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Identification of Novel Protein/DNA Interactions Within the Promoter of the Bone-Related Transcription Factor Runx2/Cbfa1

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Abstract The runt homology transcription factor Runx2/Cbfa1 is essential for bone development and osteoblast differentiation. Regulatory mechanisms that govern Runx2 transcription in osteoblasts define the osteogenic pathways that control skeletal development. In this study, we systematically examined transcription factor binding within the upstream Runx2 P1 promoter, which regulates expression of the bone-related Runx2 factor. We identified two novel protein/DNA interactions that are mediated by sequence specific factors, based on cross-competition experiments, point mutations, and gel-shift immunoassays. One complex recognizes a non-canonical Runx2 site, whereas the other factor binds to a palindromic sequence. Site-directed mutagenesis of the novel Runx2 motif (5′TCCCAC3′) within the 0.6 kb rat Runx2 promoter reduces transcription by 2-fold, indicating that this site supports enhancement of Runx2 promoter activity. Mutation of the palindromic motif (5′AGTACT3′) results in a 2–3-fold activation of the Runx2 promoter, demonstrating that the wild type sequence contributes to transcriptional repression. These studies, together with our previous findings of auto-suppression of the Runx2 promoter and negative regulation by $1,25(OH)_2$ Vitamin D3, suggest that physiological control of Runx2 gene expression is mediated by a series of intricate regulatory mechanisms.
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Key words: Runx2/Cbfa1; promoter; osteoblast; transcription; gene expression

The bone-related transcription factor Runx2 is essential for osteoblast differentiation [Merriman et al., 1995; Banerjee et al., 1997; Ducy et al., 1997; Harada et al., 1998] and chondrocyte maturation [Inada et al., 1999; Kim et al., 1999; Enomoto et al., 2000; Leboy et al., 2001]. In vitro studies have shown that Runx 2 is able to activate osteoblast phenotypic genes in various mesenchymal cell lines, indicating that it acts as a differentiation factor promoting progression along the osteoblastic lineage [Banerjee et al., 1997; Ducy et al., 1997; Lee et al., 1999]. Ablation of the Runx2 gene in mouse models results in osteoblast differentiation arrest at the earliest stages of bone formation. The skeletons of Runx2 deficient mice or knock-in mice expressing a truncated Runx2 protein are devoid of mineralized tissue and consist exclusively of non-mineralized cartilage tissue [Komori et al., 1997; Otto et al., 1997; Choi et al., 2001]. Runx2 null mice also provided evidence for the importance of this transcription factor for cartilage maturation in vivo [Inada et al., 1999]. Thus, knowledge of the activators of Runx2 transcription will provide an understanding of the complement of factors critical to formation of a mineralized skeleton.

The Runx2 gene is a downstream target of several local and systemic factors that affect osteoblast differentiation. Indeed, developmental transcription factors, signaling molecules, and other growth factors have been shown to regulate expression of Runx2 in various mesenchymal cell populations [Yamaguchi et al., 2000; Lian and stein, 2001; Prince et al., 2001; Drissi et al., 2002; Gilbert et al., 2002]. TGF-β super

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family members, such as BMP-2, accelerate osteoblast differentiation [Katagiri et al., 1990; Yamaguchi and Kahn, 1991; Yamaguchi et al., 1991] and also activate Runx2 transcription [Komori et al., 1997; Otto et al., 1997; Tsuji et al., 1998; Gori et al., 1999; Harada et al., 1999; Lee et al., 1999, 2000; Banerjee et al., 2001]. Several steroid hormones, such as dexamethasone and Vitamin D3, which are known to affect osteoblast differentiation, have also been shown to regulate Runx2 expression [Prince et al., 2001]. Our previous studies using the bone-related Runx2 P1 promoter showed that the first 600 bp of its 5′ flanking region contain several recognition motifs for distinct classes of transcription factors. We characterized multiple Runx2 sites and a negative VDRE [Drissi et al., 2002], which suppress Runx2 gene transcription. However, promoter deletion analysis of the 5′ flanking sequences of the Runx2 gene suggested that there are also several regulatory elements that positively control Runx2 transcription [Drissi et al., 2000].

Transcriptional activation of the Runx2 gene is the initial rate-limiting step in promoting osteoblast differentiation to the mature phenotype. In this study, we characterized several regulatory elements in a region of the Runx2 P1 promoter nt −351 and −92 that functionally supports transcriptional control. We show that a non-canonical Runx2 site located between nt −107 and −102 contributes to activation of Runx2 transcription. The second element mediates a novel protein/DNA interaction and overlaps with a palindromic sequence (5′AGTACT3′). Our findings suggest that both canonical and non-canonical Runx2 sites contribute to autoregulation of the Runx2 gene and that auxiliary regulatory sequences modulate expression of this bone-related transcription factor.

