Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 246-251

ISOLATION OF MOUSE AND HUMAN cDNA CLONES ENCODING A PROTEIN EXPRESSED SPECIFICALLY IN OSTEOBLASTS AND BRAIN TISSUES *

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Received October 12, 1990

Summary Using the differential hybridization screening method between osteoblastic and fibroblastic cells, a cDNA clone coding for an osteoblast specific protein, named OSF-1, consisting of 168 amino acid residues including a possible 32 amino acid long leader sequence, was isolated from murine osteoblastic cell line MC3T3-E1. The OSF-1 gene was shown by Northern blotting analysis to be expressed in mouse calvarial osteoblast-enriched cells and in mouse brain tissues, but not in thymus, spleen, kidney, liver, lung, testis or heart. The human counterpart was also found in cDNA libraries from human osteosarcoma cell line MG63 and normal brain tissues. DNA sequence analysis revealed four amino acid sequence differences between the mouse and human, of which only one is located in the mature protein. This extremely high sequence conservation suggests that OSF-1 plays a fundamental role in bone and brain functions.

Bone matrix proteins play a role in the physiology of bone tissues and this has been investigated for some time. For example, bone morphogenetic proteins(BMPs), previously proposed by Urist et al as factors which are capable of inducing cartilage and bone from mesenchymal tissue(1), have been purified from demineralized bone extract(2). Complementary DNA(cDNA) clones which encode BMP-1, BMP-2A, BMP-2B and BMP-3 have been isolated from a human osteosarcoma cell line on the basis of peptide sequence information of such proteins(3).

However, the purification of such proteins from bone matrix is rather difficult, such that there are not to date many cDNA clones available. Moreover, proteins existing in bone matrix do not necessarily function in a specific manner in bone tissues. Assuming that at least some proteins functioning in bone may be produced specifically in bone cells, we attempted to search for other possible factors involved in growth and differentiation of bone tissues by cloning cDNAs specifically expressed in osteoblasts by differential hybridization

*The nucleotide sequences reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers D90225 and D90226.

Abbreviations: BMP(s), bone morphogenetic protein(s); cDNA, complementary DNA; α-MEM, alpha modification of Eagle's medium; FBS, fetal bovine serum; D-MEM, Dulbecco's modified Eagle medium; bp, base pairs; OB, osteoblast.

0006-291X/90 $1.50

screening between mouse osteoblastic and fibroblastic cell lines, MC3T3-E1(4) and NIH3T3(5), respectively.

Here we report a new cDNA clone encoding a lysine-rich basic protein, termed OSF-1, which has partial sequence homology with MK, a differentiation factor identified in retinoic acid induced embryonal carcinoma cells(6, 7).

Materials and Methods

Cell culture. MC3T3-E1 cells were cultured in alpha modification of Eagle's medium (α-MEM, Flow laboratories) supplemented with 10% fetal bovine serum (FBS, GIBCO). Initially $2.2x10^5$ cells were seeded in 100-mm tissue culture dishes and culture medium was replaced every 3 days. After culturing for 9 days, cells were collected and used for total RNA isolation. NIH3T3 cells were similarly cultured in Dulbecco's modified Eagle medium (D-MEM, GIBCO) supplemented with 10% FBS till the semi-confluent stage for total RNA isolation. Calvarial osteoblast-enriched cells were prepared as follows. Calvaria obtained from newborn ICR mice(Shizuoka Experimental Center, Shizuoka, Japan) was minced and cultivated in 60-mm tissue culture dishes with α-MEM containing 10% of FBS. Cells which migrated from calvaria were replated and medium was replaced every 3 days. Total RNA was prepared after culturing for 9 days.

Tissues. Thymus, spleen, kidney, liver, lung, testis, heart and whole brain tissues were obtained from 4 week old C57BL/6N mice(Nihon Clea, Tokyo, Japan), and used as sources for total RNA preparation.

