Biochemical and Biophysical Research Communications **274,** 171–176 (2000)
doi:10.1006/bbrc.2000.3112, available online at http://www.idealibrary.com on IDEAL®

Identification and Expression of a Novel 3′-Exon of Mouse Runx1/Pebp2αB/Cbfa2/AML1 Gene

Kunikazu Tsuji and Masaki Noda[1]

Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10, Kanda-Surugadai, Chiyoda-ku, Tokyo, 101-0062, Japan

Received June 16, 2000

Cbfa1 (recently renamed as Runx2) is prerequisite for the differentiation of osteoblasts that play a central role in regulation of bone metabolism. Runx transcription factors belong to *Drosophila* pair rule gene "runt" family that also play critical roles during the development of hematopoietic tissues or insect bodies. To identify novel sequences related to Runx (Cbfa) genes, we screened a 11 d.p.c (days postcoitum) mouse embryonic cDNA library using a probe corresponding to Runx2 (Cbfa1) runt domain and identified a novel cDNA fragment. This cDNA (521–41) is about 2.4 kb in length and contains a sequence identical to the N-terminal half of mouse Runx1 (Pebp2αB/Cbfa2/AML1b) and a novel protein coding sequence in the C-terminal region. High expression levels of 521–41 mRNA were observed during the early mouse embryogenesis. In adult mice, 521–41 mRNA was expressed ubiquitously in lumbar vertebrae, brain, kidney, heart, muscle, and ovary. We also found that 521–41 mRNA was constitutively expressed in an osteoblast-like cell line, MC3T3E1. These data indicate that 521–41 gene is a novel splicing variant of mouse Runx1 which is expressed in skeletal tissues and osteoblast-like cells.

Osteoblasts are considered to play a central role in regulation of bone metabolism. During the remodeling cycle, osteoblasts are continuously recruited and, therefore, control of the differentiation of osteoblasts in adult bone is critical in maintaining the balance of bone mass. Runx2 (Cbfa1) transcription factor is prerequisite for the differentiation of osteoblasts (1–3). Runx2 (Cbfa1) belongs to mammalian Runx transcription factors that contain a region homologous to the DNA binding region in a *Drosophila* pair-rule gene product, Runt. This region is necessary for *Drosophila* larvae to develop (4). Mammalian Runx family consists of three related genes, Runx1, Runx2 and Runx3, which are also called as Pebp2αB/Cbfa2/AML1 (5–7), Pebp2αA/Osf2/Til-1/Cbfa1/AML3 (6, 8–10) and Pebp2αC/Cbfa3/AML2 (6, 11, 12) respectively. These mammalian Runx transcription factors are essential for the development of mouse embryos as the knock out experiments of these genes result in embryonic or neonatal lethality.

