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Distribution and expression of mRNAs for the proto-oncogenes c-fos and c-jun in bone cells *in vivo*

M. Oyama[1], J. Chiba[1], Y. Kato[2], N. Igarashi[2], M. Yoshida[1], M. Ishigami[1], S. Sugawara[1] and M. Kobayashi[2]

[1]Department of Orthopaedic Surgery, Tokyo Women's Medical College, Daini Hospital, Tokyo, Japan and

[2]Department of Pathology, Tokyo Women's Medical College, Tokyo, Japan

Summary. In this study we assessed the expression and localization of the proto-oncogenes c-fos and c-jun in normal bone so as to gain more insight into the role of these proto-oncogenes in bone tissue. Femurs of 4-week-old rats were examined by non-radioactive *in situ* hybridization. cDNA probes for c-fos- and c-jun-labeled digoxygenin were produced by Polymerase Chain Reaction (PCR).

C-fos and c-jun exhibited similar distribution in growth plate and bone tissue. Expression of c-fos and c-jun mRNAs in growth plate was observed in the proliferative zone and partly in the upper layer of the hypertrophic zone. In spongy bone, high expression of c-fos and c-jun mRNAs was observed in the osteoblast cytoplasm. However, there was little expression in bone lining cells. In the bony trabeculae, slight expression of c-fos and c-jun was observed in the premature osteocytes situated close to the bone surface, but no expression was detected in osteocytes that possessed relatively large lacunae in the center of the trabeculae. C-fos and c-jun were also slightly expressed in osteoclasts. These data strongly suggest that c-fos and c-jun are involved in regulating chondrocyte proliferation as immediate early genes, and may also be involved in the gene expression of bone matrix proteins as transcription factor (AP-1) *in vivo*. In addition, the fact that strong expression was observed in osteoblasts but hardly any expression at all in bone lining cells seems to suggest that these genes are also involved in osteoblast activation.

Key words: Osteoblast, Proto-oncogene

Introduction

The proto-oncogene c-fos is the cellular homologue of v-fos oncogene derived from murine osteosarcoma virus (Curran et al., 1982), and c-jun is the cellular homologue of the v-jun oncogene derived from avian sarcoma virus (Maki et al., 1987). C-fos and c-jun genes are included in the category of immediate early genes, a group of genes expressed initially when resting phase (G0 phase) cells are stimulated by serum or appropriate growth factors (Quantin and Breathnack, 1988). And the expression of these proto-oncogenes is controlled at transcriptional level (Ryseck et al., 1988). The c-fos and c-jun gene products form heterodimers via the leucine zipper structure, become activator protein-1 (AP-1), and are known to control transcription by binding to the AP-1 site, which is present in the target gene promotor region, as a trans-acting factor (Chiu et al., 1988). Furthermore, AP-1 sites are known to be present in the promotor region of some bone-specific genes, such as type I collagen, alkaline phosphatase, and osteocalcin (Monson et al., 1982; Matuura et al., 1990; Owen et al., 1990). Therefore, the osteoblast phenotype may be affected by the expression of c-fos and c-jun via their gene products. In fact, *in vitro*, expression of c-fos and c-jun family has been shown to be associated with bone maker genes during osteoblast proliferation and differentiation (McCabe et al., 1996). Moreover, physiological mediators and hormones such as vitamin D (Candeliere et al., 1991) and parathyroid hormone (Clohisy et al., 1992; Lee et al., 1994) have been shown to affect osteoblast phenotype via the expression of c-fos and c-jun. Interestingly, the sequence of vitamin D-responsive element overlaps AP-1site in osteocalcin gene (Schule et al., 1990).

With respect to the relationship between c-fos expression and bone-forming cells, c-fos is over-expressed in murine and human osteosarcoma (Shoen et al., 1986; Ruther et al., 1987; Wu et al., 1990) and c-fos is expressed in osteoblastic cells in the fracture healing process (Ohta et al., 1991). The AP-1 site is also present in the promotor region of collagenase (MMP-1) and stromelysin (MMP-3) (Brenner et al., 1989; Lafyatis et al., 1990; Sirum-Conney and Brinckerhoff, 1991), and c-fos and c-jun seem to be involved not only in the formation of bone matrix, but in the bone and cartilage destruction in the pathophysiology of rheumatoid arthritis.