RNA preparation and RNA analysis. Total RNA was prepared by the guanidium/ cesium chloride method (8). PolyA$^+$ RNA was prepared from total RNA by an mRNA purification kit (Pharmacia). For Northern blot analysis, 0.3 μg of polyA$^+$ RNA was subjected to formaldehyde agarose gel electrophoresis(8), and transferred to a nylon membrane by the capillary blotting method(8). For slot blot analysis, 10 μg of total RNA was fixed on a nylon membrane and subjected to hybridization. [^{32}P]-labeled DNA probes were prepared by random primed DNA labeling kit (Behringer Manheim). Human β-actin gene(9) was kindly supplied by Dr. Y.Sakaki. Hybridization was performed as previously described(8).

cDNA libraries. Double stranded cDNA was synthesized from polyA$^+$ RNA by the cDNA synthesis system plus(Amersham). Synthesized double stranded cDNA was ligated with EcoRI-NotI adaptor (Pharmacia), and cloned into λgt10 cloning vector(Stratagene). After packaging in vitro the resulting library containing approximately $1.5x10^6$ independent clones was stored in SM buffer(8) and used for screening without amplification. The cDNA libraries of human osteosarcoma cell line, MG63, and human brain tissues were purchased from Clontech.

Screening of cDNA libraries. To prepare the hybridization probes, [^{32}P]-labeled cDNAs were synthesized individually from polyA$^+$ RNAs of MC3T3-E1 and NIH3T3 cells, using AMV reverse transcriptase(Pharmacia). Clones predominantly hybridized with the cDNA probe of MC3T3-E1 against that of NIH3T3 were selected and cDNA inserts were recloned onto pUC118(Takara Shuzo, Kyoto, Japan) for DNA sequencing analysis. Human cDNA screening was performed as previously described(8) using mouse OSF-1 cDNA fragment as a probe, and cDNA inserts from positive clones were transferred to pHSG298 or pHSG398(10) for sequencing analysis.

DNA sequencing analysis. DNA sequencing analysis was performed by using an automatic DNA sequence analyzer (model 370A, Applied Biosystems) and also manually by the dideoxy chain termination method. The DNA and deduced amino acid sequences thereof were subjected to homology search against the data bank of GenBank DNA data base(release 64.0) and NBRF protein data base(release 25.0) using the programs 'FastA' and 'TFastA' in GCG Sequence Analysis Software Package.

Results

Isolation of mouse cDNA clones specific to MC3T3-E1 against NIH3T3. A cDNA library was constructed from MC3T3-E1 cells and differential screening was performed

using cDNA probes between MC3T3-E1 and NIH3T3. Approximately 300 out of $1.5x10^4$ plaques showed predominant hybridization with MC3T3-E1 cDNA probe against NIH3T3 cDNA probe, and 30 out of these 300 clones were selected after the second plaque hybridization for further investigation. Among them, 6 clones were eliminated since the size of cDNA inserts were too small(<200 bp). Each of the other 24 cDNA inserts was recloned and subjected to the DNA sequencing analysis and data base search. Out of 24, 9 clones represented previously reported genes, i.e. 3 for type-I collagen(11), 2 for osteopontin(12), 2 for ribosomal protein L39(13), one for thymosin β10(14) and one for ribosomal protein L31(15). The remaining 15 clones were subjected to differential Northern blot analysis with respective cDNA fragments between polyA$^+$ RNAs of MC3T3-E1 and NIH3T3. Consequently, one clone designated as λmOSF-1 showed rather specific hybridization to MC3T3-E1 RNA(Fig. 1). The entire DNA sequence of mouse OSF-1 cDNA was then determined, and the open reading frame encoding a 168 amino acid long peptide was identified as shown in Fig. 2A.

Tissue specific expression of OSF-1 mRNA. Subsequently, we tested the tissue specific expression of OSF-1 to confirm the expression of OSF-1 gene in osteoblasts by using primary culture of mouse calvarial osteoblasts, and to examine possible expression in any other tissues. Interestingly, it was revealed that OSF-1 gene is expressed not only in calvarial osteoblast-enriched cells, but also in whole brains(Fig. 3). No expression was confirmed, on the other hand, in thymus, spleen, kidney, liver, lung, testis or heart tissue.

