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IN VITRO AMPLIFICATION OF HUMAN BASIC FIBROBLAST GROWTH FACTOR mRNA BY RNA-SPECIFIC EXON-JUNCTION PRIMERS

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SUMMARY: Highly sensitive and RNA-specific primers for the determination of human basic fibroblast growth factor (bFGF) gene expression by RT-PCR were identified. The RNA-specific primers could amplify bFGF mRNA from 10 pg to 1 ng of total cellular RNA without interfering with the presence of genomic DNA of the cell. The feasible temperatures of the primers annealed to the template were 55°C, 60°C and 65°C. In addition, different locations of primers on the bFGF mRNA molecule yielded distinct amounts of RT-PCR products from the same concentration of RNA, suggesting that the mRNA secondary structure of bFGF affected the RT-PCR. Owing to high sensitivity and specificity of the primers to bFGF RNA, the RNA-specific primers may be potentially utilized for the determination of human bFGF gene expression by *in situ* RT-PCR.

Key Words: basic fibroblast growth factor; RNA-specific primer; RT-PCR; *in situ* RT-PCR

INTRODUCTION

Technological developments have made possible of PCR analysis to individual cells to localize DNA/RNA with non-radioactive labels at the light microscopic level. *In situ* RT-PCR (ISRP) is useful in resolving low-frequency message expression in mixed populations of cells and tissues. However, cellular RNA coexisted with homologous sequence of genomic DNA in cells eventually decreases the accuracy of result. Although DNase pretreatment and hot-start procedures are used in ISRP, direct ISRP in cells usually is not reliable because of a high background from non-specific amplification (1). Thus, it appears that ISRP may be applicable to routine diagnostics on

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paraffin-embedded biopsies as long as the oligonucleotide primer used has been proven to be specific and sensitive in a standard RT-PCR assay (2).

The fibroblast growth factors (FGF) are a group of heparin-binding growth factors which include at least nine members that have pleiotropic effects on many different cell types and at many stages of development (3). Basic FGF, a major member of FGF, is a single polypeptide of 146 amino acids with molecular weight of 18 KDa (4). It acts as a potent angiogenic factor and a mitogen for various types of mesodermal and neurodermal cells (5). It has been shown that the degree of malignancy of brain glioma and human hepatocellular carcinoma was proportional to the expression level of bFGF (6). Furthermore, recent study found that bFGF and Tat of human immunodeficiency virus type 1 (HIV-1) proteins synergized in inducing angiogenic Kaposi's sarcoma-like lesions in mice (7), and the antisense oligonucleotide against bFGF could retard the growth of Kaposi's sarcoma (8). In this communication, a highly sensitive and RNA-specific primer for the determination of human bFGF gene expression by RT-PCR was characterized. The results concluded that the RNA-specific primers may be potentially utilized for the determination of bFGF gene expression by *in situ* RT-PCR.

MATERIALS AND METHODS

Cells and Reagents

Human lung fibroblast cell (CCD-13LU), obtained from American Type Culture Collection (Rockville, MD.), were cultured in Eagle's MEM (GIBCO) medium, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin/ streptomycin. Agarose, ethidium bromide, acrylamide and Sephadex G-25 were purchased from Pharmacia LKB Biotechnology Inc. MacDNASIS software was purchased from Hitachi comp. Reverse transcriptase (*rTth*) and *Taq* DNA polymerase were obtained from Perkin Elmer Corp. All the others were of analytic grade.

Isolation of RNA and DNA

The RNA and DNA were isolated from CCD-13LU cell lines by the guanidine thiocyanate/cesium chloride gradient ultracentrifugation method (9). The RNA and DNA preparations were treated with DNase and RNase, respectively. The RNA preparation was stored in liquid nitrogen prior to use.

Oligonucleotide Primers

Deoxyoligonucleotide primers were prepared with an automated synthesizer (Applied Biosystems Model 391 PCR-MATE™, Foster, CA). The primers were deprotected from columns with concentrated NH_4OH

followed by the incubation at 55°C for 16 h. The solution was dried with a DNA concentrator (Speed-Vac, Savant), and the remainder was purified through a Sephadex G-25 (1 X 9 cm) column.

RT-PCR

RT-PCR was carried out with a 50 μl reaction buffer containing 100 mM Tris-HCl pH 8.3, 1 mM dNTP, 1 μM antisense primer and 100 ng RNA template. In the first cycle, the RNA was denatured at 94°C for 2 min, and annealed with antisense primer to the template at annealing temperatures (55, 60, 65 and 70°C) for 2 min. The reverse transcription was performed with *rTth* reverse transcriptase (2.5 U) and 2 μl of 10 mM $MnCl_2$ at 75°C for 5 min. 4 μl chelating buffer containing 50% glycerol (v/v), 100 mM Tris-HCl, pH 8.3, 1 M KCl and 7.5 mM EGTA/0.5% Tween 20 was added to stop the reaction. After the addition of 3 μl of 25 mM $MgCl_2$ and 1 μM sense primer, the amplification was proceeded on a thermocycler (Thermolyne, Barnstead, Iowa), 94°C/55°C/75°C 1 min each, for a total 35 cycles.