MATERIALS AND METHODS

Cell Culture

The rat ROS 17/2.8 osteosarcoma cells were grown in a F12 growth medium containing 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). The mouse C2C12 myoblasts and NIH3T3 fibroblasts were grown in DMEM, whereas MC3T3 osteoblasts were grown in αMEM. These media were supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cells were maintained in a humidified incubator (5% CO_2) at 37°C. For transfection experiments cells were seeded onto 6-well plates at a density of 0.1×10^6 cells/well 24 h prior to transfection.

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)

Nuclear proteins from ROS 17/2.8, NIH3T3, MC3T3, and C2C12 cells were extracted as previously described [Staal et al., 1996a]. Nuclear extracts were stored at −70°C in 20 mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol, and 0.2 mM EDTA. A 10 μl volume (8 μg) of these nuclear extracts was incubated with 10 ml of a DNA mix for 30 min at 25°C. The DNA mix for each reaction consisted of; 0.2 μl of 0.1 M DTT, 1 μg of the non-specific competitor poly dI-dC, 80,000 cpm of double-stranded DNA probe and 250–750 fmol of the relevant double-stranded unlabeled competitor oligonucleotides. Samples were separated in a non-denaturing acrylamide:bis-acrylamide (4%) gels using 0.5 × Tris-borate EDTA buffer.

Plasmid Constructs

The parental construct for our studies is a pGL3 plasmid (Promega, Madison, WI) containing 0.6 kb of the Runx2 promoter fused to the luciferase reporter gene. The mutation contained in the −116/−93 MT oligonucleotide (5′GGTAGGCA GCAAAGTT TTA CTTTG3′) was incorporated into this construct by PCR-mediated mutagenesis. The oligonucleotide (5′GGTAGGCA GCAAAGTT TTACTTTG3′) (20 picomoles) was used as a forward primer and the oligonucleotide (5′GTGAATGCTT CATTCGCCTCA3′) (20 picomoles) was used as the reverse primer in the first PCR reaction. A construct (0.25 μg) containing 0.6 kb of the rat Runx2 promoter plus its 5′UTR was used as the template. The amplification was performed in a 50 μl volume with 1 U of (exo-) Vent Polymerase (NEB, Beverly, MA) as follows; 1 min at 94°C, 30 cycles of 1 min denaturization at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C. A final extension step was carried out for 6 min at 72°C.

The purified PCR product (of approximately 100 bp) was used as a reverse primer in the second PCR reaction along with the 5′ most forward primer 5′CGGCTTGCAGCACTGTTGCTC3′ using the same template (0.25 μg) as described above. The same amplification protocol was repeated using Vent DNA Polymerase

(1 U) (New England Biolabs [NEB], Beverly, MA).

The purified 600 bp product was then digested with Hind III and Xho I restriction enzymes (NEB, Beverly, MA) then ligated into the pGL3 luciferase reporter construct. Positive clones were analyzed by automated sequencing (nucleic acid facility at UMass Medical School, Worcester, MA) to confirm the presence of the mutations. The construct consisting of pGL3 with the 600 bp promoter containing the −104/−81 MT mutation was generated in the same fashion.

Transient Transfections

ROS 17/2.8, MC3T3, C2C12, and NIH3T3 cells were grown to 50% confluency and transfected with either 0.5 μg/well of a Renilla Luciferase construct or 0.5 μg/well of β-gal construct as control for transfection efficiency, and 2 μg/well of either empty vector (pGL3) or test constructs (wild type or mutant). Cells were transfected using Superfect reagent (Qiagen, Valencia, CA) as previously described [Staal et al., 1996a; Staal et al., 1996b; Lee et al., 1999].

Luciferase assays were performed as previously described [Drissi et al., 2002]. In brief, cells were harvested 36–48 h after transfection and were lysed with 500 μl of 1 × reporter lysis buffer (Promega, Madison, Wisconsin) for 20 min. Luciferase activity was measured using the luciferase assay kit (Promega, Madison, Wisconsin) and a luminometer (monolight analytical luminescence laboratory, San Diego, CA). The activity of β-gal was measured colorimetrically.

RESULTS

Multiple Conserved Elements Support Transcriptional Control of the Runx2 Promoter in Mesenchymal Cells

Establishing functional elements in the bone-related P1 promoter of the Runx2 gene is a necessary step in defining regulatory mechanisms that can enhance or suppress transcription of Runx2 in osteoblasts. Figure 1A summarizes previously characterized, as well as putative binding sites within the Runx2 proximal promoter. These elements are conserved among rat, mouse, and human species within the first 200 bp of this promoter, and are located downstream of a purine rich region [Drissi et al., 2002]. Promoter activity of several deletion mutants was examined by transfecting a series of rat Runx2 promoter constructs into mesenchymal cell lines with distinct phenotypes, including the osteoblastic cell lines ROS17/2.8 and MC3T3 E1, C2C12 myoblasts, and NIH3T3 fibroblasts. We observed at least a 10-fold increase in luciferase activity that is mediated by a 36 nucleotide segment between the deletion constructs (−128 to −92) (Fig. 2). This increased promoter activity indicates that one or more positively acting elements are located within this region of the bone-related Runx2 promoter. Furthermore, the activity of the various deletion mutants is not tissue specific, because the same reporter gene expression profile is observed in osseous and non-osseous cells. These activating elements appear to be an important component of basal expression of Runx2 in mesenchymal cells.

