Development of Competitive mRNA PCR for the Quantification of Interleukin-6-Responsive *junB* Oncogene Expression

BioTechniques 24:854-860 (May 1998)

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ABSTRACT

The transcription factor junB belongs to the jun family of protooncogenes. The appearence of junB *mRNA in hepatic cells is an extremely early and sensitive marker of the action of proinflammatory cytokines including interleukin-6. In this study, a competitive reverse transcription (RT)-PCR assay has been developed that is suitable for the quantitative determination of* junB *mRNA expression. This nonisotopic assay compared to other methods (e.g., Northern blot) is a fast and convenient way to determine the expression of the* junB *gene and thus the immediate concentration- and time-dependent action of interleukin-6. Because interleukin-6 and interleukin-6-type cytokines play a highly important regulatory role in various pathophysiologically important processes, such as hepatic acute-phase reaction, the quantitative assay of* junB *mRNA completes the scale of laboratory approaches in inflammation and among other pathological conditions.*

INTRODUCTION

The transcription factor junB belongs to the jun family of protooncogenes (c-jun, junB and junD) that, together with the fos family of protooncogenes (c-fos, fosB, Fra-1 and Fra-2), constitutes the group of AP-1 transcription factors (2,26,31). Its function is not fully elucidated yet, but because of its lower transactivation and DNA binding capacities compared to junC, it is supposed that junB, forming a heterodimer with junC, can be an attenuator of the junC action (12).

In cells of hepatic origin, the mRNA of *junB* is a very early and sensitive marker of some cytokine action including the interleukin-6 (IL-6) family of cytokines (IL-6, IL-11, ciliary neurotrophic factor [CNTF], leukemia inhibiting factor [LIF]) and transforming growth factor β (TGFβ) (3,4,6,33). Binding of IL-6 to its receptor elicits a signal transduction cascade that leads to the phosphorylation of Janus kinase family (JAK) kinases and then of the signal transducers and activators of transcription (STAT) family of transcription factors. The STAT proteins dimerize and migrate to the nucleus where they activate different genes including *junB* (19). The promoter of the *junB* gene contains STAT, cAMP responsive element-binding protein activating transcription factor (CREB-ATF) and putative Ets-binding elements, suggesting that multiple signal transduction pathways converge on this promoter (9,15,22). The expression of *junB* mRNA but not of *junC* mRNA is highly increased in response to IL-6 (3,33). A modulating function of junB on the transcription of acute-phase protein genes has been supposed because coexpression of junB expression vectors in some hepatic cell lines reduced the transcription rates of the majority of acute-phase protein genes (3).

Because of the relatively low transcription rate of *junB*, it is difficult to determine the amount of *junB* mRNA quantitatively by Northern blot analysis. The introduction of reverse transcription polymerase chain reaction (RT-PCR) made the detection of very small amounts of mRNA possible, but because of its extraordinary sensitivity, the quantification of the results is directly not possible. There are numerous approaches to make the PCR quantitative. For example, it is possible to use: (*i*) radioactively labeled nucleotides or primers, (*ii*) fluorescent reporter molecules that are released from labeled primers (or from other components of the PCR mixture) during the amplification process, thus the amplification process can be followed by changes in physical parameters and (*iii*) internal controls (i.e., competitors that compete for the primers with the DNA to be determined), and the amount of the DNA in question can be titrated with serial dilutions of the competitor (competitive PCR) (16,24,27). We applied the latter approach. By selecting and cloning a competitor, competitive PCR for the quantitative determination of *junB* mRNA expression has been generated. Using this system, we provide a sensitive new method to follow the early action of IL-6 in in vitro experimental models of inflammation.

MATERIALS AND METHODS

Cell Culture and Treatment

HepG2 human hepatoma cells were used to study the action of IL-6. Cultures were grown in RPMI1640 medium containing 10% fetal calf serum (FCS) in 6-well plates; each well contained 3×10^6 cells. Cultures were kept at 37°C and 5% CO_2.

Cells were treated with 20, 100 and 200 U/mL IL-6 (Calbiochem-Novabiochem, La Jolla, CA, USA) (one unit is equal to 100 pg) in serum-free RPMI-1640 medium for 0.5, 2, 8 and 20 h. Following the treatments, total cellular RNA was prepared.