However, an adequate understanding of the expression and distribution of c-fos and c-jun in normal

Offprint requests to: Dr. Masaya Oyama M.D. 2-1-10 Nishiogu Arakawa-ku Tokyo, 116 Japan. FAX: 81-3-3810-9934.

bone tissue, and of their biological significance in metabolism, has not yet been defined, and thus in this present study we assessed the expression and localization of these proto-oncogenes in the bone tissue of rats during the growth process by *in situ* hybridization.

Materials and methods

Animals

Four week-old Wistar rats obtained from Japan Laboratory Animal Inc. (Tokyo, Japan) were used. Rat bone tissue was removed surgically under ether anesthesia. Tissue for RNA analysis was frozen and stored at -80 °C until used. Tissue for histological examination was fixed in 4% buffered paraformaldehyde, decalcified in 20% EDTA, and embedded in paraffin.

Experiments for *in situ* hybridization studies were performed in triplicates on three different animals.

Preparation of cDNA probes

Isolation of total cellular RNA

Cellular RNA was prepared from rat bone tissue by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomzynski and Sacchi, 1987).

Reverse transcriptation of RNA

RNA samples containing 10 μg of total cellular RNA, 50 mM of Tris-HCl pH 8.3, 75 mM KCl, 0.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 20 U of RNase inhibitor, 100 pM of random hexamer (TaKaRa, Tokyo, Japan) and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, Life Technologies, Inc., Gaitherburg, Md, USA) in a final volume of 20 μl were transcribed by incubation at 37 °C for 60 min. The samples were heated at 94 °C for 5 min to terminate the reaction, and then stored at -20 °C until used.

Preparation of oligonucleotide primers

Oligonucleotide primers were constructed from the published cDNA sequence of rat c-fos and c-jun cDNA (Kitabayashi et al., 1990). The following primers were made by TakaRa (Tokyo, Japan).

The sequences of the rat c-fos primers were 5'-TACCATTCCCCAGCCGACTC-3' (coding sense), corresponding to bases 222-241 of the cloned full-length sequence, and 5'-TGCTCTACTTTGCCCCTTCT-3' (anticoding sense), corresponding to bases 507-526.

The sequences of the rat c-jun primers were 5'-CATGGTCGCTCCCGCTGTGG-3' (coding sense), corresponding to bases 2677-2696 of the cloned full-length sequence, and 5'-CGCTTCCTCTCCGCCTTGAT-3' (anticoding sense), corresponding to bases 3020-3039. The predicted sizes of the amplified rat c-fos and c-jun products were 305 and 363 bp, respectively.

Amplification of cDNA

Each reverse transcription mixture was diluted 1:5 in RNase-free water, and 10 μl was then transferred to fresh tubes for amplification. Each sample contained the coding sense and anticoding sense primers (200 μM of each primer) spanning the given sequence for amplification, 200 mM of each digoxygenin-labelled dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl pH8.3, 10 mM $MgCl_2$, and 2.5 U of Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) in a final volume of 100 μl. The amplification profiles consisted of denaturation at 94 °C for 1.5 minutes, annealing at 58 °C for 1minute, and extension at 72 °C for 1 minute. Negative control reactions without template were routinely included in PCR amplifications with both primer sets.

In preliminary experiments, PCR was performed using dNTP (non- digoxygenin-labeled) for c-fos and c-jun. Both PCR products were electrophoressed on agarose gel and stained with ethidium bromide to detect amplified fragments, and PCR products were identified by their respective sizes.

Sequencing

Each PCR product was purified after the agarose gel electrophoresis with SUPEC-01 (TaKaRa, Tokyo, Japan), blunted by T4 polymerase and subcloned into EcoRV site of Bluescript II KS(+) plasmid. The products were sequenced by the GENESIS 2000 DNA analysis system (Du Pont Medical Products, Wilminton, Del, USA) to confirm that the PCR products obtained were the same as the published cDNA clones.