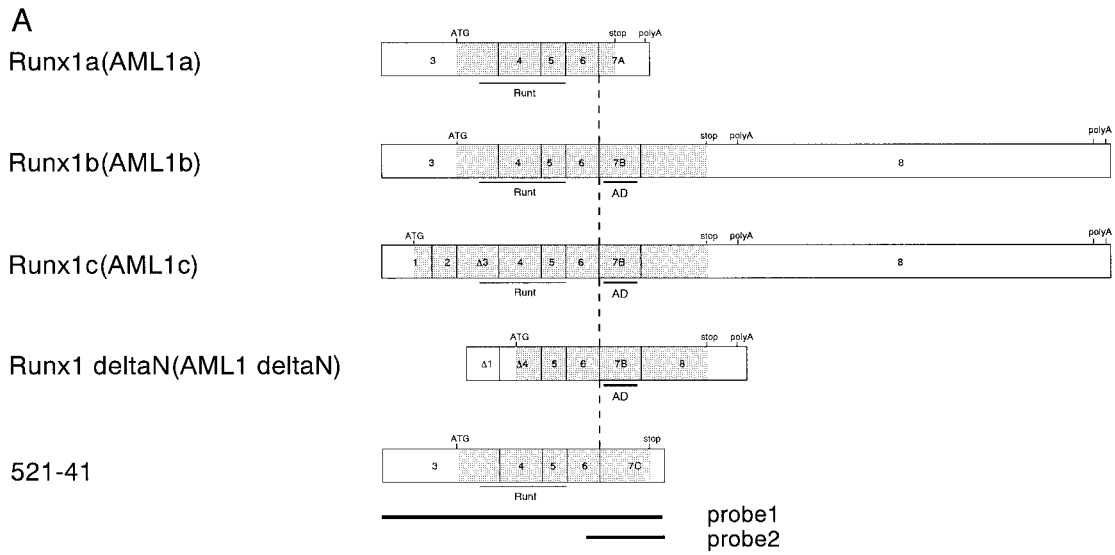
Runx2 (Cbfa1) knock out mice die immediately after birth because of the failure in breathing (1–3). In Runx2 knock out mice, skeletal patterning was normal but severe maturational arrest in osteoblasts and chondrocytes was observed (1, 13). Two major isoforms of Runx2 have been identified. Pebp2αA/Cbfa1 was found in Ras transformed NIH3T3 fibroblast cell line. This isoform is expressed in various T-cell lines (9). Pebp2αA/Cbfa1 binds to enhancer elements of T-cell-specific genes and is considered to be a regulator of T-cell development (9). Another isoform, Osf2/Cbfa1, was first identified as a binding protein of osteoblast specific enhancer element (OSE2) of osteocalcin (8). High level of Osf2 expression is detected at embryonic day 11 (E11) in mouse fetus (8). This is the time point where mouse skeletogenesis begins to occur. In fact, Osf2 mRNA is expressed in bone precursor cells. A short isoform of Osf2 (also called as Til-1 (10)), which starts from the second ATG codon of Osf2 mRNA (14), is a positive regulator of osteocalcin gene expression in various mesenchymal cell lines (15, 16). Thus, Osf2 is considered as a key transcription factor during osteoblast differentiation. However, factors upstream to Osf2 in bone cells have not yet been found.

Cloning and characterization of mouse Runx2 gene promoter (Osf2 upstream region) revealed the presence of Runx2 binding sites (OSE2) (17). Mouse Runx1 and Runx2 can bind these elements by gel electromo-

Abbreviations used: Cbfa, core binding factor a; Pebp2, polyomavirus enhancer binding protein 2; AML, acute myeloid leukemia; α MEM, minimum essential medium α modification; PTH, parathyroid hormone; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BMP2, bone morphogenetic protein 2; FGF, fibroblast growth factor; TGFβ, transforming growth factor β.

[1] To whom correspondence should be addressed. Fax +81-3-5280-8067. E-mail: noda.mph@mri.tmd.ac.jp.

0006-291X/00 $35.00



B

human	Runx1a(AML1a)	EEDTAPWRC
human	Runx1b(AML1b)	DTRQIQPSPPWSYDQSYQYLGSIASPSVHPATPISPGRASGMTTLSAELSSRLS
human	Runx1c(AML1c)	DTRQIQPSPPWSYDQSYQYLGSIASPSVHPATPISPGRASGMTTLSAELSSRLS
	521-41	GKNPTEPTTLCLCWSPRRRKHRGCQAFLGALRELLKPRSISWEPNEENAVPSAEYLYSEKCGC

FIG. 1. Structure and novel 3′-amino acid sequence of clone 521–41. (A) Schematic structures of clone 521–41 and human Runx1 isoforms, Runx1a (AML1a), Runx1b (AML1b), Runx1c (AML1c), and Runx1 delta N (AML1 delta N). The coding region is indicated by gray box. Runt domain and transactivation domains are indicated by underlines. Exon numbers are indicated in each box. (B) Amino acid sequence of the novel 3′-codiong region of clone 521–41.

bility shift assay and therefore Runx2 regulates its expression as a positive feedback (18). To search for a novel Runx gene which may regulate the expression of Runx2 gene during mouse embryogenesis, we screened a E11 mouse-embryo-derived cDNA library using a cDNA fragment of runt domain of mouse Runx2 as a probe. We identified a novel alternative splicing variant of mouse Runx1 which is highly expressed during mouse embryogenesis and lumbar vertebra in adult mice.