RNA Preparation

Total cellular RNA was prepared according to the method of Chomczynski and Sacchi (8). Briefly, cells were lysed with guanidium isothiocyanate, and the lysates were then subjected to phenol/chloroform extraction. The aqueous phase was removed and precipitated with isopropanol, and after washing with 70% ethanol, the pellet was dissolved in sterile diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was measured with a Spectronics Genesys 2 Photometer (Milton Roy, Rochester, NY, USA) at 260 nm. RNAs were kept at -80°C until use.

To check the purity of RNA and the accuracy of the measurement of the RNA concentration, 2 µg RNA were run on a 1% agarose gel (Sigma Chemical, St. Louis, MO, USA) containing 10% formaldehyde and 40 µg/mL ethidium bromide (EtdBr) for 2 h at 40 V. Only samples that proved to contain the same amount of RNA (indicated by the 28S RNA band) were used for further analysis.

Reverse Transcription

RT was carried out on an Ataq Gene Controller (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction mixture (total 20 µL) contained 5 mM Mg^{2+} (Perkin-Elmer, Norwalk, CT, USA), 2 µL 10× PCR Buffer II (Perkin-Elmer), 0.25 mM 4× dNTPs (Amersham Pharmacia Biotech), 20 U RNase inhibitor (Promega, Madison, WI, USA), 0.5 µM oligo(dT) primer (Perkin-Elmer), 25 U Moloney murine leukemia virus (MuLV) Reverse Transcriptase (Perkin-Elmer) and DEPC-treated H_2O. RNA (1 µg) was added to the reaction mixture. Samples were incubated at 42°C for 30 min and 99°C for 5 min. cDNAs were stored at -80°C until use.

Standard RT-PCR for *junB*

Specific primers (synthesized by Amersham Pharmacia Biotech) were used to amplify the *junB* sequence (20). These primers are located in the 3′ noncoding region of the *junB* mRNA. The sequences are sense: CCAGTCCTTCCACCTCGACGTTTACAAG and antisense: GACTAAGTGCGTGTTTCTTTTCCACAGTAC.

The PCR was carried out using the following program on the Ataq Gene Controller: an initial denaturation at 94°C for 4 min then 35 cycles at 95°C for 30 s, 57°C for 45 s and 73°C for 45 s, then a final extension at 72°C for 5 min. The reaction mixture (total 50 µL) contained 5 µL 10× buffer (Promega), 1.5 mM Mg^{2+} (Promega), 1 mM 4× dNTPs (Amersham Pharmacia Biotech), 50 pmol sense and antisense primers, H_2O, 1 U *Taq* DNA polymerase (Promega) and 2 µL sample (cDNA). Mineral oil (20 µL; Sigma Chemical) was layered on top of the reaction mixture. The amplified PCR product was 257 bp long.

Generation of the Internal Control by Mismatch Protocol

To make the PCR competitive, a competitor (a DNA molecule that is amplified by the same primers as the cDNA to be determined but that can be distinguished from the original cDNA by its different molecular weight) was constructed.

To generate such a competitor, the standard PCR for *junB* was applied with the following modifications to facilitate mismatch priming: (*i*) the annealing temperature was lowered to 37°C (from 57°C); (*ii*) instead of 94°C for 4 min, it was begun at 72°C for 4 min; (*iii*) the PCR was assembled on ice; and (*iv*) 3 mM instead of 1.5 mM Mg^{2+} was used. This method, using low-stringency primer annealing PCR, is much faster than conventional methods including digestion of PCR products with restriction enzymes and gives reproducible results (14). In this reaction, 2 µg of genomic DNA instead of cDNA were used.

Genomic DNA was purified from the Hep2 human laryngeal carcinoma cell line with the Tris/sodium dodecyl sulfate (SDS)/proteinase K lysis, phenol/chloroform/isoamyl alcohol extraction, ethanol precipitation method (17). From the purified nucleic acids, RNA was removed with a 1-h RNase (Boehringer Mannheim GmbH, Mannheim, Germany) treatment at 37°C. This was followed by repeated phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The DNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Aliquots of the genomic DNA were denatured by incubation for 10 min at 95°C, then immediately chilled on ice before addition to the reaction mixture. After assembling the reaction mixtures but before the amplification process, samples were incubated for 15 min at room temperature to facilitate the annealing of the primers to the denatured template.

