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# Analysis of Type-I and Type-II RUNX2 Protein Expression in Osteoblasts

Sivasubramaniam Sudhakar, Michael S. Katz, and Narayanasamy Elango[1]
*Geriatric Research, Education, and Clinical Center, South Texas Veterans Health Care System, Audie L. Murphy Division, and Division of Geriatrics and Gerontology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284*

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**Runt-related transcription factor-2 (RUNX2) is expressed as two isoforms (type-I and type-II) differing only in their amino terminal sequences. The amino terminus of type-I contains MRIPV instead of MASNSLFSAVTPCQQSFFW in type-II. Although type-II mRNA has been considered osteoblast specific, the RUNX2 protein isoforms expressed in osteoblasts have not yet been identified. Using antisera generated against the two different amino terminal sequences of type-I and type-II RUNX2, we show the expression of both isoforms in cells with the mature osteoblast phenotype (fetal rat calvarial cells, and ROS 17/2.8, SaOS-2 and U2OS osteosarcoma cell lines), but only type-I in partially differentiated osteoblast-like cells (the UMR-106 osteosarcoma cell line). Since UMR-106 cells express both type-I and type-II mRNAs, our results suggest that the translation of type-II mRNA is repressed in these cells. No RUNX1 and RUNX3 proteins are detected in any of the osteoblastic cells tested. The antisera we have generated will be useful for studies relating expression of RUNX2 isoforms to control of osteoblast differentiation.** © 2001 Academic Press

***Key Words:*** **osteoblast differentiation; transcription factor; RUNX2 specific antibodies; translational regulation.**

Differentiation of osteoblasts from pluripotent mesenchymal stem cells is a multistep process that is beginning to be unraveled. Core binding factor a1 (Cbfa1), an osteoblast specific transcription factor, has recently been cloned and implicated as a major regulator of osteoblast differentiation and gene expression (1–7). Cbfa1 is a member of a family of three transcription factors containing a common DNA binding runt domain (8–10). The three transcription factors were cloned by different groups and designated by different names (reviewed in 11). The nomenclature committee of the Human Genome Organization has recently named these factors as runt-related transcription factors (RUNX), and their new designations are RUNX1 [Cbfa2/acute myeloid leukemia factor 1 (AML1)/polyoma enhancer binding protein αB (PEBPαB)], RUNX2 (Cbfa1/AML3/PEBP2αA), and RUNX3 (Cbfa3/AML2/PEBPαC). The three RUNX proteins have extensive homology in addition to the common runt domain (12).

RUNX2 is expressed as two isoforms, type-I and type-II, with different amino terminal regions. The amino terminus of type-I protein contains MRIPV in place of MASNSLFSAVTPCQQSFFW present in the amino terminus of type-II protein; the remaining 508 amino acids of the two transcription factors are identical. An isoform termed type-III, encoded by the same reading frame that codes for type-II RUNX2 but initiated at an upstream translational start site, has been described in mouse and rat (1, 13, 14). However, the upstream translational start site does not contain the Kozak consensus sequence (15) and the type-III protein is inefficiently translated (16). Moreover, there is no open reading frame corresponding to type-III in human mRNA (14). These findings call into question the existence of the type-III isoform.

Type-I RUNX2 was initially thought to be T-cell specific (17), while type-II RUNX2 has been considered osteoblast specific (1, 3, 4). Evidence for the expression of type-I mRNA in osteoblastic cells and bone is contradictory (13, 14, 18). The RUNX2 protein isoforms expressed in osteoblasts have not yet been identified. The antibodies used in previous studies to identify RUNX2 proteins were generated against regions common to both type-I and type-II isoforms (4, 16, 19, 20). There is no antibody currently available to distinguish type-I and type-II RUNX2 proteins. It remains unclear whether type-I, type-II or both RUNX2 proteins are expressed in osteoblasts. Here we report the genera-

[1] To whom correspondence should be addressed at GRECC (182), Audie L. Murphy Memorial Veterans Hospital, 7400 Merton Minter Boulevard, San Antonio, Texas 78284. Fax: (210) 617-5312. E-mail: elangon@uthscsa.edu.

