CONSTRUCTION OF EUKARYOTIC EXPRESSION VECTOR WITH GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR GENE

ZHENG Qiu-hong 郑秋红, ZHENG Tian-rong 郑天荣, XIE Yun-qing 谢云青
LU Lin 卢林, CHEN Hui 陈晖,

Department of Tumor Molecular Biology, Fujian Tumor Hospital, Fuzhou 350014, China

ABSTRACT

Objective: To construct the eukaryotic expression vector that express human granulocyte-macrophage colony-stimulating factor (hGM-CSF) gene for making highly express in mammalian cells. Methods: Extract totally RNA from the induced human fetal lung (HFL) cell line. HGM-CSF cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR), and then directionally subcloned into the HindIII and EcoRI site on the pcDNA3.1 plasmid, which was controlled by the CMV promoter, to form the recombinant expressing vector pcDNA3.1-GM-CSF. Results: The PCR amplification was identified and the sequence was analyzed, the results showed that hGM-CSF was properly inserted into the vector and the sequence was correct.

Key words: Human granulocyte-macrophage colony-stimulating factor (hGM-CSF), Reverse transcription and polymerse chain reaction (RT-PCR), Eukaryotic expression

Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) has been widely used in clinic since it was cloned in 1985.[1] It is not only an effective hematopoiesis growth factor, but also a cytokines that can stimulate the powerfully efficient and persistent antitumor activity in all body.[2] In this study, the human GM-CSF gene was amplified by RT-PCR, and then reconstructed into eukaryotic expression plasmid to make it efficiently and persistently express in mammalian cells. These results provided a basis for study of GM-CSF in cancer gene therapy.

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Correspondence to: Zheng Qiu-hong, Department of Tumor Molecular Biology, Fujian Tumor Hospital, Fuzhou 350014, China; Phone: (0086-591)-3660063 ext. 8416; Fax: (0086-591)-3660011; E-mail: ZQH@pub2.fz.fj.cn

MATERIALS AND METHODS

Cell Culture and Isolation of RNA

The human fetal lung (HFL) cells (a gift from Prof. Wei He, Institute of Basic Medical Sciences, Chinese Academy of medical Sciences) were cultured in RPMI-1640 medium supplemented with 70% coverage.[3] Total cellular RNA was extracted from the activated cells according to the reference of Trizol™ Kit (GIBCO-BRL).

Synthesis of Oligonucleotide Primers

The primers were designed according to the sequence of hGM-CSF cDNA reported by Wrong,[1] from the started AUG coding the signal peptide. Two pairs of primers were synthesized here. Primer 1 was designed according to the 5' end sequence of GM-CSF cDNA, added up HindIII restrict endonuclease site and several guard base pairs, and primer2 to the 3' end sequence, added up EcoRI site and several base pairs as well. The sequence of each was:

primer 1: 5'-GCTCAAGCTTCTGGAGGATGTGGCTGC-3';
primer 2: 5'-GGACGAATTCACTCCTCGAATGGCTCCC-3'.

Primer 3 and primer 4 were tow inner primers which were designed according to the internal sequence of GM-CSF cDNA and used in nested primer PCR(NP-PCR) for further identified. The sequence of them were:

primer 3: 5'-CTCCTGGGCACTGTGGC-3';
primer 4: 5'-GCTCTTAGCAGTCAAAGG-3'.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was reversely transcripted to first cDNA chains according to the reference of Super Script™ Kit (GIBCO-BRL). The product was

amplified by PCR. Each cycle was set for 3 minutes at 94°C firstly, then followed by 1 minute at 95°C, 1 minute at 60°C, 5 minutes at 72°C for 30 cycles, and 5 minutes at 72°C finally. This reaction was gone on at PCR heating block (PE). The production was electrophosised on 1.5% agarose gel with EB.

Nested Primer PCR (NP-PCR)

According to PRINCIPLE OF GENETIC ENGINEERING[4] and PCR OPERATING AND APPLICATION GUIDE,[5] pcDNA3.1-GM-CSF was used as template in the first PCR. In this reaction, only primer 1 and primer 2 were used. After 20 cycles, the reaction was stopped. And then using the production (5μl) as template in the once more PCR. Primer 3 and primer 4 were used here, and 30 cycles were needed in this reaction. The final production was elelctrophoresised on 1.5% agarose gel with EB.

Construct pcDNA3.1-GM-CSF Eukaryotic Expression Plasmid

Referring to MOLECULAR CLONING,[6] the reconstructed plasmid was extracted, digested and purified. The GM-CSF gene was inserted into pcDNA3.1 (a gift from Dr. Li Xue, Institute of Basic Medical Science, Chinese Academy of Medical Sciences). The PCR production digested by EcoRI/HindII (promega) was directionally subcloned into EcoRI/HindIII site of pcDNA3.1 with the action of T_4 ligase (promega). The recombined plasmid pcDNA3.1-GM-CSF was transferred into $DH_{5\alpha}$ (supplied by Dr. Li Xue). The positive colonies were picked out randomly from LB solid medium with 60μg/ml Ap. The plasmid was extracted, and digested by EcoRI/HindIII, the identified by electrophosis on 1.5% agarose gel with EB. The pcDNA3.1-neo was set as negative control.

GM-CSF Gene Sequence Analysis

The recombinant plasmid was extracted, and the sequencing template was purified. Nucleotide sequences were determined from T_7 promoter by using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit.