Isolation of human counterpart of OSF-1 cDNA. The human counterpart of OSF-1 was isolated from the cDNA libraries of human osteosarcoma cell line, MG63, and one of the human brain tissues, superior temporal gyrus. Positive clones were also found in cDNA libraries of other brain tissues, such as nucleus accumbens, caudate, amygdala,

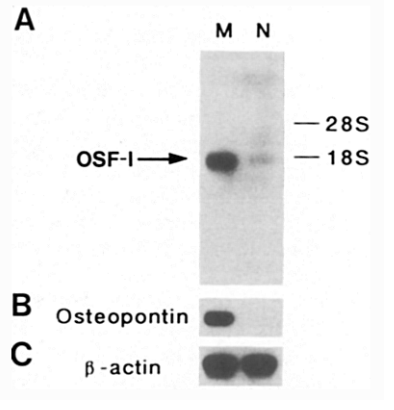

Fig. 1. Northern blotting analysis of mRNAs from MC3T3-E1 and NIH3T3 cells. Volumes of 0.3μg of polyA$^+$ RNA of MC3T3-E1(lane M) and NIH3T3(lane N) were fixed on a nylon membrane after formaldehyde agarose gel electrophoresis and hybridized with various [^{32}P]-labeled DNA probes, such as mouse OSF-1 cDNA(panel A), mouse osteopontin cDNA(panel B) and exon 4 DNA of human β-actin gene(panel C). Positions of ribosomal RNAs are indicated in panel A by 28S and 18S, respectively. The arrow indicates the position of mRNA of OSF-1, at the approximate position of 1,800 nucleotides.

A

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Mouse: ATCCCGCCAAGGAAGCCCCAGAGCACAAGAAAACCCAAAGTGGAGAGAGG   50

GGAAGAAAGAAAGCACTGAGTCATCCATCCAGAAGGGGGGAGAGCAGAGCGCAGCCGCCC  110
                                Human: ..........C----...G....   20

AGGCAGGAGCATCAGCCAG--CGATACCTGGAGTCTGCAGAAACCTCGCCCGCACTTTGC  168
.A.....TG....G..CCG.G.....C..A..G.T.G.AA.-.T...C.C....        79

AACAAAGGCAGCCAGCTAGTCAGCGAGGACCTCTGCAAGCCAAAAAATGTCGTCCCAGCA  228
..........A.T.AGC.C...A.....G.T.C...-.TC....[...]CA.G.T..A..  138

ATATCAGCAGCAACGTAGAAAATTTGCAGCTGCCTTCCTGGCATTGATTTTCATCTTGGC  288
G..C.....G...C.....T.....C.....AC....                         198

AGCTGTGGACACTGCTGAGGCCGGGAAGAAAGAGAAACCTGAAAAAAAGGTGAAAAAGTC  348
.....T.....A..A.....A.....A....G.....                         258

TGACTGTGGAGAATGGCAGTGGAGTGTGTGCGTGCCTACCAGCGGGGACTGTGGATTGGG  408
.....T.....C....T..A.....GC....                               318

CACCCGGGAGGGCACTCGCACTGGCGCCGAGTGCAAACAGACCATGAAGACTCAGAGATG  468
...A.....G.....A..T.....G..A.....C.....                       378

TAAGATCCCTTGCAACTGGAAGAAGCAGTTTGGAGCTGAGTGCAAGTACCAGTTCCAGGC  528
.....C.....A.....C..G.....A.....                              438

TTGGGGAGAATGTGACCTCAATACCGCCTTGAAGACCAGAACTGGCAGCCTGAAGCGAGC  588
C.....G..C..A...C.....A..T.....                               498

TCTGCACAATGCTGACTGTCAGAAAACTGTCACCATCTCCAAGCCCTGTGGCAAGCTCAC  648
C.....C..A..C.....G.....A..G..                                558