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tion of type-I, type-II and type-I/type-II RUNX2 specific antisera, and their application to evaluate RUNX2 protein expression in osteoblastic cells.

## MATERIALS AND METHODS

*Cells and cell culture.* Monkey kidney COS-7 cells and osteoblast-like osteosarcoma cell lines from rat (ROS 17/2.8 and UMR-106) and human (SaOS-2 and U2OS) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Osteoblast-enriched fetal rat calvarial cells were isolated by the method of Cohn and Wong (21) and grown in DMEM with 10% FBS.

*Generation of antisera.* The antiserum against type-I RUNX2 was generated against a five amino acid peptide MRIPV that is the amino terminus of type-I RUNX2 as well as RUNX1 and RUNX3 (12). The antiserum against type-II RUNX2 was raised against a 19 amino acid peptide MASNSLFSAVTPCQQSFFW, the amino terminus of type-II RUNX2 (AA1 to AA19). A 21 amino acid peptide DTATSDFCLWPSSLSKKSQAG, common to both RUNX2 isoforms (type-I, AA335 to AA355; type-II, AA349 to AA369) but not present in RUNX1 and RUNX3 (12), was used to generate the antiserum against both type-I and type-II RUNX2 proteins. The peptides were synthesized and conjugated to keyhole limpet hemocyanin, and the conjugated peptides were used to immunize New Zealand White rabbits. Genemed Synthesis, Inc. (South San Francisco, CA) carried out the peptide synthesis and antiserum production.

*Transient transfection.* COS-7 cells were seeded at $2.5 \times 10^5$ cells per well in a six well plate and cultured for 24 h. Cells were transfected with pcDNA3.1 vector alone or pcDNA3.1 containing RUNX2 cDNA (type-I or type-II) using lipofectamine (GIBCO-BRL Products, Gaithersburg, MD) according to the manufacturer's instructions. Three hours after transfection, the transfection medium was replaced by DMEM with 10% FBS. At 48 h post-transfection, cells were processed for Western blot analysis or electrophoretic mobility shift assay.

*Western blot analysis.* Transfected COS-7 cells and osteoblastic cells grown to about 95% confluency in 10-cm dishes were washed with PBS and lysed with lysis buffer containing 100 mM Tris–HCl, pH 6.8, 2% SDS, 2% $\beta$-mercaptoethanol and 10% glycerol. Cell lysates, after incubating at 100°C for 5 min, were centrifuged at 14,000 rpm for 5 min in an Eppendorf and stored at −70°C. Protein concentration was determined using the Non-Interfering Protein Assay Kit from Geno Technology, Inc. (St. Louis, MO). Samples were resolved on 10% SDS–polyacrylamide gels, and transferred to PVDF membranes (Schleicher and Schuell, Keene, NH) using a Hoefer Semi-Phor Semi-Dry transfer unit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The membranes were blocked with 4% nonfat milk in PBS/0.1% Tween 20 for 1 h at room temperature, washed in PBS three times (15 min each), and incubated for 1 h in PBS/0.1% Tween 20/RUNX2 antibody (anti-type-I, anti-type-II or anti-type-I/type-II). The membranes were then washed twice (30 min per wash) with PBS/0.1% Tween 20, incubated in PBS with horseradish peroxidase conjugated anti-rabbit antibody from donkey for 1 h, and washed three times (15 min each) in PBS/0.1% Tween-20. The signals were detected by chemiluminescence using an ECL kit from Amersham-Pharmacia Biotech, Inc. according to the manufacturer's instructions.

*Electrophoretic mobility shift and supershift assays.* Electrophoretic mobility shift assay (EMSA) was performed by a modification of the method of Chodosh (22). Transfected COS-7 cells and osteoblastic cells grown to about 95% confluency were washed with Tris–HCl buffered saline, resuspended in extraction buffer containing 20 mM Hepes, pH 7.4, 450 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and protease inhibitors, and sonicated. The cell extracts were centrifuged, and the supernatants were stored at −70°C. Protein concentrations were determined using the Bradford Protein Assay Kit.