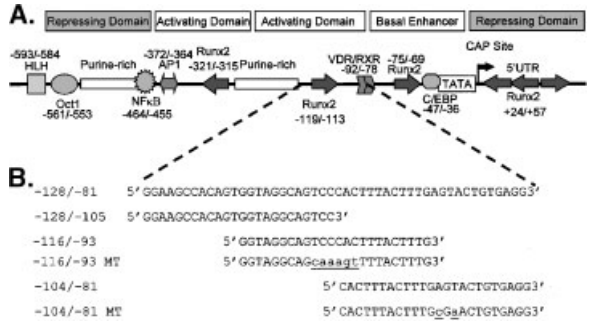

Fig. 1. Organization of transcriptional regulatory elements on the rat Runx2 promoter. **Panel A**: Schematic illustration of the Runx2 promoter. Enhancing and repressing domains, separated by two purine rich stretches, carry several putative recognition elements for well known transcription factors. Runx2, CREB, C/EBP, VDR/RXR, Oct-1, NFkB, AP-1, and HLH consensus sequences are indicated. Previously reported functional Runx2 sites within the promoter and downstream of the TATA box in the 5′UTR are shown. **Panel B**: Oligonucleotides that were used for electrophoretic mobility shift assays (EMSA) span the region between nt −128 and −81. For each probe of 24 bp in length, an overlap of 12 nucleotides with the adjacent probe was included. A full-length oligonucleotide (−128/−81) was also generated for competition experiments. Mutated motifs are underlined and shown in lower case.

Two Novel Protein/DNA Interactions Occur Within the −128 to −81 Transcriptional Regulatory Domain

We focused on novel protein/DNA interactions within the −128 to −81 region that is responsible for enhancement of Runx2 promoter activity. To determine sequence specific protein/DNA interactions, three overlapping oligonucleotides

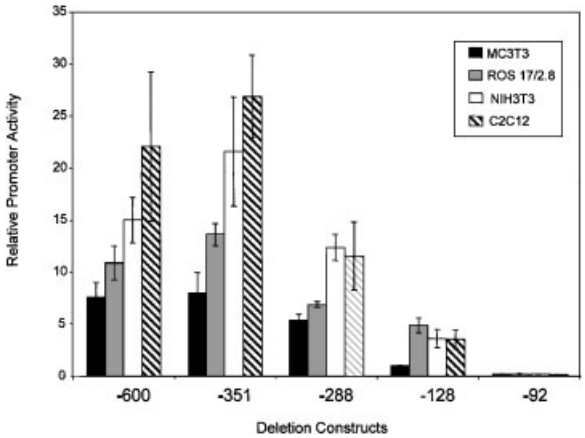

Fig. 2. The proximal segment of the rat Runx2 promoter contains several activating domains between nt −351 and −92. MC3T3 (black bars), ROS 17/2.8 (gray bars), NIH3T3 (white bars), and C2C12 (striped bars) cells were transfected with the indicated promoter luciferase deletion constructs (−600, −351, −288, −128, and −92). Histograms represent means of luciferase values over empty vector of n = 3 samples ± standard deviation. Fold activity increase in the −128 deletion mutant transfected into ROS 17/2.8, C2C12, and NIH3T3 cells and MC3T3 cells.