RESULTS AND DISCUSSION

Amplification of Human GM-CSF DNA

GM-CSF can be secreted by mitogen activated T lymphocytes and macrophages. Owing to the lower mRNA level, it was a heavy work that the GM-CSF gene used to be isolated from DNA libraries by hybridization. In this study, using the RT-PCR method, human GM-CSF was specifically amplified. Total RNA was extracted by Trizol™ Reagent from HFL cells, which were induced by TNF-α to get abundant mRNAs. From the distinct 18s and 28s RNA band, it can be concluded that the extracted RNA was total, shown in Figure 1. cDNA was obtained from the total RNA by RT-PCR. The amplified product was obtained by PCR in which primer 1 and primer 2 were used. The size of the production was 462 bp which was just the same as the predict, shown in Figure 2. To ensure that the hGM-CSF sequence was correct, the amplified production was identified by BglI enzymatic digestion firstly, the result showed 203bp and 241bp fragment, according with prediction, shown in Figure 3.

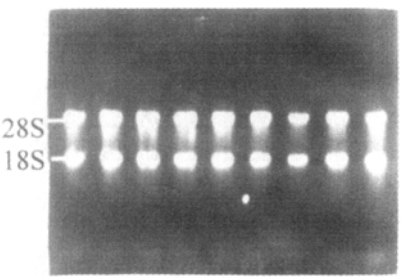

Fig. 1. Gel electrophoresis of total RNA extracted from HFL cell

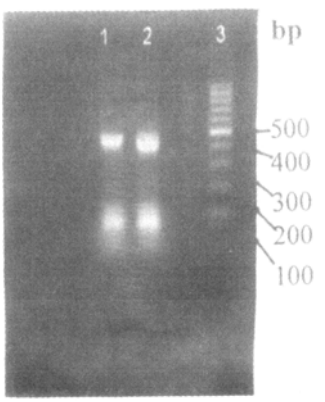

Fig. 2. Gel electrophoresis of amplified rh GM-CSF cDNA (2.0% agarose gel)
1-2: amplified hGM-CSF DNA;
3: 100bp Lamda DNA Marker

Nucleotide Sequence of the Amplified hGM-CSF DNA

The DNA contains a single open reading frame including all codons, encoding the amino acids in hGM-CSF mature protein and signal peptide with the first ATG located at 11th nucleotide from the 5' end followed by 144 codons before the termination triplet

TGA at nucleotide positions 441-443.

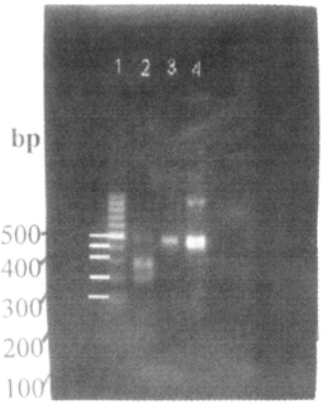

Fig. 3. Restriction enzymatic analysis of the PCR production. 1: 100bp Lamda DNA Marker 2: PCR product/BglI 3. PCR product/EcoRI, HindIII 4. PCR product

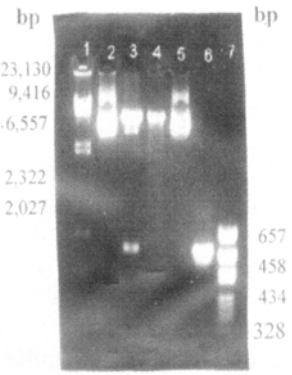

Fig. 4. Restriction enzymatic analysis of the pcDNA3.1-GM-CSF plasmid. 1: λ DNA/HindIII marker; 2: pcDNA3.1-GM-CSF; 3: pcDNA3.1-GM-CSF/EcoRI, HindIII; 4: pcDNA3.1/EcoRI/HindIII; 5: pcDNA3.1; 6: PCR production; 7: PGEM-ZF DNA/HaeIII marker

Analysis of pcDNA3.1-GM-CSF Eukaryotic Expression Vector

Though GM-CSF DNA can be efficiently expressed in prokaryote, it is still limited by lackness of the system of regulation and modification, which the eukaryotic vector possesses. So the mammalian cells were expected as a system to express GM-CSF gene into natural and glycosylated protein. pcDNA3.1 is an efficient eukaryotic expression vector, controlled by the CMV promoter. The amplified production possessed EcoRI/HindIII site as well as pcDNA3.1 vector, so it could be directionally inserted into the vector after digestion by EcoRI/HindIII. The recombinant pcDNA3.1-GM-CSF plasmid was transferred into $DH_{5\alpha}$, selected by LB solid medium with 60μg/ml Ap. Because there is no other screened sign in pcDNA3.1, the positive colonies were picked out randomly, amplified in LB liquid medium and the plasmid was extraced, then identified by EcoRI/HindIII digestion. In this course, the pcDNA3.1-neo was set as negative control. The positive recombinant plasmid was digested into 442bp and 4.95kb fragment, but the negative only one linear band, shown in Figure 4. It was shown that the GM-CSF cDNA had been directionally subcloned into EcoRI/HindIII site on the pcDNA3.1 vector. By the same PCR in which the recombinant plasmid was used as template it was further identified that GM-CSF cDNA had been inserted into pcDNA3.1 as shown in Figure 5.

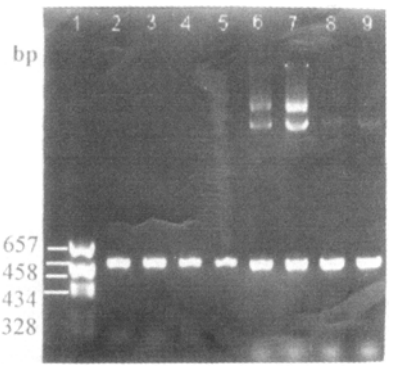

Fig. 5. Gel electrophoresis of NP-PCR production. 1: PGEM-7ZF DNA/HaeIII marker; 2-5: NP-PCR production; 6-9: PCR production from recombinant vector

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