Preparation of tissue sections

Serial 5 μm sections were cut from each block, and one slide was stained with hematoxylin and eosin for histological interpretation. The sections for the *in situ* hybridization studies were bound to aminopropyltriethoxysiline-coated microscope slides and incubated at 50 °C for 2 hours.

in situ hybridization

Sections were deparaffinized in xylene, dehydrated through ethanol, and air dried. Basic protein was removed by incubation for 15 minutes in 0.2N HCl at room temperature. After rinsing in 2xSSC (20xSSC is 3M NaCl and 0.3M sodium citrate, pH7.0) at room temperature, the sections were treated with 10 μg/ml proteinase K (Amresco, Ohio, USA) in 20 mM Tris-HCl pH7.4 and 2 mM $CaCl_2$ at 37 °C for 1 hour, rinsed in phosphate-buffered saline (PBS), and refixed

for 5 minutes in 4% paraformaldehyde-PBS. The sections were then rinsed in PBS and acetylated for 10 minutes in freshly made 0.1M triethanolamine (pH 8.0) solution containing 0.25% acetic anhydride. The slides were washed in 2xSSC for 5 minutes, three times, dehydrated through ethanol, and then air dried.

The hybridization mixture contained 50% formamide, 5xSSC, 5xDenhardt's solution (100x Denhardt's solution is 2% Ficoll, 2% polyvinyl-pyrrolidone [PVP], 2% bovine serum albumin [BSA]), 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA (sssDNA), and 10% dextran sulfate. Each section was covered with 100 μl hybridization solution containing 200ng DIG-labeled cDNA probe. Hybridization was carried out in a moist chamber at 37 °C for 15 hours. The slides were subsequently washed twice in 2xSSC at room temperature for 30 minutes, and then washed twice with 50% formamide/1xSSC at 45 °C for 10 minutes. The color of the sections was then developed with 5 mM levamizole according to the manufacturer's instructions (Boehringer Mannheim, Germany). Negative control experiments were performed by either pretreating tissue sections with RNase A (100μg/ml in 2xSSC) at 37 °C for 1 hour before hybridization or by incubating tissue sections with an excess amount of unlabeled cDNA.

Results

The epiphyseal growth plate and primary bony trabeculae of the femur of 4-week-old rats develop well, and numerous osteoblasts are found at the surface of the primary trabeculae. We assessed the expression of the proto-oncogenes c-fos and c-jun in bone tissue during this developmental process by *in situ* hybridization. Digoxygenin-labeled cDNA probes were used to identify c-fos and c-jun transcription products. The results are outlined in Table 1. No signal was obtained by *in situ* hybridization in the RNase-treated sections prepared as controls. By *in situ* hybridization, c-fos and c-jun exhibited similar distribution in growth plate and bone tissue.