CAAGCCCAAGCCTCAAGCGGAGTCAAAGAAGAAGAAAAAGGAAGGCAAGAAACAGGAGAA  708
.....A.....A..A..T.....                                       618

GATGCTGGAT[TAA]AAGACGCCACCGTCTGTGGACCAGGAAAAGGGCATCAGCAAACAGGA  768
.....[...]....T.T....---.....A..TA.....A.....                675

TCAATTAATTATTCCATTTATACCTACTGTAGGCTTTTTATTCAACAGTTATCTGTAGCT  828
...G....C....G.....TG...C.....G.....A.A.....A....-            734

TAAGTACATGATAGGCAAAAACAAAGAGAAAAGAAATGTTTTTGTAGTAGTGGTTTTTTT  888
.....CA...A.....A.....-A.....C.T.....AA                       793

GTTTTTGTTTTTGTTTTTGTTTTTTTTAATGTATACCATAGTACCAGTAGGGGCTTATAA  948
A.G.A.AC.A.A..ACCA..AGG                                       816

TAAAGGATTGTAATACTATTTAGGAAGTTGAACTCTGTAGTACATAATAGGAGGTAGGAT 1008
TGAGGTAAGTTTTTTGGTGTTGTTATTTTGTTTTGTTTCATTTTGGTTTGGTTTGGTTTT 1068
TGAAGTTATGTGATATTTCACATTTAAATCTTTTTTCTTTTTTACATGTTTTCTCTTGTG 1128
CATCAATTTAAATGTTACAACCATGTAAACTACTTCTCTTGTTAGATAGATTTTCACCTA 1188
GACTTTTTTTCCCAAATCAGAAAAAAAATACACACTAAATAAAGCAGCAATAAAATATAA 1248
ATCATTCTATTGGAGAGAAATGCATTGTTTTCTGCCAGTGGATATTTTCTTTGAAAGTTT 1308
GCAGACTGAGAGGAGAGAGGCAGAGCAACGATGTAGTGAAATGTTGATCTTTGTTTTTTT 1368
TTTTTTTTTAAGATAAGATTGAAACATGAAATCCTTTCACTTTGGCAGAAAAACATTTG 1428
TTTTCTTGATGAAATTATTTTTACATCTGAGGAAAAAAATCTAGGAAAATAAAACAAGTG 1488
ATGCTGAATTAAAAAAAAAAAAAAAA                                    1514
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B

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Mouse: MSSQQYQQQRRKFAAAFLALIFILAAVDTAEAGKKEKPEKKVKKSDCGEW   50
Human: .QA.....F.....                                       50

QWSVCVPTSGDCGLGTREGTRTGAECKQTMKTQRCKIPCNWKKQFGAECKYQFQAWGECD 110
.....                                                       110

LNTALKTRTGSLKRALHNADCQKTVTISKPCGKLTKPKPQAESKKKKKEGKKQEKMLD   168
.....E.....                                                 168
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Fig. 2. A) Mouse and human OSF-1 cDNA sequences. The mouse OSF-1 cDNA sequence, 1,514 nucleotides long, is shown on the upper line. Nucleotides which differ in the human OSF-1 cDNA sequence, 816 nucleotides long, are indicated below the corresponding positions of the mouse sequence. Dots represent identical bases and dashes indicate gaps or deletions. The initiation and termination codons are boxed. The possible poly adenylation signal sequence is shown by the underline located at positions 1476 to 1481 in the mouse cDNA sequence. B) Mouse and human OSF-1 protein sequences deduced from respective cDNA sequences. The OSF-1 protein sequences are shown, including the first methionine followed by possible signal sequences (up to the arrow head), defined according to amino terminal sequencing data(17,18). Four amino acid replacements in human OSF-1 are shown below the mouse sequence. Dots represent identical amino acid residues in human. A cluster of amino acid residues having a partial sequence homology to that of MK is underlined.