Two complementary oligonucleotides containing the OSE-2/RUNX element (23) (5′GATCCGCTGCAATCACC**AACCACA**GCA and 5′GATCTGC**TGTGGTT**GGTGATTGCAGCG; OSE-2/RUNX element is designated in bold) were annealed by heating at 100°C for 3 min followed by 65°C for 5 min and then slowly cooling to room temperature. The double stranded (ds) DNA was radiolabeled by filling the overhang with dATP, dGTP, TTP and [$\alpha$-$^{32}$P]dCTP using the Klenow fragment of *Escherichia coli* DNA polymerase I. Ten micrograms of cell lysate was incubated overnight at 4°C in a reaction mixture containing 2 $\mu$g of the nonspecific competitor poly(dI-dC) (Amersham-Pharmacia Biotech, Inc.) and 300 $\mu$g/ml BSA in binding buffer [12% glycerol, 12 mM Hepes–NaOH (pH 7.9), 4 mM Tris–HCl (pH 7.9), 60 mM KCL, 1 mM EDTA and 1 mM DTT] in a total volume of 19 $\mu$l. One microliter of ds oligonucleotide probe (about 20,000 cpm) was then added to the reaction mixture and incubated for 30 min at room temperature. In supershift experiments, rabbit preimmune serum or antipeptide antiserum was preincubated with the reaction mixture for 30 min before the addition of the $^{32}$P-labeled probe. Protein-DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel and visualized using a Molecular Dynamics Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

### *Characterization and Validation of Type-I, Type-II, and Type-I/Type-II RUNX2 Antisera*

We used the 5 amino acid amino terminal peptide MRIPV of RUNX1, type-I RUNX2 and RUNX3 (12), and the 19-amino acid amino terminal peptide MASNSLFSAVTPCQQSFFW that is specific to type-II RUNX2 (3, 4, 14), to generate type-I and type-II specific RUNX2 antisera, respectively. These antisera were tested on lysates of COS-7 cells overexpressing type-I or type-II RUNX2 protein by transient expression. The COS-7 cells do not express any RUNX proteins (24). Type-I specific antiserum reacted with a 65 kDa protein overexpressed in COS-7 cells transfected with pcDNA3.1 containing type-I RUNX2 cDNA, but not with proteins in COS-7 cells transfected with type-II cDNA expression plasmid. In contrast, type-II specific antiserum reacted with a protein of 65 kDa overexpressed in COS-7 cells transfected with pcDNA3.1 containing type-II cDNA, but not with proteins in cells overexpressing type-I RUNX2. Neither type-I nor type-II specific antiserum reacted with any protein from COS-7 cells transfected with control vector (Fig. 1). An antiserum that recognizes both RUNX2 proteins has also been shown to react with a 65-kDa protein in COS-7 cells transfected with an expression plasmid containing type-II RUNX2 cDNA (16).

A synthetic peptide of 21 amino acids, DTATSDFCLWPSSLSKKSQAG, was used to raise antiserum against both type-I and type-II RUNX2 proteins. This peptide is specific to type-I (AA335 to AA355) and type-II RUNX2 (AA349 to AA369) proteins, and is not present in RUNX1 or RUNX3 proteins (12). The type-I/type-II RUNX2 antiserum—unlike the type-I specific