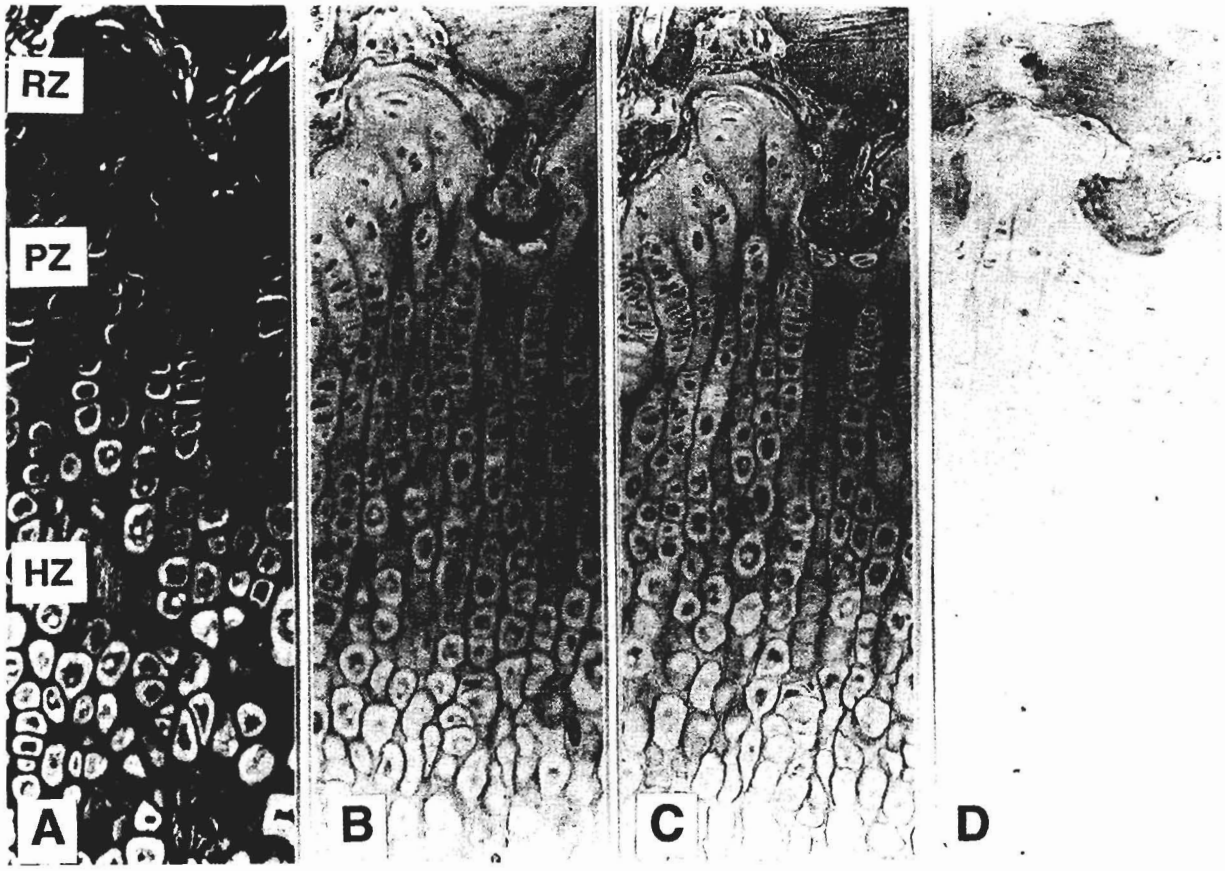

Fig. 1. Expression of c-fos and c-jun mRNAs in growth plate of distal femurs. **A.** Histology showing region of enchondral ossification: resting zone (RZ); proliferaring zone (PZ); hypertrophic zone (HZ). H-E staining. Serial sections hybridized with digoxygenin labeled c-fos probe (**B**) and c-jun probe (**C**); Both signals are predominantly present in proliferating chondrocytes and partly hypertrophic chondrocytes; resting chondrocytes are negative. **D.** Negative control treated with RNase. A-D x 50

Epiphyseal growth plate

The growth plate of the distal femur has a three-zone structure consisting of a resting zone, a proliferating zone, and a hypertrophic zone, proceeding toward the diaphysis. The hypertrophic chondrocytes were 3 to 10 times larger than the cells in the resting and proliferating zones, and the cells were also in close contact with each other. The boundary between the hypertrophic zone and the primary spongiosa was distinct; the two zones were separated by an almost perfect straight line. C-fos and c-jun mRNAs were highly expressed in this epiphyseal growth plate in the proliferating zone and partially in the upper layer of the hypertrophic zone (Fig. 1).

Bone

Many osteoblasts containing abundant cytoplasm and Golgi apparatus were seen along the surface of trabecular bone, and there were also some flat bone lining cells. C-fos and c-jun mRNAs were observed intensely in the cytoplasm of these osteoblasts that exhibited cubical and fusiform shapes (Fig. 2). In contrast, there was very little expression of either c-fos or c-jun mRNA in the flat bone-lining cells (Fig. 3).