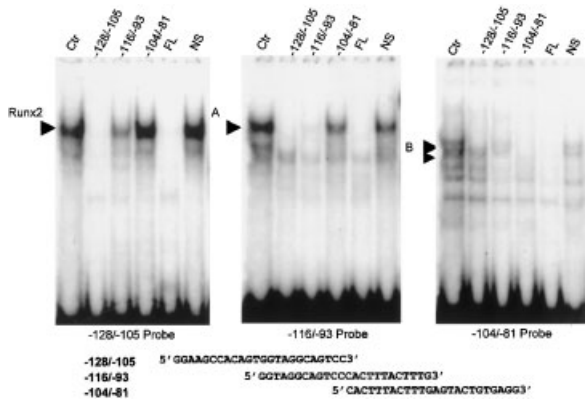

Fig. 3. Multiple protein/DNA interactions in the principal regulatory region of the Runx2 promoter. Gel-shift assays were performed with the three overlapping oligonucleotides (−128/−105, −116/−93, −104/−81) as probes using ROS 17/2.8 nuclear extracts (8 μg) and in the presence of unlabeled oligonucleotide competitors including −128/−105, −116/−93, −104/−81, full length −128/−81 (FL), and an E2F consensus element (nonspecific competitor, NS) as indicated. **Left Panel**: The prominent complex formed with the −128/−105 probe competes with self and full length (FL) oligos, whereas it only partially competes with the −116/−93 oligo and neither competes with the −104/−81 nor a nonspecific (NS) E2F oligonucleotide. Runx2 was previously shown to mediate this complex (arrow). **Middle Panel**: The −116/−93 oligonucleotide is used as a probe. The specific complex designated A competes with the −116/−93 and the full length (FL) oligonucleotides, whereas the nonspecific E2F unlabeled competitor (NS) does not affect complex A binding. Both unlabeled −128/−105 and −104/−81 oligonucleotides fail to compete with the complex A. **Right Panel**: The −104/−81 oligonucleotide is used as a probe. The full length (FL) and the −104/−81 oligonucleotides compete with the −104/−81 probe. The −128/−105 oligonucleotide fails to compete for the binding of the protein/DNA complex B, while the −116/−93 oligonucleotide does compete for binding of complex B.

between nt −128 and −81 were designed (see Fig. 1B), and used with nuclear extracts from rat osteoblastic ROS 17/2.8 cells for mobility shift assays. Figure 3 shows that unique protein/DNA interactions occur with each probe between nucleotides −128 and −81. All protein interactions exhibited self-competition wit the unlabeled probe and competition with an disonucleotide spanning the entire −128 to −81 region, whereas a non-specific oligonucleotide did not compete for binding. These results indicate that at least three major protein/DNA complexes (Runx2, complex A, and complex B) are specific. The prominent complex (labeled Runx2) formed with the −128/−105 probe (Fig. 3, left panel), partially competed with the −116/−93 oligonucleotide but not with the −104/−81 oligonucleotide. This Runx2 complex was characterized in previous studies and binds to nt −119/−113 [Drissi et al., 2002].

Several protein/DNA interactions are also formed on the −116/−93 probe, which cross competes with the −128/−105 oligonucleotide for the specific complex A (Fig. 3, middle panel). However, the −104/−81 oligonucleotide fails to compete for the complex A protein/DNA interaction. This result suggests that complex A interacting with the −116/−93 probe is related to the Runx2 complex in probe −128/−105. The third probe oligonucleotide (−104/−81) (Fig. 3, right panel) competes with itself for a DNA/protein complex designated B. However, limited cross-competition was observed with both the −128/−105 and the −116/−93 oligonucleotides (Fig. 3, right panel). This observation suggests that the specific interaction designated complex B is not located within the 12 nt overlap between the −116/−93 and −104/−81 probes. Thus, our competition analyses identify two novel protein/DNA interactions within the transcriptional regulatory domain of the Runx2 promoter.

A Unique Runx Motif Mediates Activation of the Runx2 Promoter

Our studies above suggest that complex A is formed only with the −116/−93 oligonucleotide and may be related to Runx2. To further

establish specificity of this complex, we generated a mutant (−116/−93 MT) oligonucleotide in which the six center nucleotides (5′TCCCAC3′) within the −116/−93 oligonucleotide were altered (5′TGaaAC3′). Gel-shift assays using the −116/−93 wild type probe combined with increasing amounts of unlabeled self and mutant oligo competitors show that complex A does not compete with the −116/−93 MT nucleotide containing the mutated motif (5′TGaaAC 3′) (Fig. 4A,B). This result indicates that the middle six nucleotides (5′TCCCAC3′) of the −116/−93 probe which resemble the Runx consensus binding motif (see Fig. 4E) are essential for this interaction. We, therefore, confirmed that the protein(s) involved in complex A are interacting with the Runx2 motif by performing competition experiments using a previous established Runx site as the unlabeled competitor (Fig. 4C). Increasing amounts of this Runx oligonucleotide completely abolish detection of complex A.

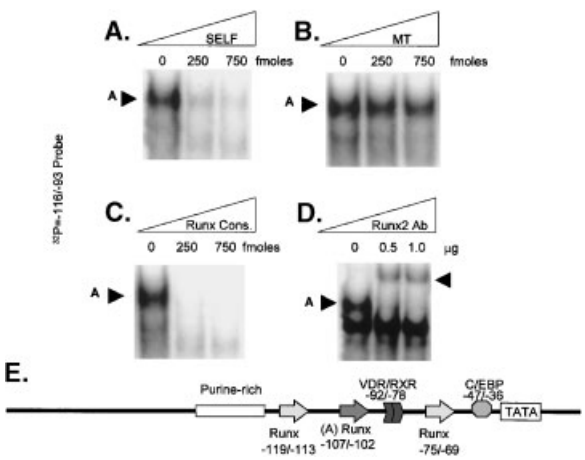

Fig. 4. Nucleotides −107/−102 are esssential for protein/DNA interaction of complex A. A mutation generated in the center of the −116/−93 oligonucleotide sequence (nt −107/−102) disrupts protein/DNA interaction within complex A. **Panel A–D**: The −116/−93 oligonucleotide is used as a probe. Panel A: The −116/−93 oligonucleotide competes with itself for complex A, when increasing amounts (0–750 fmoles) are added. Panel B: Increasing amounts (0–750 fmoles) of the mutated −116/−93 oligonucleotide used as a cold competitor for competition with the −116/−93 labelled probe failed to compete for specific protein/DNA binding. Panel C: Increasing amounts (0–750 fmoles) of a Runx consensus sequence (shown at the bottom) competes with complex A for protein/DNA binding. Panel D: Gel-shift immunoassay using increasing amounts of the Runx2 specific antibody causes an upward shift of complex A, formed with the −116/−93 oligonucleotide probe. **Panel E:** Representation of the novel positive Runx site (A) on the Runx2 promoter at position −107/−102 between the negative VDRE and an upstream Runx2 site.