The resulting PCR products were run on a 2% agarose gel (Sigma Chemical) in 1× TBE buffer (8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA, pH 8.0) containing 0.5 mg/L EtdBr, and the bands different in size from that of the original product (257 bp) were excised. The gel slices were subjected to the freeze-squeeze method (29) to remove the PCR products from the gel. The PCR products obtained were used for a second PCR after a 1:100 dilution (30) applying the standard PCR program for *junB* (high-stringency annealing) to test whether the products can be re-amplified. The re-amplified products were purified with subsequent phenol/chloroform/isoamyl alcohol extraction and precipitated with ethanol, and the resulting pellet was dissolved in TE buffer.

Cloning of the PCR Products

Two PCR products judged to be suitable were introduced into the DH5α™ *E. coli* strain (Life Technologies, Gaithersburg, MD, USA) with the help of the pGEM®-T Vector System (Promega). The ligation reaction was performed according to the instructions of the manufacturer. Transformation of the cells was achieved using the calci-

um chloride method (10). The transformed cells were plated on agarose containing 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 10 mg/mL ampicillin. Blue-white screening was applied to identify transformed colonies. Ten white colonies were selected for the two suitable PCR products each and grown in 5 mL LB medium (tryptone and yeast extract from Oxoid, Basingstoke, Hampshire, England, UK). Plasmid minipreps were produced using the alkaline lysis method (5) with the modification that after adding potassium acetate, the supernatant was subjected to a phenol/chloroform/isoamyl alcohol-extraction and ethanol-precipitation procedure for further purification. PCRs were performed with the 100-fold dilution of the minipreps to check for the presence of the desired product. One clone was tested for competition with HepG2 cDNA with 8 dilutions ranging from 10^2 to 10^9 of the plasmid, and cDNA was applied in the same volume (2 µL). This clone, which proved to be competitive, contained a competitor DNA of approximately 370 bp vs. the 257-bp product for *junB*. The clone was grown in 50 mL TB medium to produce plasmid midiprep. Plasmid midiprep was produced using the Recovery™ Plasmid Midi Prep Kit (Hybaid, Middlesex, England, UK). The plasmids were linearized during an overnight incubation in the presence of *Apa*I restriction enzyme (Boehringer Mannheim GmbH) to make the competition reaction more reproducible. The concentration of the linearized plasmid stock solution was 0.075 µg/mL.

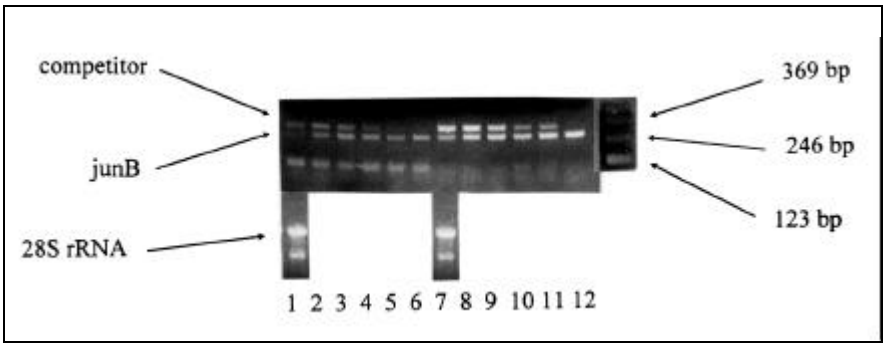

Figure 1. Illustration of a competitive RT-PCR detecting *junB* mRNA expression on HepG2 cells. Two sets of experiments are shown. Lanes 1–6: untreated control; lanes 7–12: cultures treated with 100 U/mL IL-6. Lanes 6 and 12 show the results of noncompetitive RT-PCRs (in the absence of competitor). Lanes 1–5 and 7–11 represent four serial twofold dilutions of the competitor beginning from 10^5-fold and 10^4-fold dilutions of the competitor plasmid stock solution (0.075 µg/mL) in the case of the untreated control and IL-6 treatment, respectively. 100 U/mL IL-6 induced the expression of *junB* mRNA tenfold as compared to the untreated control. The identical intensity of the 28S RNA bands indicates that the same amount of RNA was included in all reactions.