In the bony trabeculae, slight expression of c-fos and c-jun was present in premature osteocytes located close to the surface of the trabecular bone and partially embedded in bone. No expression was detected in

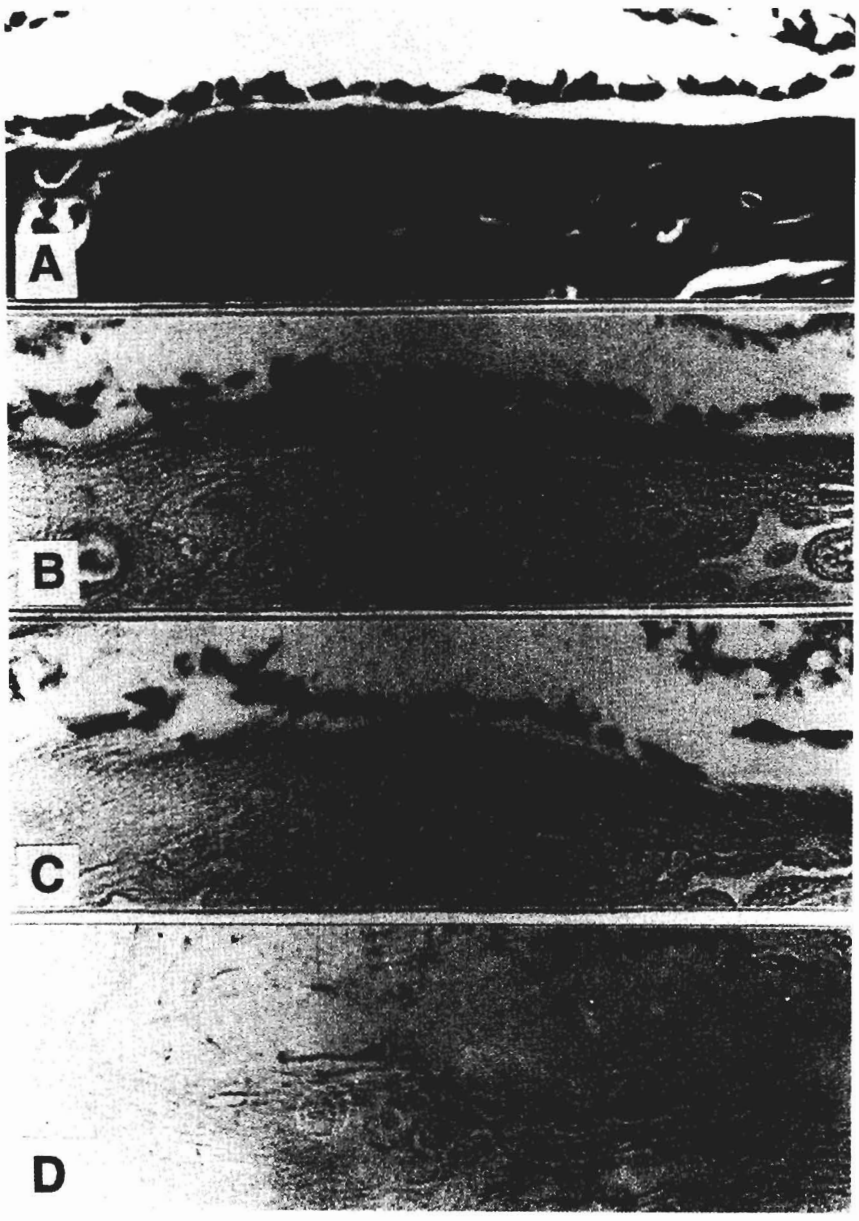

Fig. 2. Expression of c-fos and c-jun mRNAs in osteoblasts of secondary spongiosa. (**A**) Histology showing well developed Golgi apparatus in cytoplasm of osteoblasts. H-E staining. Serial sections showing strong expression and co-localization of c-fos (**B**) and c-jun (**C**) in cytoplasm of osteoblasts. Both mRNA are slightly expressed in premature osteocyte (arrowhead) and no expression was seen in mature osteocytes (arrows). (**D**) Negative control treated with RNase. x 100

the enlarged mature osteocytes within the bone (Fig. 2).

There were scattered typical multinucleated osteoclasts possessing relatively large cell bodies in places where the surface of the bony trabeculae was depressed. These cells showed very little expression of c-fos and c-jun mRNAs (Fig. 4). Some of the bone marrow cells also showed slight expression of c-fos and c-jun mRNAs.