Using an antibody specfic for Runx2, we further established by gel-shift immunoassay that Runx2 is a major component of complex A (Fig. 4D). This newly identified non-canonical Runx2 binding site (Fig. 4E) increases to seven the total number of experimentally validated Runx2 sites within the first 600 bp plus the 5′UTR of the bone-related Runx2 P1 promoter (see Fig. 1A).

To assess the function of this novel Runx2 site, we introduced the same mutation that abolished complex A protein/DNA interactions into the 0.6 kb Runx2 P1 promoter. Both the wild type and mutant promoter-luciferase constructs were transiently transfected into ROS 17/2.8, MC3T3, NIH3T3, and C2C12 cells to analyze the effects of this mutation in vitro. Figure 5A shows that a 40–50% decrease in promoter activity is observed when the 5′TCCCAC3′ site is mutated in all four cell lines. These results suggest that this protein/DNA interaction element mediates enhancement of Runx2 promoter activity.

To compare this protein/DNA interaction among the various cell lines, we performed gel-shift experiments with the −116/−93 probe and nuclear proteins extracted from ROS 17/2.8, MC3T3, NIH3T3, and C2C12 cells. Figure 5B shows that different complex binding patterns are observed between osseous and non-osseous cells. This new Runx2 complex is, as expected, enriched in ROS 17/2.8 nuclear extracts and not prominent in MC3T3 osteoprogenitor cells and non-osteoblastic cell lines. Together, our results show that this regulatory element (5′TCCCACT3′), which maintains only the core of the typical Runx consensus sequence (5′AGTGGT3′), is a novel positive Runx2 site that enhances activity of the rat Runx2 promoter.

A Novel Protein/DNA Interaction Located Between the New Runx2 Site and the VDRE Represses Runx2 Promoter Activity

We used a similar approach with the −104/−81 oligonucleotide to identify complex B and the contribution of the corresponding recognition sequence to promoter activity. We systematically introduced a series of point mutations into the −104/−81 oligonucleotide and found that transforming a 5′GAGTACT3′ motif into 5′GcGaACT3′ produced an oligonucleotide that fails to compete for protein/DNA binding, whereas the wild type −104/−81 oligonucleotide successfully binds complex B (Fig. 6A,B). This

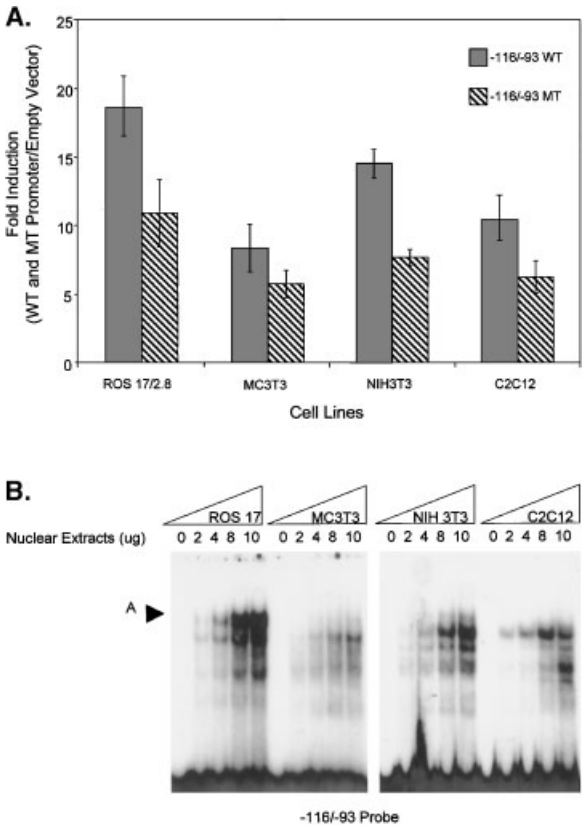

Fig. 5. The region between nt −107 and −102 is important for activation of the Runx2 promoter. **Panel A**: ROS 17/2.8, C2C12, MC3T3, and NIH 3T3 cells were transfected with both the wild type (gray bars) and mutated −116/−93 motif (striped bars) within the 0.6-kb fragment of the rat Runx2 promoter. This mutation causes a 40–50% decrease in promoter activity in each of these cell lines. **Panel B**: Comparison of nuclear extracts from ROS 17/2.8, NIH3T3, MC3T3, and C2C12 cells, interacting with the −116/−93 probe. Gel-shift assays using the −116/−93 oligonucleotide as a probe, and increasing amounts (0, 2, 4, 6, 10 μg) of nuclear extracts from osseous (ROS 17/2.8, MC3T3) and non-osseous (NIH3T3, C2C12) cell lines. Complex A is predominantly present in the rat osteosarcoma cell ROS 17/2.8 nuclear extracts.