MATERIALS AND METHODS

Reagents. 11-day embryonic mouse cDNA library was purchased from Clontech Laboratories (Palo Alto, CA). Alpha MEM medium and fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). Human recombinant BMP2 was kindly supplied by Genetics Institute (Cambridge, MA).

Plaque hybridization screening. To obtain novel cDNA fragments containing runt domain, λ gt11 mouse embryo-derived cDNA library was prepared on ten 22 × 22 cm^2 Luria-Bertani plates and 2 × 10^6 plaques were screened after transferring to nylon filters (GeneScreen, NEN Research Products, Boston, MA). Filters were hybridized with a ^{32}P-labeled NcoI-HindIII fragment of mouse Runx2 (Pebp2αA1/Cbfa1) cDNA which contains all of runt domain.

Cell culture. Clonal mouse osteoblastic MC3T3E1 cells were kindly provided by Dr. Kodama (Oh-U University, Japan) and were maintained in alpha MEM supplemented with 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. To examine the effects of cytokines and hormones on the expression of the cloned gene, the cells were plated at 3 × 10^4 cells/cm^2 one day prior to the treatment and then the media were replaced next day with fresh ones containing cytokines, hormones or vehicle.

RNA preparation and Northern blot analysis. Total cellular RNA from MC3T3E1 cells or mouse tissues was extracted according to acid guanidine phenol chloroform method (19). Ten micrograms of total RNA per lane was fractionated in agarose gel (1% agarose, 20 mM MOPS, pH 7, 5 mM sodium acetate, 0.5 mM EDTA, 2.3% formaldehyde) and was transferred onto nylon membranes (GeneScreen, NEN Research Products, Boston, MA). The membranes were incubated at 42°C for six hours in a hybridization buffer (50% formamide, 250 mM sodium phosphate, pH 7.2, 25 mM sodium chloride, 0.5% SDS, 0.2 mg/ml sheared herring sperm DNA, 10% polyethylene glycol MW 6000, 10× Denhardt's solution containing 0.4% Ficoll type 400, 0.4% polyvinyl-pyrolidone, 0.4% BSA fraction V). One of the obtained DNA fragments was labeled with ^{32}P-dCTP using BcaBEST random primer labeling kit (Takara Shuzo Co. Ltd., Tokyo, Japan) and was used for hybridization. Hybridization was performed at 42°C for 24 h in a fresh hybridization buffer containing 1 × 10^6 cpm/ml of the labeled probe. After hybridization, membranes were rinsed at 65°C for 40 min in 0.2× SSC, 0.5% SDS solution and were exposed to X-ray film.

RESULTS

Isolation of a Novel cDNA Fragment Containing Runt Homology Domain

To identify a novel gene of Runx family, we screened a cDNA library derived from E11 mouse embryo and 12

independent clones were isolated. Out of the 12 clones, 9 clones were identical to the mouse Runx1, one was identical to the mouse Runx2 and two clones contained novel DNA sequences which were found to be linked to the 3′ end of the runt homology domain. One of the two clones, named as 521–41, was further analyzed. Analyses of the sequence indicate that 521–41 is about 2.4 kb in length and contains a novel protein coding sequence in the C-terminal region (Fig. 1B). This novel 360-bp exon, which we refer to exon 7C, contains a 189-bp protein coding sequence and 171bp 3′UTR. The conjugation site of this novel mouse sequence, exon 7C, corresponds to that between exon 6 and exon 7A/exon 7B in human Runx1 (AML1a, 1b, 1c and delta N in Fig. 1A) gene (20). No sequence homologous to this novel exon 7C has been found in the human Runx1. N-terminal region of 521–41 contains a sequence identical to the region corresponding to exon 3 to 6 of mouse Runx1 (Pebp2αB1) which is homologous to human Runx1 (AML1b) (20) (Fig. 1A).