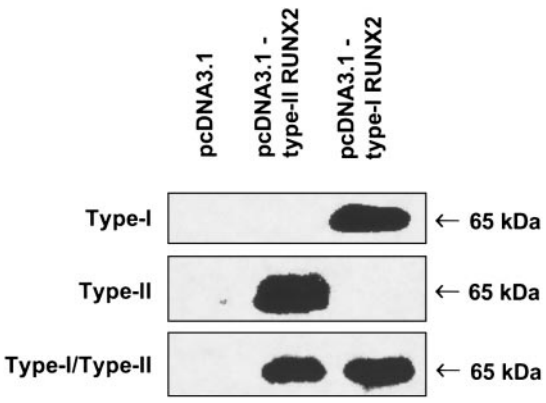


**FIG. 1.** Specificity of RUNX2 antisera. Western blot analysis of lysates from COS-7 cells transfected with pcDNA3.1 (control vector) or pcDNA3.1 containing rat type-I or type-II RUNX2 cDNA. Proteins (100 $\mu$g each) were resolved on 10% SDS-polyacrylamide gels and blotted onto PVDF membranes. The membranes were probed with antisera to type-I RUNX2 (upper panel), type-II RUNX2 (middle panel), or type-I/type-II RUNX2 (lower panel). Signals were detected by chemiluminescence as described under Materials and Methods.

and type-II specific antisera—reacted with a 65-kDa protein in lysates of transfected COS-7 cells overexpressing either type-I or type-II isoform; the type-I/type-II antiserum did not, however, react with any protein in COS-7 cells transfected with the control vector pcDNA3.1 (Fig. 1).

EMSA and supershift assay were performed using lysates from ROS 17/2.8 cells or COS-7 cells transfected with pcDNA3.1 (negative control) or pcDNA3.1 with RUNX2 cDNA (type-I or type-II), and the radiolabeled dsDNA containing the OSE-2/RUNX element. The results shown in Fig. 2 indicate that RUNX protein present in lysates of ROS 17/2.8 cells (lane 2) and COS-7 cells overexpressing type-II RUNX2 (lane 6) shifted the mobility of the dsDNA containing OSE-2/RUNX element, whereas proteins in lysates of COS-7 cells transfected with control vector (lane 9) did not. Moreover, antiserum against the 19 amino acids of type-II RUNX2 supershifted the dsDNA-protein complex containing RUNX protein from ROS 17/2.8 cells (lane 3) as well as COS-7 cells overexpressing type-II RUNX2 (lane 7). In contrast, control non-immune serum did not cause a supershift (lanes 4 and 8). The type-II RUNX2 antiserum also did not supershift the radiolabeled dsDNA in the absence of any lysate (lane 5). These findings confirm the reactivity of anti-type-II antiserum with type-II RUNX2 protein and implicate the presence of the type-II RUNX2 isoform in ROS 17/2.8 cells. In experiments not shown, the antiserum against the type-I isoform supershifted dsDNA-protein complex containing RUNX proteins from ROS 17/2.8 cells and COS-7 cells transfected with type-I RUNX2 expression plasmid; dsDNA-protein complex containing either the type-I or type-II protein were supershifted by the type-I/type-II antiserum.

### *Identification of the RUNX2 Protein Isoforms Expressed in Normal Osteoblasts and Osteoblast-like Osteosarcoma Cells*

To examine which isoform of RUNX2 protein is expressed in osteoblastic cells, we used the type-I, type-II and type-I/type-II RUNX2 antisera in Western blot analysis of lysates from normal osteoblasts (fetal rat calvarial cells) and rat and human osteoblast-like osteosarcoma cells (ROS 17/2.8, UMR-106, SaOS-2 and U2OS). As shown in Fig. 3, lysates from fetal rat calvarial cells as well as ROS 17/2.8, UMR-106, SaOS-2 and U2OS osteosarcoma cells reacted very strongly with the antiserum against type-I RUNX2 and also the anti-type-I/type-II antiserum. On the contrary, cell lysates from all cell types excluding UMR-106 reacted with the type-II RUNX2 specific antiserum (Fig. 3). These results implicate the expression of both type-I and type-II RUNX2 proteins in all osteoblastic cell types tested except UMR-106 cells, which express only type-I RUNX2 protein.