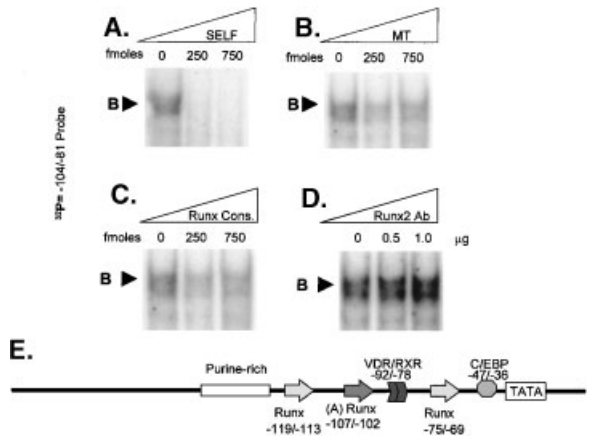

Fig. 6. Competition of the −104/−81 probe with self and mutant competitor. Gel-shift assays using the −104/−81 oligonucleotide as a probe and ROS 17/2.8 nuclear extracts. **Panel A**: Competition with increasing amounts (0–750 fmoles) of unlabeled wild type oligonucleotide (self). **Panel B**: Competition with increasing amounts (0–750 fmoles) of the oligonucleotide (MT) carrying a mutation introduced within the 5′TGAGTAC3′ motif of the −104/−81 oligonucleotide. **Panel C**: Competition with increasing amounts (0–750 fmoles) of the Runx consensus sequence oligonucleotide (Runx cons.) for complex B binding. **Panel D**: Gel-shift immunoassay using 0.5 and 1 μg of the Runx2 specific antibody (Runx2 Ab). **Panel E**: Relative location of the novel complex B on the Runx2 promoter at position −92/−90 between the novel positive Runx site and the negative VDRE.

result establishes nucleotides that are essential for complex B formation. This protein binding motif overlaps the 5′ portion of the previously characterized negative vitamin D responsive element (nt −92/−78) [Drissi et al., 2002]. Competition experiments using a disonucleotide spanning −95 to −75 do not compete for binding of the novel complex B (data not shown). Thus, complex B is not related to protein/DNA interaction, and apart from the 5′AGTACT motif requires base pairs between −104 to −95. Given the proximity of this new site to two Runx2 sites (Fig. 6E), a Runx consensus sequence was used for cross-competition and found to compete partially with the probe for binding of complex B (Fig. 6C). However, gel-shift immunoassay using increasing amounts of a Runx2 specific antibody reveals that complex B is not immunologically related to Runx2 (Fig. 6D). These results indicate that this is a usual protein/DNA interaction between −104 and −85 that is related neither to VDRE nor to Runx2.

To determine the functional implications of complex B in mediating promoter activity, we introduced the mutated binding site into the Runx2 promoter-luciferase construct and transfected both wild type and mutant promoters into ROS 17/2.8, MC3T3, NIH3T3, and C2C12 cells. Figure 7A shows that mutation of the complex B binding site causes a 2–3-fold enhancement of promoter activity compared to the wild-type promoter. This result indicates that the complex B protein/DNA interaction is responsible for down-regulating the Runx2 promoter. We further demonstrate that complex B is present in each of the cell types examined (Fig. 7B). Our results suggest that the factor(s) involved in mediating repression of