Discussion

In this study, we assessed the expression and localization of the proto-oncogenes c-fos and c-jun by *in situ* hybridization in bone cells *in vivo*. As summarized in Table 1, transcripts of c-fos and c-jun were expressed in the growth plate, predominantly in the proliferating zone, and partially in the upper layer of hypertrophic zone. There was also high expression in spongy bone, predominantly in the osteoblasts. The two proto-oncogenes had similar distributions in the bone tissue. Co-localization of c-fos and c-jun appears to be based on the fact that the products of these genes form hetero-dimers, become AP-1, and may act on bone-specific genes such as type I collagen, alkaline phosphatase, and osteocalcin.

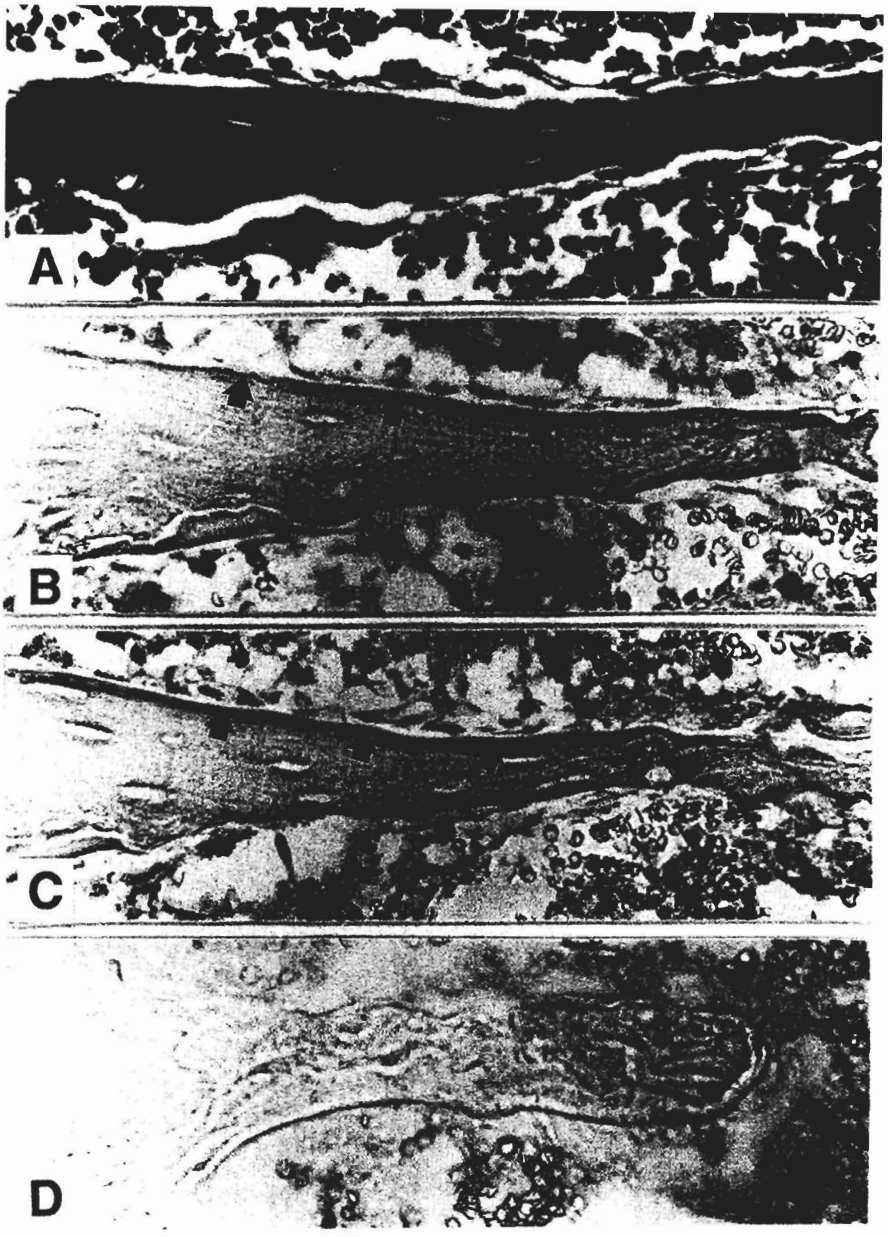

Fig. 3. Expression of c-fos and c-jun mRNAs in lining cells of secondary spongiosa. (**A**) Histology showing flat lining cells on the surface of trabecular bone (H-E staining). Serial sections hybridized with digoxygenin-labeled c-fos probe (**B**) and c-jun probe (**C**). Both signals are weak or negative in lining cells (arrows). **D.** Negative control treated with RNase. x 100

Table 1. The expression of the messenger RNAs for c-fos and c-jun in rat bone tissue.