Expression of the Clone 521–41 during Embryonic Development

To examine the possibility that 521–41 gene could be expressed earlier than Runx2 during embryogenesis, we performed Northern blot analysis using mRNAs prepared from mouse embryos at different stages. As shown in Fig. 2, high expression levels of a 3.8kb mRNA species which hybridizes to the novel exon 7C sequence of 521–41 were observed in mouse embryos on day 6 and 8 postcoitum. Then the levels decreased on day 12 (Fig. 2).

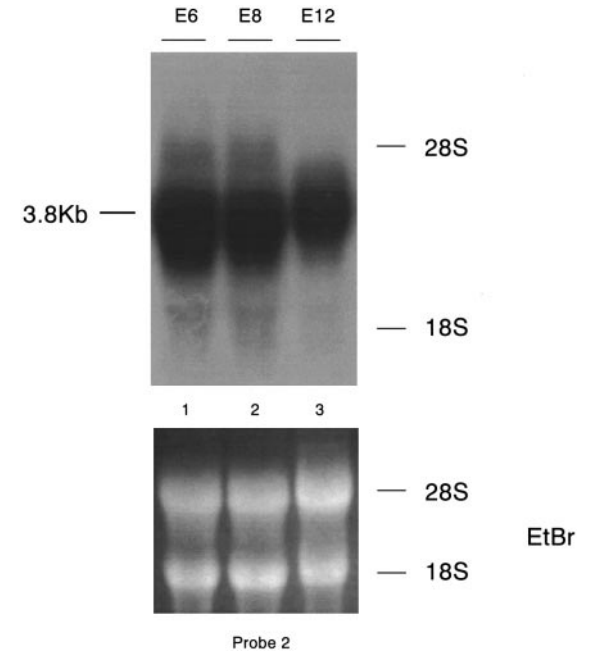


FIG. 2. Northern blot analysis of the mRNA expression of 521–41 during mouse embryogenesis. Northern blot analysis was performed as described under Materials and Methods. Probe 2 indicated in Fig. 1A was used for hybridization to the RNA prepared from mouse embryos at days 6, 8, and 12. This probe contains entire exon 7C, which is specific to clone 521–41 and 3′-114 bp of the exon 6 sequence. 28S and 18S rRNA levels serve as control.

Expression of the Clone 521–41 in Adult Tissues

Tissue distribution of mRNA species hybridized to the full length of cDNA clone 521–41 (probe 1 in Fig. 1A) was examined in adult tissues. Northern blot analysis using a full size 521–41 indicated that the presence of four major bands (7.5, 6.3, 3.8, and 2.1 kb) corresponding to Runx1b (AML1b) and Runx1c (AML1c) in adult lumbar vertebra (Fig. 3A). These four bands were also detected in mouse ovary coinciding with the previous findings on the expression of human Runx1 (AML1) (20, 21). Among those four bands, the 7.5-kb species was more abundant in lumbar vertebrae than that of in ovary. This band corresponds to Runx1b (Pebp2αB/AML1b) (21), which contains transactivation domain, exon 7B and 8, and acts as a positive regulator of target genes containing runt binding sequences in their enhancer region.

We further examined the expression of mRNA specifically hybridized to the novel exon 7C located in the 3′ region of 521–41 (probe 2 in Fig. 1A). As shown in Fig. 3B, a single 3.8-kb band was detected in various tissues including bone, brain, heart, kidney, spleen, ovary and muscle. Among these, the expression level was slightly high in lumbar vertebra.

Expression and Regulation of Clone 521–41 in Osteoblastic MC3T3E1 Cells

To elucidate the possibility whether 521–41 transcript is expressed in osteoblast-like cells and whether its expression is regulated by calciotropic cytokines or hormones, we performed Northern blot analysis. As shown in Fig. 4A, three major bands (6.3, 3.8, and 2.1 kb) were detected when probe 1 was used as a probe. In contrast, a single 3.8-kb band was served in control MC3T3E1 cells when the novel exon 7C-specific probe 2 was used (Fig. 4B). The intensity of the 3.8-kb band was relatively strong in the RNA obtained from MC3T3E1 cells cultured in either the presence or absence of 500 ng/ml BMP2 (Fig. 4B) or other cytokines or hormones tested (TGFβ, basic FGF, retinoic acid, vitamin D_3, dexamethasone, PTH or TPA, data not shown).