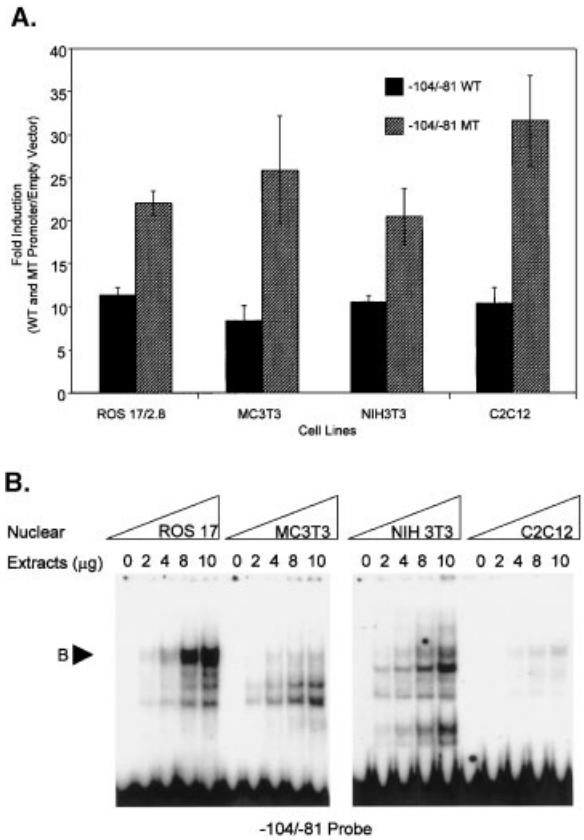

Fig. 7. A novel protein/DNA interaction mediates repression of Runx2 promoter activity. **Panel A**: Transient transfection of ROS 17/2.8, MC3T3, C2C12, and NIH3T3 cells with the rat 0.6 kb Runx2 promoter containing the mutated 5′TGcGaAC3′ motif. Promoter activity in these various cells is upregulated by 2–3-fold in the mutant (striped bars) compared to the wild type promoter (black bars). **Panel B**: Comparison of ROS 17/2.8, MC3T3, NIH3T3, and C2C12 nuclear extracts with the −104/−81 probe. Protein/DNA interaction of complex B was detected in ROS 17/2.8, MC3T3, NIH 3T3, and C2C12 cells. The −104/−81 oligonucleotide was used as a probe and incubated with increasing amounts (0, 2, 4, 8, 10 μg) of nuclear proteins extracted from all these cells. Complex B is present in each of these cell lines, albeit at different levels.

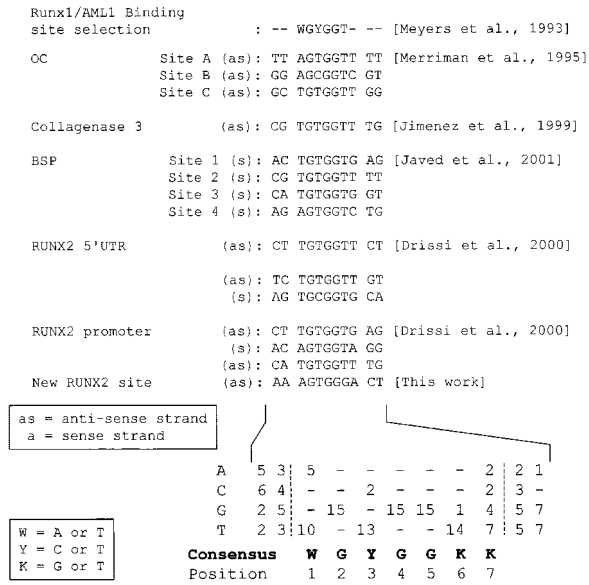

Fig. 8. Compilation of Runx2 binding sites in bone-related promoters. The Runx2/Cbfa1 consensus element shown in the last line is based on experimentally validated binding sites in bone-related promoters and is similar to that initially established by binding site selection experiments using Runx1/AML1. The sequences of Runx2 binding sites in the osteocalcin (OC), bone sialoprotein (BSP) and Runx2 promoters were aligned and the frequency of occurrence of specific nucleotides at each position was determined (lower panel).

the Runx2 promoter are present in a broad spectrum of mesenchymal cells.

Definition of a Runx2 Consensus Element

Previous studies have identified Runx2 binding sites in the promoters of several bone-related genes (Fig. 8). Most of these promoters contain multiple Runx2 binding sites that are frequently positioned with the same orientation relative to the sense- or antisense-strand. Alignment of these experimentally established Runx2 binding sites reveals a Runx2 consensus recognition motif (Fig. 8). This Runx2/Cbfa1 response element which is based on naturally occurring Runx2 binding sites is remarkably similar to the Runx1/AML1 recognition sequence defined by binding site selection analysis of random oligonucleotides. The Runx2 and Runx1 consensus sequences are similar but Runx2 binding sites exhibit redundancy of the core GGT-motif (positions 4, 5 and 6) that is essential for Runx1/AML1 binding , and a nucleotide preference for G or T at position 7. The Runx2 consensus, we define here may be useful for the identification of Runx2 sites in other bone-specific genes.

DISCUSSION

In this study, we assessed transcriptional mechanisms related to activation of the Runx2 gene, focusing on the proximal region of the Runx2 P1 promoter that has been shown to enhance Runx2 transcription. Using a panel of overlapping oligonucleotides, we demonstrated by gel-shift assays that Runx2 proximal promoter activity is associated with several novel protein/DNA interactions. Our results reveal that two specific DNA elements residing

between nt −128 and −81 have regulatory functions. One of these elements is a novel Runx2 related motif (5′TCCCAC3′) and is responsible for enhancement of promoter activity. A second sequence specific element (5′AGTACT3′) forms a complex with a protein present in all cell phenotypes and appears to contribute to down-regulation of Runx2 transcriptional activity. Thus, multiple protein/DNA interactions contribute to the transcription of the Runx2 gene.