	C-FOS	C-JUN
Growth plate		
Resting zone	-	-
Proliferating zone	++	++
Hypertrophic zone		
Upper	+	+
Lower	-	-
Bone		
Osteoblast	++	+++
Lining cells	±	±
Premature osteocytes	+	+
Mature osteocytes	-	-
Osteoclasts	±	±
Bone marrow cells	+	+

A visual assessment was made of the relative intensity of the hybridization signals obtained using the cDNA probes when compared with the controls. -: absent; +: week; ++: moderate; +++: strong.

Growth plate

Growth plates are the sites responsible for the growth in length of long bones by endo-chondral ossification. In view of the fact that vigorous cell proliferation occurs in the proliferating zone (Robertson, 1990), our findings, i.e., that c-fos and c-jun mRNAs are present in the proliferating zone, are consistent with the basic property of the c-fos and c-jun genes being involved in the cell proliferation and differentiation. Moreover, in the mouse, in which the c-fos gene is deficient because of gene targeting, the thickness of the proliferating zone is greatly reduced and the columnar arrangement along the longitudinal axis is irregular (Wang et al., 1992). This suggests that c-fos is an essential element in the differentiation and proliferation of normal chondrocytes in the proliferating zone, and the results of our *in situ* hybridization study paradoxically seem to support the finding of c-fos expression in normal proliferating zones.