DISCUSSION

Identification of a Novel 3′-Exon of Mouse Runx1 Gene

We identified a novel 3′-exon of mouse Runx1. This exon contains a 189-bp fragment of protein coding se-

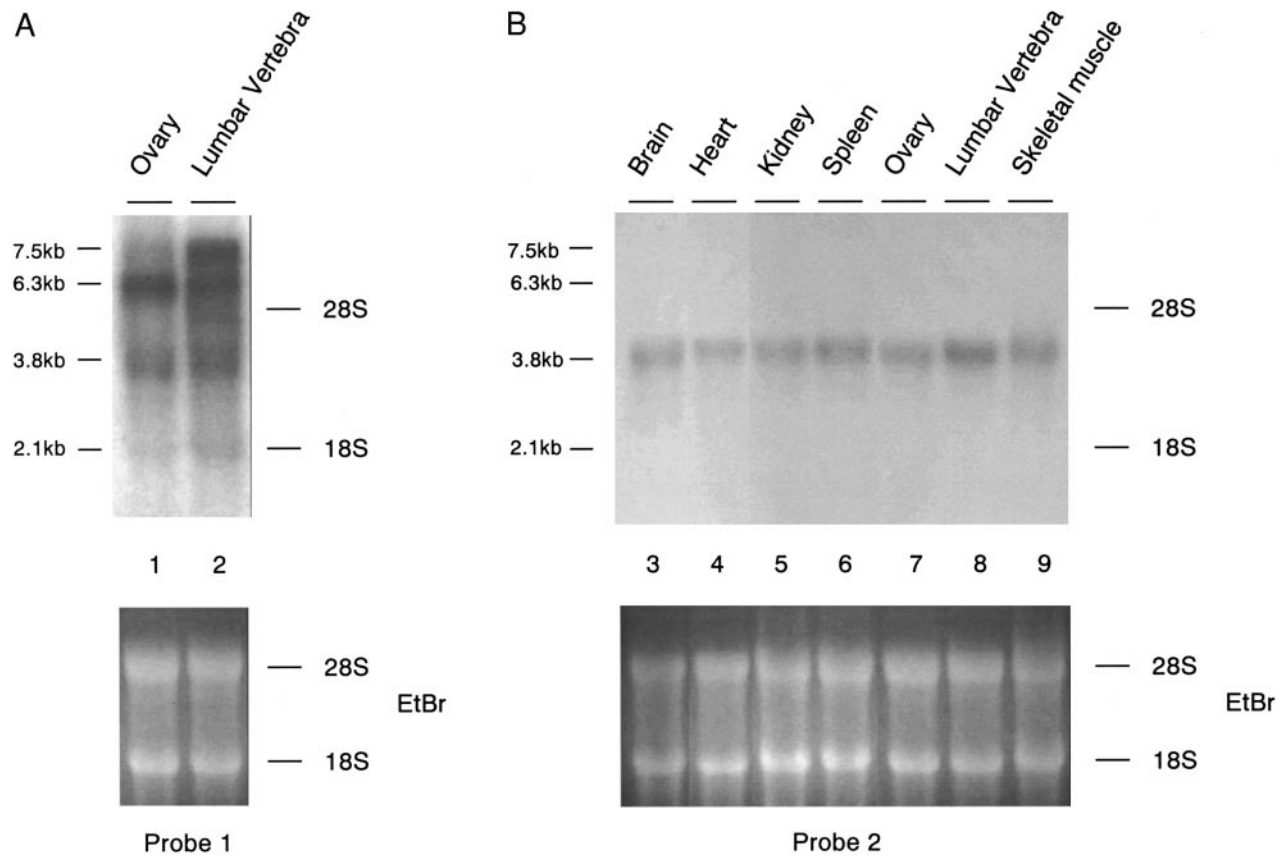


FIG. 3. Northern blot analysis of the 521–41 transcripts in various adult mouse tissues. Ten micrograms of total RNA samples extracted from various adult mouse tissues was fractionated and blotted onto nylon membrane. Northern blot analysis was performed as described under Materials and Methods. (A) Probe 1 indicated in Fig. 1A was used for hybridization. Four bands, about 7.5, 6.3, 3.8, and 2.1 kb in length, were detected. (B) Probe 2 indicated in Fig. 1A was used for hybridization. This probe contains entire exon 7C, which is specific to clone 521–41 and 3′-114 bp of the exon 6 sequence. 28S and 18S rRNA levels serve as control.

quence and a 171 bp of 3′UTR fragment conjugated at exon6/7 boundary. Human Runx1 gene contains several alternative splicing variants which are classified into four subfamilies (Runx1a (AML1a), Runx1b (AML1b), Runx1c (AML1c) and Runx1 delta N (AML1 delta N)) (20). As shown in Fig. 1A, Runx1b, Runx1c, and Runx1 delta N contain the same exons in their 3′ region (exons 6 and 7B), while Runx1a contains a unique exon 7A. The novel exon 7C in the clone 521–41 does not have homology to any of the other 3′-exons (7A and 7B) in previously known Runx1 (AML1) splicing variants and computer analysis of homology search (BLAST) did not find any sequences related to this exon 7C sequence, indicating that 521–41 belongs to a novel subfamily of Runx1 which we suggest as Runx1d.