It is interesting to note that the three novel antisera identified a 65 kDa band but no other proteins in all cell lysates tested. Even the anti-type-I antiserum generated against a common peptide present in the amino termini of RUNX1, type-I RUNX2 and RUNX3 proteins reacted with a single band of 65 kDa. The protein detected by the anti-type-I antiserum is not RUNX1 or RUNX3 because the molecular weights of RUNX1 and RUNX3 proteins are at most about 53 and 50 kDa, respectively (25, 26). In addition, RUNX1 and RUNX3 proteins are not expected to react with the type-I/type-II RUNX2 antiserum, which was generated against a peptide common to the two RUNX2 isoforms but not present in RUNX1 and RUNX3. Thus the Western blot results described here demonstrate that only RUNX2, but no RUNX1 or RUNX3, proteins are expressed in normal osteoblasts and osteoblast-like osteosarcoma cells.

## DISCUSSION

RUNX2 protein regulates the differentiation of osteoblasts and the expression of specific genes characteristic of the osteoblast phenotype (1, 2, 4, 27, 28). There are two isoforms of RUNX2, type-I and type-II. Thus far, no studies have shown which protein isoform is expressed in osteoblasts. In the current study we have generated three antisera, one that reacts specifically with type-I RUNX2 protein, another reacting specifically with type-II RUNX2 protein, and a third that recognizes both RUNX2 proteins. Using these antisera we have demonstrated for the first time that both

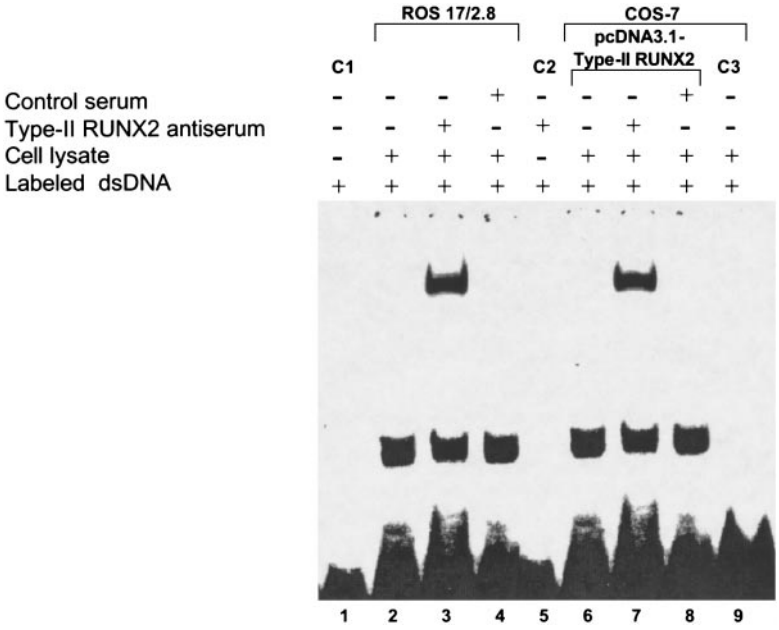


**FIG. 2.** EMSA and supershift assay of cell lysates containing type-II RUNX2 protein. Cell lysates (10 $\mu$g) and/or antiserum (1:20,000 dilution) were incubated with $^{32}$P-labeled dsDNA containing the OSE-2/RUNX element, and DNA-protein complexes were separated on a 4% native polyacrylamide gel as described under Materials and Methods. Control 1 (C1), radiolabeled dsDNA alone (lane 1); Control 2 (C2), radiolabeled dsDNA incubated with type-II RUNX2 specific antiserum (lane 5); Control 3 (C3), radiolabeled dsDNA incubated with lysate from COS-7 cells transfected with control vector pcDNA3.1 (lane 9).

type-I and type-II RUNX2 proteins are expressed in normal osteoblasts derived from fetal rat calvaria and in ROS 17/2.8, SaOS-2 and U2OS osteoblast-like osteosarcoma cell lines, whereas only type-I RUNX2 protein is expressed in UMR-106 osteoblast-like osteosarcoma cells. Neither RUNX1 nor RUNX3 is detected in osteoblastic cells, consistent with previous results in mouse calvaria (29).