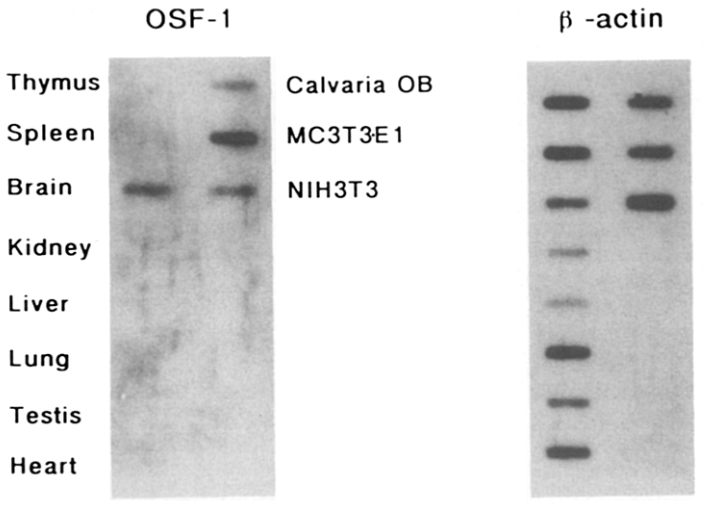

Fig. 3. Tissue specificity of OSF-1. Volumes of 10μg of total RNA prepared from thymus, spleen, brain, kidney, liver, lung, testis, heart, calvarial osteoblast-enriched cells(Calvaria OB), MC3T3-E1 and NIH3T3 were fixed on a nylon membrane and hybridized with radioactive probes of mouse OSF-1 cDNA and exon 4 DNA fragment of human β-actin gene.

hippocampus, putamen and substantia nigra(data not shown). The DNA sequence of human OSF-1 cDNA was determined(Fig. 2A) and it was revealed that 4 out of 168 amino acid residues have been changed during the course of evolution(Fig. 2B). In the region of possible signal sequence(see Fig. 2B), serine, serine and leucine residues at amino acid positions 2, 3 and 20 are replaced by glutamine, alanine and phenylalanine residues in human, respectively, when the first methionine residue is defined to be at position 1. On the other hand, in the mature protein region, only one change was found at amino acid position 130, from aspartic acid residue to glutamic acid residue. We calculated values of nucleotide difference for this gene between mouse and human as defined by Miyata and Yasunaga(16). These values were 0.48 and 0.013 for silent positions and amino acid replacement positions, respectively. This suggests that although the mutation rate of this gene itself was not particularly low at DNA level after the divergence of mammals, the OSF-1 gene may have been under strong constraint at the protein level during mammalian evolution .

Discussion

By differential hybridization screening between mouse osteoblastic cells, MC3T3-E1, and fibroblastic cells, NIH3T3, we obtained a cDNA clone encoding a 168 amino acid long peptide, including the first methionine, which is expressed in osteoblasts and brain tissues, but not in other tissues examined(Fig. 3). Protein sequence homology search revealed that OSF-1 shares partial sequence homology with MK, a differentiation factor identified in embryonal teratocarcinoma cells induced by retinoic acid(6, 7)(Fig. 2B). In spite of such homology, the manner of tissue specific expression seems to be

distinguishable. While MK is said to be expressed specifically in kidney in the adult mouse, OSF-1 is not expressed in kidney but specifically in osteoblasts and brain tissues.

We also found that the first 14 amino acid sequence from N-terminus of heparin binding neurite growth factor purified from rat brain(17) and the first 25 amino acid sequence of heparin binding growth factor isolated from bovine uterus(18) coincide with amino acid sequences of OSF-1-between 33rd and 46th positions and between the 33rd and 57th positions, respectively. Considering other similarities such as molecular weight and protein basicity as well, we postulate that OSF-1 identified in this work and these heparin binding factors may prove to be identical and that this highly conserved protein may play an important role in both bone and brain metabolism in mammals.

Acknowledgments: We thank Dr. Y.Otawara-Hamamoto for critical reading of this manuscript, Dr.Y.Sakaki for human β-actin gene and Mr.K.Higashino for culturing MC3T3-E1 cells.

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