Functions of the Runx1 Isoforms

Runx1 knockout mice die between embryonic days 11.5 and 12.5 due to hemorrhage in the central nervous system and lack of erythropoiesis and myelopoiesis in the liver. Homozygous Runx1-deficient ES cells failed to contribute to the hematopoiesis in chimeric mice. Thus, Runx1 is considered to be essential for definitive hematopoiesis (22, 23). Four major isoforms of Runx1 gene transcripts are produced by alternative splicing of N-terminal or C-terminal exon (20). Runx1 delta N (AML1 delta N) isoform does not have N-terminal half of runt domain and Runx1a (AML1a) isoform lacks C-terminal activation domain. Transfection of these isoforms results in suppression of target gene expression in 32Dcl3 myeloid cell line or hematopoietic differentiation of K562 leukemia cells (20, 24). On the other hand, hematopoietic defects in Runx1 knock out mice are rescued by Runx1b (AML1b/Pebp2αB1) isoform expressed under the control of Runx1 (AML1)-regulatory sequences through a knock-in approach (25). Therefore, Runx1b (Pebp2αB1/AML1b) isoform plays a central role in definitive hematopoiesis.

Function of 521–41 during embryogenesis is not clear at this point. It has been shown that transactivation domains of mouse Runx1 gene exists in exons 7B and 8, and forced expression of Runx1a but not Runx1b inhibits differentiation of K562 leukemia cells (24). In analogy to