The unique protein/DNA complex formed between nucleotides −116 and −93 and was shown to require a non-canonical 5′TCCCAC3′ Runx motif. When this site was mutated, the protein/DNA interaction was completely abolished. Our functional data clearly show that this mutation results in reduction of reporter gene expression, thus demonstrating that this new site indicates activation of Runx2 promoter activity. This is the first experimental evidence that Runx2 is capable of enhancing activity of the Runx2 gene. Previous studies have shown that at least three other perfectly matching Runx2 consensus elements are located within this promoter. These three Runx sites were characterized and found responsible for mediating auto-suppression of the Runx2 gene. The novel Runx2 binding site, we identified in this study differs from the known Runx consensus sequence 5′AGTGGT3′, as previously described [Meyers et al., 1993; Merriman et al., 1995]. However, both canonical and our non-canonical sequences contain the 5′CCA3′ core motif which is required for Runx2 binding [Meyers et al., 1993]. Runx2 protein forms multimeric complexes with many co-regulatory factors and interacts with other proteins to support synergistic responsiveness in the vicinity of other regulatory motifs. The uniqueness of this novel Runx site may specify assembly of a Runx-containing complex mediating enhanced transcriptional activity, in contrast to repressive activity mediated at other Runx sites. There is ample precedence for promoter context dependent activation or repression through Runx2 sites [Ji et al., 1998; Javed et al., 2001; Mengshol et al., 2001; D'Alonzo et al., 2002].

Downstream of the novel Runx site, we have identified another novel protein/DNA complex residing in the −104/−81 segment of the Runx2 gene. This complex B binding site is located between the new Runx2 element and a previously characterized negative vitamin D responsive element [Drissi et al., 2002]. We have established by mutagenesis that this site mediates up to 3-fold repression compared to its neighboring negative VDRE that mediates repression to only approximately 50% of control [Drissi et al., 2002]. The complex B binding site encompasses a perfect palindromic sequence (5′AGTACT3′), and partially overlaps, but is distinct from the previously described negative VDRE. Mutational analysis and cross competition with this VDRE further confirms that complex B binds independently of the VDRE sequence [Drissi et al., 2002]. Thus, the −104/−78 region of the promoter can interact with multiple proteins that confer negative regulation of Runx2 promoter activity.

The overall responsiveness of the proximal region of the Runx2 promoter that we have studied here is a net effect of both suppression and activation. This interrelationship between enhancing and suppressing elements underscores the tight regulation of Runx2 gene expression and the regulatory duality to maintain precise control of Runx2 transcripts [Drissi et al., 2002]. Runx2 expression is developmentally regulated during osteoblast differentiation and most abundantly expressed in mature osteoblasts. The bone-related Runx2 protein is also expressed in mesenchymal progenitor cells committed to the osteoblast or chondrocyte phenotype [Banerjee et al., 2001]. We propose that the function of the −128/+1 proximal promoter region in both osseous and non-osseous cells may control basal expression of the Runx2 gene to attenuate Runx2 expression.

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REFERENCES

Banerjee C, McCabe LR, Choi J-Y, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML-3/CBFA1 is a major component of a bone specific complex. J Cell Biochem 66:1–8.

Banerjee C, Javed A, Choi J-Y, Green J, Rosen V, van Wijnen AJ, Stein JL, Lian JB, Stein GS. 2001. Differential regulation of the two principal Runx2/Cbfa1 N-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Endocrinology 142:4026–4039.

Choi J-Y, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB,

Stein JL, Jones SN, Stein GS. 2001. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. Proc Natl Acad Sci, USA 98:8650–8655.

D'Alonzo RC, Selvamurugan N, Karsenty G, Partridge NC. 2002. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for Collagenase-3 promoter activation. J Biol Chem 277:816–822.

Drissi H, Luc Q, Shakoori R, Chuva de Sousa Lopes S, Choi J-Y, Terry A, Hu M, Jones S, Neil JC, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2000. Transcriptional autoregulation of the bone-related CBFA1/RUNX2 gene. J Cell Physiol 184:341–350.

Drissi H, Pouliot A, Koolloos C, Stein JL, Lian JB, Stein GS, van Wijnen AJ. 2002. $1,25(OH)_2$Vitamin D3 suppresses the bone-related Runx2/Cbfa1 gene promoter. Exp Cell Res 274:323–333.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T. 2000. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 275:8695–8702.

Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, Lian JB, Stein GS, Nanes MS. 2002. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/PEBP2αA) is inhibited by tumor necrosis factor-α. J Biol Chem 277:2695–2701.

Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL. 1999. Differentiation of human marrow stromal precursor cells: bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res 14:1522–1535.

Harada H, Taniguchi T, Tanaka N. 1998. The role of interferon regulatory factors in the interferon system and cell growth control. Biochimie 80:641–650.

Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. 1999. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 274:6972–6978.

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, Komori T. 1999. Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 214:279–290.

Javed A, Barnes GL, Jassanya BO, Stein JL, