneous order.

MATERIALS AND METHODS

Enzymes, kits and chemicals

Pfu DNA polymerase, DNA marker I, DH5α competent cells were obtained from Takara Biotech Co., Ltd. (Dalian, China). RNAprep pure Blood kit, TIANamp swab DNA kit, TIANscript RT Kit were purchased from Tiangen Biotech Co., Ltd (Beijing, China). pGEM®-T easy vector, Wizard SV Gel and PCR Clean-up System were purchased from Promega Biotech Co., Ltd (Madison, WI). IPTG, X-Gal, and other chemicals were obtained from BaoXin Scientific Equipment Co., Ltd (Yangling, China).

Primer design

The human leukemia inhibitory factor (hLIF) mRNA sequence was obtained from NCBI Genbank database. The hLIF sequence which is 609 bp and composed of 3 exons, so two pairs overlapping primers must be designed. Oligonucleotides were synthesized and purified by Genscript Co., Ltd (Nanjing, China).

Isolation of total RNA and genomic DNA

With the approval by the Institution Review Board for ethical conditions, the total RNA of whole blood collected from a healthy donor was extracted with RNAprep pure Blood kit (TIANGEN) according to the manufacture's instructions, which was used in the following overlap-PCR; the genomic DNA was extracted with TIANamp swab DNA kit (TIANGEN) from the oral mucosa for the RT-PCR. The purified RNA or DNA was dissolved in 30 µl TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and its concentration was determined by a spectrophotometer-ND-1000.

cDNA clone of hLIF using overlap-PCR

First, the optimal annealing temperature was determined by gradient PCR, and then completed exons amplification using the following primers:

P1 (exon 2): 5'-ATGAAGGTCTTGGCGGCAG-GAGTTGTGCCCCTGCTGTTG-3',

P2 (exon 2): 5'-*CCCCTGGGCTGTGTA-ATAGAGAATAAAGAG*GGCATTGG-3';

P3 (exon 3): 5'-*CTCTTTATTCTCTAT-TACACAGCCCAGGGG*GAGCCG-3',

P4 (exon 3): 5'-GCTAGAAGGCCTGGGCCAACACGGCG-3'.

The italic 30 bases are the overlapping region at the exon-exon joining sites, which made it easier to splice the exons. For each exon, a standard PCR was performed with the following 50 µl reaction mixture, which included 0.4 µg genomic DNA extracted from the oral mucosa, 1.0 µM of each primer, 5 µl dNTP mix (0.2 mM of each), 5 µl 10 x Pfu Buffer with $MgSO_4$, 0.6 µl Pfu DNA polymerase (2.5 u/µl). Exons amplification was completed in individual tubes on a Bio-RAD PCR system using the following thermal cycling conditions: initial denaturation at 95ºC for 3 min, followed by 30 cycles of denaturation at 95ºC for 30 sec, annealing at 58ºC for 30 sec, and extension at 72ºC for 30 sec, and a final extension for 8 min at 72ºC.

The PCR products were purified and mixed, and used as a template in the followed overlap-PCR. In the overlapping PCR, the primers were the forward primer of the second exon and the reverse primer of the third, and the other components were the same with above, except for the extension time adding to 45 sec. After the full-length ORF sequence was amplified and all the PCR products were subjected to 1.5% (w/v) agarose gel electrophoresis. The gel containing appropriate DNA segments were excised and the segments were purified with Wizard SV Gel and PCR Clean-up System (Promega).

The hLIF clone by RT-PCR

The purified RNA was the first-strand of cDNA in the process of reverse transcription by TIANscript RT Kit. The first-strand of cDNA was used as the template in the RT-PCR to obtain the coding sequence of hLIF gene, and the PCR components and parameters were as the above.

Cloning and sequencing

The resultant hLIFs were cloned into pGEM®-T easy vector. DNA sequencing was carried out by Genscript Company.

RESULTS

cDNA clone of hLIF using overlap-PCR

Exon 2 and exon 3 of hLIF were amplified using a standard PCR, and the 30 databases overlapping region were taken into each exon following the exons amplification, which made it easier to splice the exons. Primers P1 and P4 were used to perform another universal PCR to splice the exons. The PCR product, and two of the exons (exons 2 and 3 were demonstrated on 1.5% agarose gel electrophoresis (Figure 1). The predicted length of exon 2, exon 3 and the hLIF gene are 213 bp, 426 bp, and 609 bp respectively. These segments were sequenced and blasted with Genbank database, and the results indicated that coding sequence of the hLIF was harbored correctly. Also, exons 2 and 3 were successfully spliced, and there were an average of 0.5 point mutations per clone, which were silent mutations (Figure 2a).

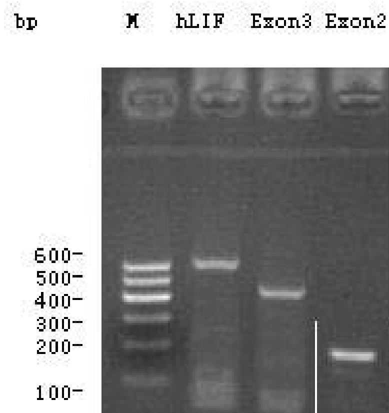

Fig. 1 Amplification and splicing of hLIF coding sequence by exon-splicing. The exons were amplified by the standard PCR with the exons own primers, and that hLIF was performed by the overlapping PCR with P1 and P4. The length of the performed fragments of exon2, exon3 and the full-length hLIF is 213 bp, 426 bp, and 609 bp respectively. “M” is DNA marker I.

The hLIF clone by RT-PCR

We performed RT-PCR to obtain the coding sequence of hLIF gene, which was demonstrated on 1.5% agarose gel electrophoresis (Figure 3). DNA sequencing of the resultant hLIFs was carried out by Genscript Company. The mutation rate was higher in the hLIF gene by reverse transcription comparing with overlap-PCR, and the mutations resulted in the changes of amino acid, even ORFs. The results were showed in Figure 2b to blast with Genbank database.

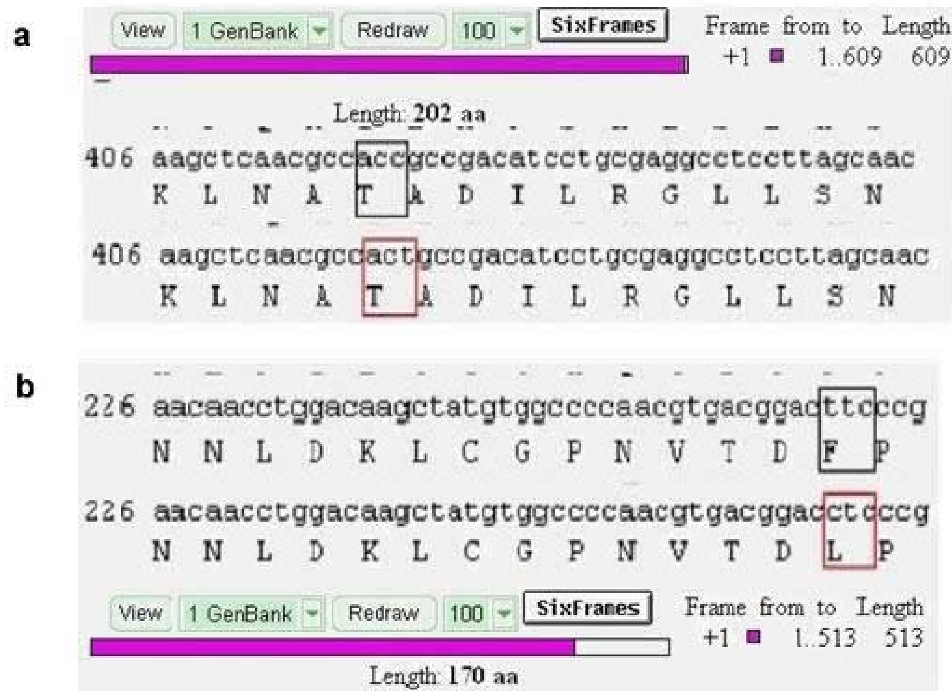

Fig. 2 The results blasted with Genbank database. (a) The cDNA of hLIF obtained using the overlap PCR. The length of ORF of hLIF is 609 bp, which is correct. Even though the C become to T, the amino acid is unchanged, and that is the silent mutation. (b) The resultant hLIF cDNA by RT-PCR. The figure indicated the amino acid and the ORF were changed because there existed the mutation.