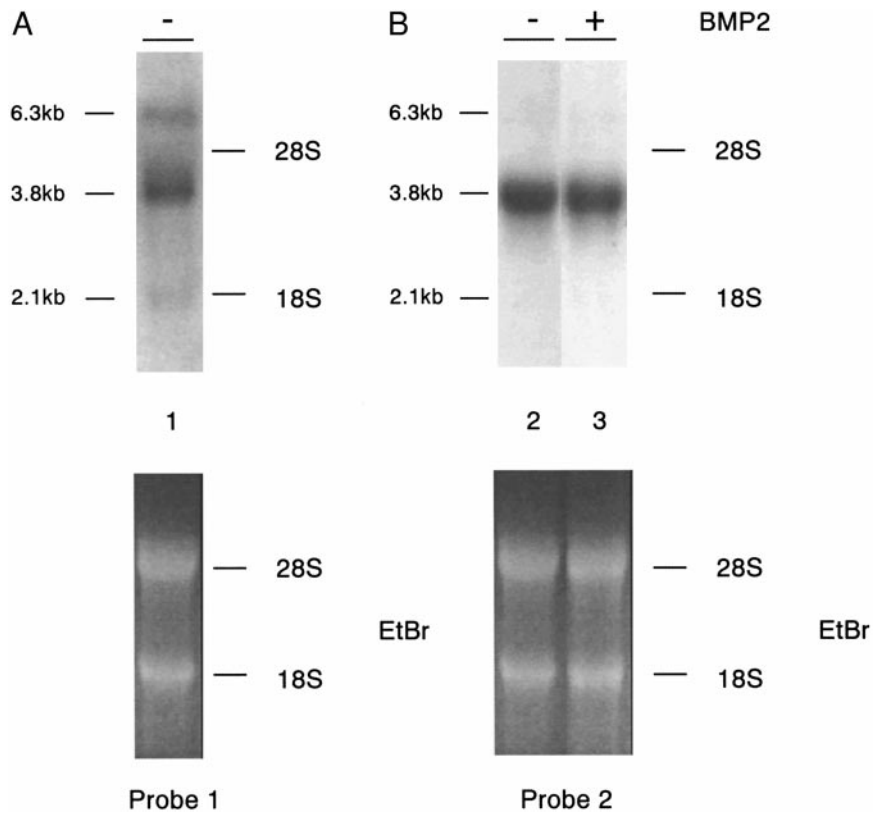


FIG. 4. Expression of 521–41 in osteoblast-like MC3T3E1 cells. Osteoblast-like MC3T3E1 cells were cultured in the presence or absence of 500 ng/ml recombinant human BMP2. Northern blot analysis was performed as described under Materials and Methods. (A) Probe 1 indicated in Fig. 1A was used for hybridization. (B) Probe 2 indicated in Fig. 1A was used for hybridization. 28S and 18S rRNA levels serve as control.

this observation, a possible function of 521–41 would be also a negative transcriptional modulator.

Relative Abundance of Runx mRNAs

Ten out of the 12 clones (including 521–41) contained identical sequences to mouse Runx1 gene and one was identical to mouse Runx2 gene. This ratio is reasonable compared to the relative abundance of the species in E11 mouse embryos. This observation raises a possibility that the major population of the positive regulator of mouse Runx2 gene expression could be mouse Runx1 family, however, this notion needs further elucidation.

Expression of 521–41 in Tissues and Cells

Northern blot analysis of mRNA species hybridized to the exon 7C-specific probe obtained from 521–41 clone indicates that a 3.8 kb transcript is expressed during the early stages of mouse development. Its expression levels were high on days 6 and 8, but were down regulated in day 12 embryos indicating the presence of developmental regulation of the expression. 521–41 is expressed in various adult tissues including bone such as lumbar vertebra. We also examined the expression of 521–41 in osteoblast-like cell line, MC3T3E1, and found that 521–41 was constitutively expressed in these cells. The abundance of 7.5-kb species which corresponds to Runx1b was relatively high in adult lumbar vertebra. These findings raise a possibility that 521–41 may act as a modulator of Runx1b. Although further study is needed to elucidate the function of 521–41 in bone and bone cells, identification of the novel species of AML1-isoform and its expression in osteoblasts are intriguing in the light of the importance of Runx family member, Runx2, as a major regulatory transcription factor in osteoblasts.

ACKNOWLEDGMENTS

This research was supported by the grant from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS) (96I00205), the grants-in-aid received from the Ministry of Education, Culture, Science and Sports (12557123, 12026212, 12215060, and 0930734), the Inamori Foundation, and the grants from CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST) and NASDA.