The expression pattern of type-II RUNX2 in the cells we have tested coincides with that of osteocalcin, a marker of the terminally differentiated osteoblast phenotype (30, 31, our unpublished results). Osteocalcin expression is demonstrable in all osteoblastic cell types examined except UMR-106 (32–34), the only cell type lacking type-II RUNX2 protein. Since type-II RUNX2 regulates the expression of osteocalcin (1), the absence of type-II RUNX2 protein in UMR-106 cells may account at least in part for the lack of osteocalcin expression in these cells (33). Of note, the expression of osteocalcin by ROS 17/2.8 cells (32) but not UMR-106 cells (33) suggests that, of the two rat osteosarcoma cell lines, ROS 17/2.8 cells represent a more mature osteoblast phenotype (33).

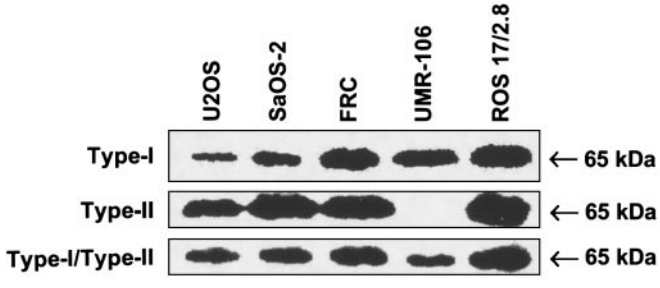


**FIG. 3.** RUNX2 protein expression in normal osteoblasts (fetal rat calvarial cells, FRC) and osteoblast-like osteosarcoma cell lines. Whole cell lysates (100 $\mu$g) were separated on 10% SDS–polyacrylamide gels and blotted onto PVDF membranes. The blots were hybridized with type-I RUNX2 antiserum (1:10,000 dilution; upper panel), type-II RUNX2 antiserum (1:500,000 dilution; middle panel) or type-I/type-II RUNX2 antiserum (1:500,000 dilution; lower panel). Signals were detected by chemiluminescence as described under Materials and Methods.

Interestingly, UMR-106 cells express both type-I and type-II RUNX2 mRNA (4, 7, our unpublished results), although until now the type of RUNX2 protein expressed in UMR-106 cells has not been described. In the present work, we show that the RUNX2 protein expressed in UMR-106 cells is the type-I isoform (Fig. 3). Even though type-II RUNX2 mRNA is expressed in UMR-106 cells, no immunoreactive type-II RUNX2 protein was demonstrable with the type-II specific antiserum (Fig. 3), suggesting that the type-II RUNX2 mRNA is not translated in these cells.

The two isoforms of RUNX2, type-I and type-II, are encoded by two mRNAs with entirely different 5′ untranslated regions (UTRs) (type-I mRNA, 1015 nucleotides; type-II mRNA, 210 nucleotides) (7, 14, 17, 35). In addition, type-II mRNA differs from type-I mRNA by having 57 nucleotides encoding the amino terminal 19 amino acids MASNSLFSAVTPCQQSFFW in place

of the 15 nucleotides in type-I mRNA encoding the amino terminal five amino acids MRIPV. The remaining sequences of the two mRNAs, containing 508 amino acid coding sequence and 3′ UTR of 3964 nucleotides, are identical. Since the two RUNX2 mRNAs differ in their 5′ but not 3′ UTRs, we predict that the translation of type-II RUNX2 mRNA in UMR-106 cells is repressed by an as yet unidentified mechanism involving the 5′ UTR.

In conclusion, we have generated three specific antisera to demonstrate for the first time that cells with the mature osteoblast phenotype (fetal rat calvarial cells; ROS 17/2.8, SaOS-2 and U2OS osteosarcoma cells) express both type-I and type-II RUNX2 proteins and no RUNX1 and RUNX3 proteins. Moreover, we have shown that UMR-106 cells, which may represent an earlier stage of differentiation along the osteoblast lineage (33), express type-I, but not type-II, RUNX2 protein. Previously UMR-106 cells were found to express type-II RUNX2 mRNA at a level comparable to that observed in ROS 17/2.8 cells (7). On the basis of these results, we propose that development of the mature osteoblast phenotype may be controlled, at least in part, by translational regulation of type-II RUNX2 mRNA.

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