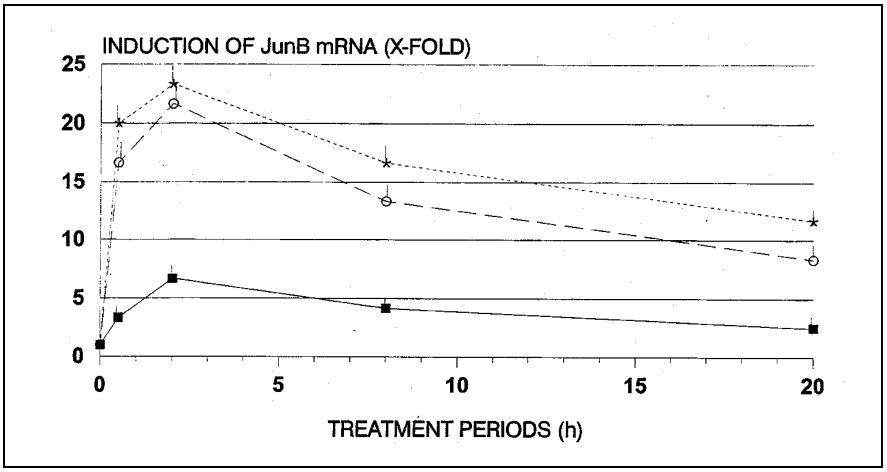

Figure 2. Kinetics of the induction of *junB* mRNA in response to IL-6 on HepG2 cells (determined by competitive RT-PCR). 20 (-■-), 100 (-O-) and 200 U/mL (-*-) IL-6 were applied. Treatment periods were 0.5, 2, 8 and 20 h. Standard error is indicated (n = 3).

Protocol for the Competitive PCR

cDNAs were always applied in a volume of 2 µL. The competitor was included in the same reaction mixtures as the cDNAs. Two microliters of the serial dilutions (ranging from 10^2 to 10^9 dilution factors) of the linearized plasmids were used.

Because the competitor and *junB* DNA are in the same reaction mixture, they compete with each other for the primers (competitive PCR). The less competitor (greater dilution) added to the reaction mixture, the weaker the competitor band will be and the stronger the *junB* band will be. The dilution factor where the band of *junB* becomes more intensive than that of the competitor indicates the amount of *junB* cDNA. This can be called the equivalence zone (analogy to a titration) (13,34). To determine the equivalence zone more precisely, 4 twofold dilutions were made from the dilution factor preceding the above equivalence zone, and a PCR was performed as before. The position of the equivalence zone was determined as before.

The products of the competitive PCR (8 µL product + 2 µL loading buffer) were run on 2% agarose gels in 1× TBE buffer containing 0.5 mg/L EtdBr.

Protocol for Sequencing the Competitor

For generating the competitor by a PCR facilitating mismatch priming, it seemed to be desirable to know the sequence of the isolated DNA product. For this purpose, the competitor plasmid (not the linearized form) has been sequenced using a Sequenase® Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK). The products of the labeling reaction were run on urea-containing polyacrylamide gels.

Homology analysis of known sequences was performed using the Basic Local Alignment Search Tool (BLAST) (**http:\\www.ncbi.nlm.nih.gov/BLAST/**) (1).

RESULTS

Results of the Competitive RT-PCR Assay

Results of a competitive RT-PCR are shown in Figure 1. As can be seen from Figure 1, following the serial dilutions of the competitor, the band of the competitor weakens, while the band of *junB* gets stronger. The dilution factor where the band of *junB* gets stronger than the band of the competitor indicates the amount of *junB* cDNA (equivalence zone). Using this protocol, the extent of *junB* mRNA induction following treatments relative to the untreated control (x-fold induction) has been determined.

The results of the competitive RT-PCRs carried out for treatments with different concentrations of IL-6 and different treatment periods are shown in Figure 2. As can be seen from Figure 2, IL-6 induced the expression of *junB* in a time- and concentration-dependent manner with maximal induction at 2 h and 200 U/mL IL-6 concentration. The different concentrations of IL-6 showed similar kinetics of *junB* induction in the function of the treatment periods.

Sequence of the Competitor

The sequence of the cloned competitor is shown in Figure 3. The competitor is 100 bp longer than the original *junB* PCR product. Results of the sequence homology analysis with BLAST indicate that the sequence of the competitor does not show significant homology to *junB* and other known genes.

DISCUSSION

IL-6 is a pleiotropic cytokine regulating B-cell development, hematopoiesis and the acute-phase reaction (18,21). IL-6 is the central regulator of the second phase of the acute-phase reaction. It induces the transcription of many acute-phase protein genes in the liver (4). The early events occuring in the signal transduction of IL-6 are subjects of intensive investigation.