REFERENCES

1. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) *Cell* **89,** 755–764.
2. Mundlos, S., Otto, F., Mundlos, C., Mulliken, J. B., Aylsworth, A. S., Albright, S., Lindhout, D., Cole, W. G., Henn, W., Knoll, J. H., Owen, M. J., Mertelsmann, R., Zabel, B. U., and Olsen, B. R. (1997) *Cell* **89,** 773–779.
3. Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. (1997) *Cell* **89,** 765–771.
4. Lawrence, P. A., and Johnston, P. (1989) *Development* **105,** 761–767.
5. Bae, S. C., Yamaguchi-Iwai, Y., Ogawa, E., Maruyama, M., Inuzuka, M., Kagoshima, H., Shigesada, K., Satake, M., and Ito, Y. (1993) *Oncogene* **8,** 809–814.
6. Levanon, D., Negreanu, V., Bernstein, Y., Bar-Am, I., Avivi, L., and Groner, Y. (1994) *Genomics* **23,** 425–432.
7. Miyoshi, H., Shimizu, K., Kozu, T., Maseki, N., Kaneko, Y., and Ohki, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10431–10434.
8. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) *Cell* **89,** 747–754.
9. Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake, M., Shigesada, K., and Ito, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6859–6863.
10. Stewart, M., Terry, A., Hu, M., O'Hara, M., Blyth, K., Baxter, E., Cameron, E., Onions, D. E., and Neil, J. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8646–8651.
11. Bae, S. C., Takahashi, E., Zhang, Y. W., Ogawa, E., Shigesada, K., Namba, Y., Satake, M., and Ito, Y. (1995) *Gene* **159,** 245–248.
12. Wijmenga, C., Speck, N. A., Dracopoli, N. C., Hofker, M. H., Liu, P., and Collins, F. S. (1995) *Genomics* **26,** 611–614.
13. Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., Sato, M., Yamagiwa, H., Kimura, T., Yasui, N., Ochi, T., Endo, N., Kitamura, Y., Kishimoto, T., and Komori, T. (1999) *Dev. Dyn.* **214,** 279–290.
14. Thirunavukkarasu, K., Mahajan, M., McLarren, K. W., Stifani, S., and Karsenty, G. (1998) *Mol. Cell. Biol.* **18,** 4197–4208.
15. Harada, H., Tagashira, S., Fujiwara, M., Ogawa, S., Katsumata, T., Yamaguchi, A., Komori, T., and Nakatsuka, M. (1999) *J. Biol. Chem.* **274,** 6972–6978.
16. Xiao, Z. S., Hinson, T. K., and Quarles, L. D. (1999) *J. Cell. Biochem.* **74,** 596–605.
17. Fujiwara, M., Tagashira, S., Harada, H., Ogawa, S., Katsumata, T., Nakatsuka, M., Komori, T., and Takada, H. (1999) *Biochim. Biophys. Acta* **1446,** 265–272.
18. Drissi, H., Shakoori, R., Hu, M., Neil, J. C., Stein, J. L., Lian, J. B., Stein, G. S., and van Wijnen, A. J. (1999) *J. Bone Miner. Res.* **14**(Suppl. 1), S154.
19. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
20. Zhang, Y. W., Bae, S. C., Huang, G., Fu, Y. X., Lu, J., Ahn, M. Y., Kanno, Y., Kanno, T., and Ito, Y. (1997) *Mol. Cell. Biol.* **17,** 4133–4145.
21. Miyoshi, H., Ohira, M., Shimizu, K., Mitani, K., Hirai, H., Imai, T., Yokoyama, K., Soeda, E., and Ohki, M. (1995) *Nucleic Acids Res.* **23,** 2762–2769.
22. Okuda, T., Takeda, K., Fujita, Y., Nishimura, M., Yagyu, S., Yoshida, M., Akira, S., Downing, J. R., and Abe, T. (2000) *Mol. Cell. Biol.* **20,** 319–328.
23. Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3444–3449.
24. Niitsu, N., Yamamoto-Yamaguchi, Y., Miyoshi, H., Shimizu, K., Ohki, M., Umeda, M., and Honma, Y. (1997) *Cell Growth Differ.* **8,** 319–326.
25. Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996) *Cell* **84,** 321-330.