RESULTS AND DISCUSSION

Up to date, several efforts have been made to resolve the problems of primer design for RT-PCR (10). However, some designed primers were not specific or incapable to amplify particular mRNA for uncertain reasons. Since in situ RT-PCR (ISRP) has been evidenced more sensitive than *in situ* hybridization (2), a clear background is essential for faithful interpretation of result. The specimen for *in situ* RT-PCR contains RNA and DNA in the same reaction. Although genomic DNA in cells can be treated with DNase prior to RT-PCR, a co-amplification of genomic DNA template usually may not be completely eliminated by *in situ* RT-PCR.

RNA secondary structure influences the determination of gene expression by RT-PCR (10). The exon-junction primer has provided an optimal RNA-specific primer for the detection of human tumor necrosis factor receptor I (11). To design optimal primers for the determination of human bFGF RNA expression by RT-PCR, the bFGF mRNA secondary structure was predicted with DNA and protein sequence analysis software (MacDNASIS Pro™) according to the lowest folding energy (12-14). The overall structure of bFGF mRNA was then participated into every 400 bp domains. The exon-junction primers where exhibited less RNA secondary structure of bFGF were designed. The primers used in the experiment were located in the RNA domain of 400-800 nt (Fig. 1). The primer sequences of four combinations (symbolized M1, M2, M3 and M4) are listed in Table 1. As shown in Fig. 2, the primer combinations specifically amplified bFGF cDNAs

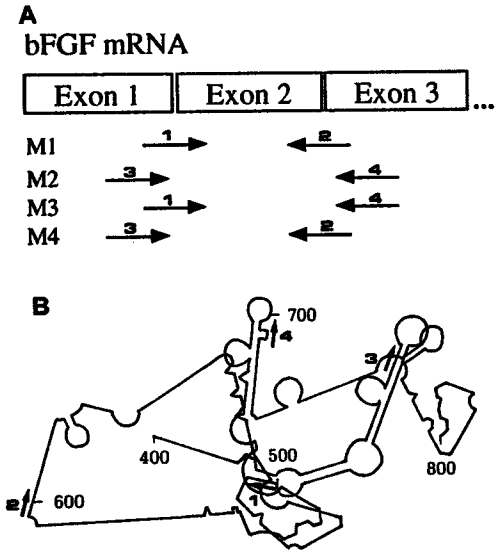

Fig. 1. Illustration of the primers in the RNA secondary structure of bFGF domains. The overall RNA folding structure was partitioned into every 400 bp domains. A, The primers used in the experiment are located in the exon 1, 2 and 3 of bFGF RNA domain of 400-800 nt. B, The arrows indicate positions and directions of the primers in the RNA secondary structure.

Table 1. The primer sequences for the determination of human bFGF mRNA

Primers	5'-	Sequence	Sense/Antisense
M1	498	5'GGAGAAGAGCGACCCTCACATCAAG3'	Sense
	615	5'ACACTCATCCGTAACACATTTAGAAGCC3'	Antisense
M2	440	5'GCAAAAACGGGGGCTTCTTCCTGCG3'	Sense
	705	5'CCAGTTCGTTTCAGTGCCACATACCAA3'	Antisense
M3	498	5'GGAGAAGAGCGACCCTCACATCAAG3'	Sense
	705	5'CCAGTTCGTTTCAGTGCCACATACCAA3'	Antisense
M4	440	5'GCAAAAACGGGGGCTTCTTCCTGCG3'	Sense
	615	5'ACACTCATCCGTAACACATTTAGAAGCC3'	Antisense