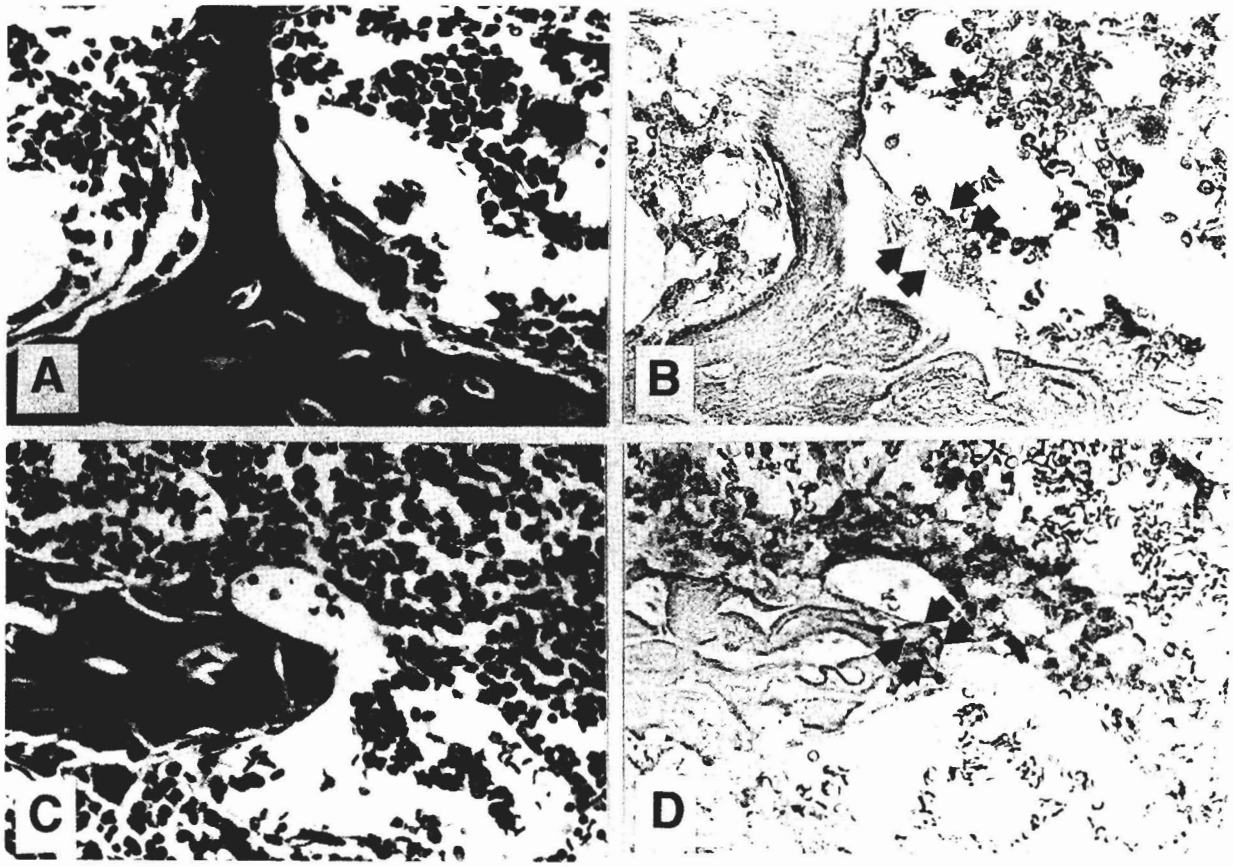

Fig. 4. Expression of c-fos and c-jun mRNAs in osteoclasts of secondary spongiosa. (**A, C**) Histology showing well differentiated and multi-nucleated osteoclasts (H-E staining). (**B**) Serial section to (A), treated with digoxigenin labeled c-fos probe, cytoplasm of osteoclast are weak or negative (arrows). **D.** Serial section to (C), treated with digoxigenin labeled c-jun probe, osteoclast is also weak or negative (arrows). x 100

Bone

Intense expression of c-fos was observed in osteoblasts and osteocytes in the bone matrix of long bones before the onset of osteosarcoma in transgenic mice in which overexpression of c-fos was induced (Grigoriadis et al., 1993). Because of this, the authors suggested that the transgenes were expressed predominantly in cells of the osteoblastic lineage. The c-fos and c-jun expression in cells of the osteoblastic lineage detected by *in situ* hybridization in the present study was also intense in osteoblasts, but there was little expression in bone lining cells or immature osteocytes, and mature osteocytes were negative. These findings suggest that these proto-oncogenes may be involved in the differentiation and proliferation of osteoblastic cells *in vivo*. Our data is also in good agreement with *in situ* hybridization showing c-fos expression in mechanically- loaded and control osteoblasts (Inaoka et al., 1995) and embryonic mouse bones (Carrasco et al., 1995). Intense expression is observed in osteoblasts, in which Golgi apparatus development and vigorous bone matrix protein synthesis are thought to occur simultaneously, and since there is little expression in bone-lining cells, c-fos and c-jun also seem to be involved in osteoblast activation during physiological bone remodeling.

However, overexpression of c-fos inhibits the production of type I collagen by osteoblasts (Kuroki et al., 1992), and leads to deregulated bone development (Ruther et al., 1987). Thus, as shown by the results of *in situ* hybridization in the present study, proper c-fos expression is required for physiological bone metabolism, and failure to do so leads to disease, such as tumors and rheumatoid arthritis.

On the other hand, since osteopetrosis develops as a result of gene targeting (Wang et al., 1992), c-fos also appears to be involved in the proliferation and differentiation of cells in the osteoclast lineage. Moreover, there has been a report that non-receptor type tyrosine kinase, c-src, is involved in ruffled border formation and bone resorption activation in differentiated osteoclasts (Boyce et al., 1992). The *in situ* hybridization data presented here showed that the c-fos and c-jun mRNA expression was weak or negative in multinucleated typical cells of osteoclasts. Interestingly, c-fos expression is required for osteoclast progenitor development and proliferation, but not for maturation *in vitro* (Udagawa et al., 1996) and also c-fos expression is considered to be required in the commitment of osteoclast progenitors, the common differentiation unit of osteoclasts with monocytes/macrophages, to osteoclasts (Kukita and Kukita 1996). These data are in good agreement with the absence of c-fos expression in mature osteoclasts in the present study. These findings suggest that these proto-oncogenes are not involved in mature osteoclast activation and may affect the osteoclastic lineage of early stages of differentiation *in vivo*.

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