

IDENTIFICATION OF A NOVEL GENE ISOLATED FROM A FRACTURE CALLUS

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Abstract -We have previously hypothesized that a vast number of genes are expressed during the fracture repair. Past work in our laboratory identified many of these genes, involved specifically during fracture healing, some being functionally unknown (identified as ESTs (Expressed Sequence Tags) or as completely novel). One such EST was selected for further studies based on its expression pattern (from microarray experiments), its highest level of mRNA expression on post-fracture (PF) day 10 and 14. Since a partial sequence is known, a "contig" approach was utilized to construct the full-length gene. A final contig of 1686 bp was constructed. Primers were designed to amplify 1121 bp of the contig using RT-PCR. However, no band was obtained. Simultaneously, primers were designed for the amplification of the original EST which was obtained. This fragment was subcloned, sequenced, and its identity verified. Further, northern analysis was performed using RNA isolated from intact bone as well as PF day 3, 5, 7, 10, 14, and 21 callus. Results indicate this gene was differentially expressed, with high levels on PF days 7-21, and its mRNA size was ~6500 bp. The task remains to obtain the full-length gene and characterize its structure and function.

Keywords- fracture healing, gene expression

I. INTRODUCTION

Fracture healing is a complex process, consisting of four stages. These include inflammation, chondrogenesis, ossification, and remodeling. Each of these stages is controlled by a vast number of genes displaying a specific temporal and spatial pattern [1]. Through Suppressive Subtractive Hybridization (SSH), we have identified thousands of these genes that show a differential expression pattern during the fracture repair process [2]. Further, the construction of custom cDNA microarrays, allowed the determination of the temporal expression of all identified genes during the early stages of fracture repair (PF day 3-21) [3]. Since there were many EST's present in our subtracted cDNA library, their structure and function remains unknown.

In order to probe the nature of some of these ESTs, we took advantage of their short known sequences (using the accession number) and attempted to obtain the full-length cDNA clone. One particular EST was chosen for further studies based on its expression pattern during the repair process. Identification of a full-length gene with a specific expression pattern during fracture repair will enable us to conduct structure-function experiments to facilitate understanding the role of this novel gene in the healing process

II. METHODS

Contig Construction

A 553 bp EST was selected from a previously generated microarray data set that indicated highest level of expression on PF days 10 and 14. The fragment was blasted in GenBank, a database containing a collection of cDNAs. One homologous DNA fragment overlapping the 5' region and another fragment overlapping the 3' region was found, thereby extending the original EST in both directions. Next, the newly generated sequence was blasted, and a homologous cDNA fragment was found that overlapped the 5' end. The final contig consisted of four different fragments and measured 1686 bp in length.

Reverse Transcription-PCR

Using the final contig, primers were designed to amplify a 1121 bp cDNA fragment. PCR was carried out using QIAGEN One Step RT-PCR Kit. PCR conditions consisted of 40 cycles, in which DNA was denatured at 94°C for 30 sec, annealed at 61°C for 45 sec, and extended at 72°C for 60 sec. Simultaneously, primers were designed based on the original EST to amplify 476 bp of the total length. PCR conditions consisted of 40 cycles, in which DNA was denatured at 94°C for 30 sec, annealed at 60°C for 45 sec, and extended at 72°C for 60 sec. Gel electrophoresis (agarose) was performed to visualize results. Upon confirmation of a band, the fragment was subcloned using AdvanTAGE PCR Cloning Kit. Finally, the subcloned cDNA fragment was sequenced.

Northern Blot

Total RNA (20 µm) was electrophoresed on a 1% formaldehyde/agarose gel and transferred to a nylon membrane. cDNA probes were labeled with ³²P-dCTP and hybridized to the membrane overnight at 65°C in a solution containing 15% formamide, 0.2M NaPO₄ (pH 7.2), 1mM EDTA, 7% SDS, and 1%BSA. After completion of hybridization, the blot was washed in a solution of 2X SSC/ 1% SDS at 50°C for 30 min, 0.2X SSC/ 1%SDS at 50°C for 30 min, and 0.2X SSC/ 0.1% SDS at 65°C for 30 min. The blot was exposed to Kodak film at -80°C.

III. RESULTS

Contig Construction

From the original EST, a final contig was constructed, consisting of four overlapping rat EST fragments. The length was 1686 bp (Fig 1).

PCR

Based on the contig, we performed RT-PCR using a primer combination that should have yielded an 1121 bp

Northern Blot

Following confirmation of the sequence, we then examined the expression of this gene using northern analysis. Results from the Northern blot indicate that this gene was indeed differentially expressed, as indicated by the microarray data. More specifically, its expression levels were higher levels on PF days 7-21 (Fig 2a). The size of the mRNA was determined to be approximately 6500 bp.

IV. DISCUSSION

Results described in this paper indicate the identification and expression of a novel gene, with a role in the fracture repair process. Initial attempts to clone the full-length gene, failed but we are optimistic that with a few modifications of our PCR conditions, we will be able to obtain it. Further, Northern analysis revealed that the expression levels of this gene were high on PF days 7-21, which is almost in agreement with the microarray data that indicated that this EST displayed high levels of expression on PF days 10-21. Since, northern blot analysis is more accurate than microarray data, we strongly believe that this data is much more.

The observation that the highest levels of mRNA expression was on PF day 7, corresponds with the onset of cartilage formation, known to occur and forms the basis of the soft callus [4]. Further, the expression of this EST decreases slightly on day 10, more so on day 14, and expression increases to higher level again on day 21. Based on this data, it is possible that this gene is involved in chondrogenesis and endochondral ossification. Obviously, more work is required to conclusive determine the structure and function of this gene in the repair process.



Fig. 1. Final contig construct. The original EST is represented in yellow and the other 3 overlapping ESTs in green, blue, and purple

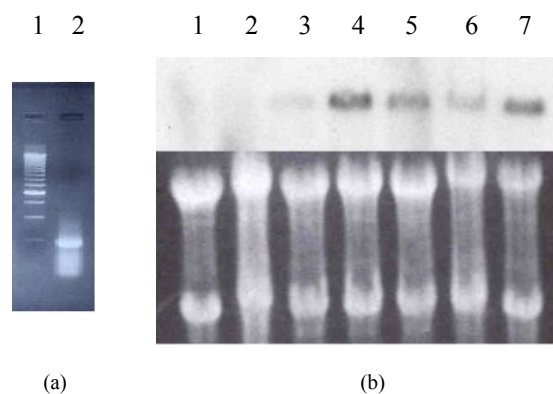


Fig. 2. (a) PCR results for the original EST fragment. Lane 1 represents 1000bp ladder, lane 2 represents band at 429 bp. (b) Northern blot, lane 1 intact bone compared to lane 2 PF day 3, lane 3 PF day 5, lane 4 PF day 7, lane 5 PF day 10, lane 6 PF day 14, lane 7 PF day 21

V. CONCLUSION

The expression of this gene has been confirmed through this set of experiments. It has been observed that the gene is differentially expressed, with high levels at PF days 7-21. However, the task remains to obtain the full-length gene, as well as to determine its spatial (cell/tissue) expression.

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