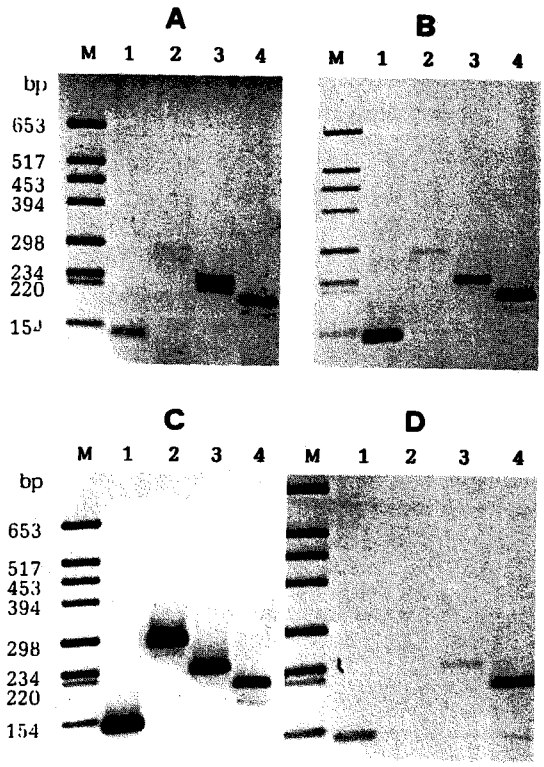

Fig. 2. Determination of human bFGF RNA by RT-PCR at various annealing temperatures. M, DNA markers. The numbers 1, 2, 3 and 4 denote M1, M2, M3 and M4, respectively. The annealing temperatures of primers: A, 55°C; B, 60°C; C, 65°C and D, 70°C.

from cellular RNA by RT-PCR at annealing temperatures of 55°C, 60°C, 65°C and 70°C, and no band was visualized for M2 at 70°C. All the bFGF cDNA bands were verified by DNA sequencing. In addition, the clear background of the RT-PCR indicated no cross-reaction with another templates, suggesting a high specificity of the primers. Moreover, different locations and annealing temperatures of the primers yielded distinct amounts of RT-PCR products from the same quantity of RNA, implying that the RNA secondary structure of bFGF might affect RT-PCR.

An RNA-specific primer may be able to eliminate background of *in situ* RT-PCR. In the present paper, RNA specificity of the primers was evaluated by RT-PCR of cellular RNA, genomic DNA and combination of RNA and DNA of CCD-13LU cells. As shown in Fig. 3,

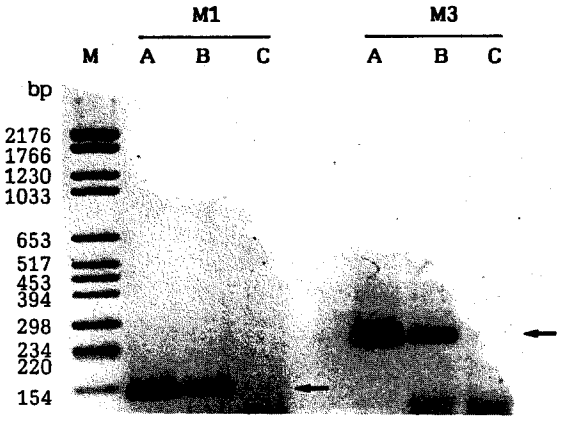

Fig. 3. RNA specificity of the primer. The RNA specificity of the primers was analyzed by RT-PCR. A, cellular RNA (100 ng); B, combination of the cellular RNA (100 ng)/genomic DNA (100 ng) and C, genomic DNA (100 ng). M, DNA markers. The predicted sizes of bFGF cDNA are indicated by arrows.

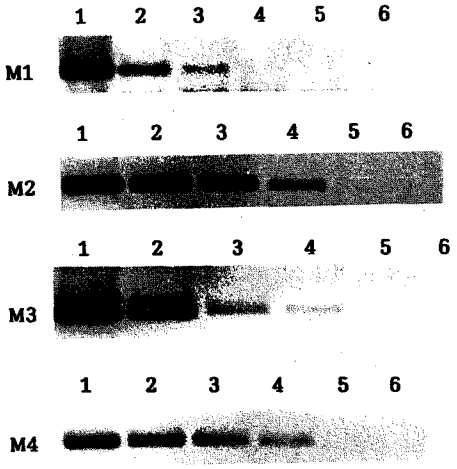

Fig. 4. Comparison of the sensitivity of primers for bFGF by RT-PCR. Serial dilutions of cellular RNA, ranged from 250 ng to 1 pg, were employed for RT-PCR. The number represents: 1, 250 ng; 2, 10 ng; 3, 1 ng; 4, 100 pg; 5, 10 pg; 6, 1 pg of total cellular RNA.

M1 and M3 primers specifically amplified cDNA from the cellular RNA and the combination of cellular RNA and genomic DNA by RT-PCR, and no band was visualized in the group of genomic DNA. However, M2 and M4 generated several bands of genomic DNA templates, and the bands were shown no correlation with bFGF (data not shown). The result indicates that M1 and M3 primers are RNA specific. The lowest limitation of detection by the M1, M2, M3 and M4 primers were 1 ng, 100 pg, 10 pg and 100 pg of total cellular RNA, respectively (Fig. 4).

In conclusion, this paper provides detail information of the practical primers for the determination of bFGF gene expression by RT-PCR. In view of the results of high sensitivity and RNA specificity of the designed bFGF primers, the primers were ideal for the determination of gene expression of human bFGF by RT-PCR, and it could be potentially utilized for *in situ* RT-PCR in cells.

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