IL-6 (and other cytokines belonging to the IL-6 family) induces the expression of the *junB* gene, which is a very sensitive and early marker of IL-6 action. In contrast to the assays measuring the action of IL-6 by the determination of the amount of acute-phase proteins (e.g., α_2-macroglobulin, haptoglobin, fibrinogen), the determination of *junB* mRNA expression makes it possible to judge the action of IL-6 by an early phase in its action. It is indicated by the finding that the expression of *junB* mRNA increases immediately after the addition of IL-6 to cultures of hepatoma cells, and it approaches maxi-

mum induction as quickly as 15–30 min after treatment (3), while at least 18 h are needed for acute-phase proteins (e.g., by enzyme-linked immunosorbent assay [ELISA]) to detect differences.

The development of competitive RT-PCR made the quantitative measurement of mRNA expression possible in a fast and comfortable way that avoids the use of radioactivity. This technique is suitable for the determination of samples with a very low amount of the mRNA in question because of the sensitivity of the PCR (7,16). The sensitivity of other methods for mRNA quantification, such as Northern blot hybridization and RNase protection, is not high enough for detection of low-abundance transcripts in samples with limited cell numbers (11). RT-PCR is 1000-fold–10 000-fold more sensitive than RNA blot techniques (27). In our other studies we were able to detect the effect of IL-6 on primary murine hepatocytes (unpublished observations) and experiments are in progress to use this technology in primary human embryonic hepatocytes, which are available only in limited number.

However, because of the high number of factors influencing the reaction the PCR makes the quantitative interpretation of the results directly not possible (32). The application of housekeeping genes (actin, glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]) provides only semiquantitative results. The application of an internal control for the PCR can be used to solve this problem (11).

A competitive PCR assay for the determination of *junB* cDNA has been developed by applying a competitor that was produced with a PCR facilitating mismatch priming (low-stringency primer annealing). We preferred this method to others using restriction enzymes because of the lack of known specific restriction enzyme sites on the 257-bp segment of the *junB* cDNA amplified by the standard RT-PCR.

The sequence homology analysis of the competitor DNA did not show significant homology to either the sequence of the *junB* gene or that of the *junB* cDNA. Siebert and Larrick (28), however, did show that the efficiency of amplification is mainly influenced by the sequence of the primers and not by the intervening sequence; a competitive DNA fragment with a different base sequence is amplified with similar efficiency to the PCR fragment to be determined. This result, however, is not accepted unanimously. Pannetier et al. (23) claim that this finding cannot be generalized because the amplification process might depend on the quality of the cDNA to be amplified. An advantage of this system over others that use competitors with high homology to the sequence to be determined is the lack of formation of heteroduplexes between the competitor DNA and the cDNA in question (14).

We considered the results of RNA running, specifically, the identical intensities of the 28S rRNA bands, as indicators of the identical amounts of RNA included in the RT reactions. We also performed parallel RT-PCRs for β-actin as a housekeeping gene to indicate the efficiency of the RT reaction. We did not detect differences between the two methods; the identical intensities of the 28S rRNA bands were always accompanied by identical intensities of β-actin bands. The expression of housekeeping genes, however, can be influenced by treatments [e.g., the expression of β-actin decreases with increasing concentrations of anti-CD3 in Jurkat lymphoma cells (25)].

By this method, we determined the extent to which IL-6 induces the expression of *junB* mRNA compared to the untreated control. The results shown in Figure 2 indicate that the induction of *junB* mRNA in response to IL-6 on HepG2 cells is time- and concentration-dependent. Comparison of the RT-PCR results with Northern blot analysis shows close similarities (3). The kinetics of induction were almost the same in both assays with maximal induction between 0.5 and 2 h. In both assays, the expression of *junB* mRNA remained elevated even 20 h after treatment.

We also performed experiments on liver biopsies from patients suffering from various diseases to test the clinical applicability of this method. Preliminary results indicate that the expression

Figure 3. Nucleotide sequence of the competitor. Underlined sequences correspond to the primers.

of *junB* can be correlated with some diseases of the liver.

This new method seems to be very useful in various in vitro (and possibly clinical) studies measuring pharmacological, endocrinological and other influences on the effect of IL-6 on hepatocytes.

ACKNOWLEDGMENTS

This work was supported by grants from the OTKA of the National Academy of Sciences (Nos. T-022065 and T-020603.

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Received 19 September 1997; accepted 11 December 1997.

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