DISCUSSION

The gene synthesis based PCR is now frequently used in molecular biology laboratories worldwide, but there were a few reports about cDNA cloning of Bioactive cytokine directly from genomic DNA. So we employed a novel strategy to clone the human leukemia inhibitory factor (hLIF) gene cDNA from genomic DNA, directly isolated from the mucosa membrane of mouth.

In the traditional RT-PCR, it is necessary to extract total RNA and in this case from a human source, so we must consider how to collect these samples. Here we acquired the genomic DNA using TIANamp swab DNA kit, which uses swabs in order to obtain cells from the oral mucous membrane for further DNA extraction. Also, the genomic DNA was easily extracted within 90 min. So, the method to obtain the genomic DNA is simple, rapid, reliable and stable and omits the complex process when extracting RNA.

An *in vitro* exon ligation method was proposed by Lebedenko et al. (1991), and the entire process included two-step PCR to amplify the exons, cloning of all exons separately, and introduction of specific restriction enzymes and ligation of multiple restriction fragments. An et al. (2007) proposed a novel PCR mediated Genomic DNA Splicing (GDS strategy) for cloning any eukaryotic cDNA or coding sequence from a genomic DNA preparation, but it needed to design the exons primers, overlapping primers and outmost primers separately. Also, it was proposed a method to synthesis cDNA based on the use of the class IIS restriction enzymes to acquire the coding sequence of Hoxa7 gene, so the matching restriction enzymes must be taken into account at the exon-exon joining sites (Xiao-Xia et al. 2008). Due to the specificity of the gene, the coding sequence (19 bp) of the first exon can be spliced to the second exon fragment in the form of the deliberately designed primers, like adding the restriction enzyme sites.

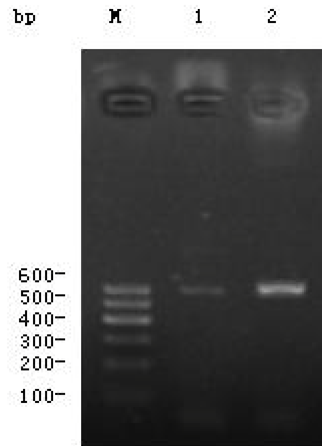

Fig. 3 Amplification of hLIF coding sequence by RT-PCR. The coding sequence of hLIF gene was successfully reverse transcribed and amplified, which is 609 bp (lanes 1), and lanes 2 was the full-length hLIF by exon-splicing. The figure indicated that it is more easily to obtain the coding sequence of target gene by exon-splicing in terms of quantity, compared with the traditional reverse transcription. "M" is DNA marker I.

Although the coding sequence of hLIF gene is composed of only three exons and only 19 bp is the coding sequence, located in the first exon, two pairs of overlapping primers must be designed. The coding sequence of 19 bp in the first exon should be designed in the primer like adding the recognition sequence of the restriction enzymes. Then the cDNA sequence (only 19 bp) of the first exon can be connected to the other spliced fragments. Only a few hours are needed in the whole splicing process, which employs two rounds of PCR amplification.

LIF derives its name from its ability to induce the terminal differentiation of myeloid leukaemic cells. LIF is normally expressed in the trophectoderm of the developing embryo, with its receptor LIFR expressed throughout the inner cell mass. Therefore the gene only exists in the blood and the embryo, making it difficult to obtain the cDNA of hLIF by RT-PCR. The gene synthesis strategy provides a feasible way to clone the gene of bioactive cytokine. It is not necessary to consider the expression level, the quantity of mRNA and any specific tissue for a particular target gene (An et al. 2007)

In conclusion, with a new gene synthesis strategy, “PCR-based genomic exons splicing”, we successfully acquired the full-length 609 bp coding sequence of hLIF gene, which consist of three exons. The approach is simple, rapid, with high fidelity, highly efficient and cost-effective without RNA preparation and cDNA synthesis. Also, it does not consider tissue specificity and the expression of the target gene, and unassociated segments without restriction enzymes and DNA ligase.

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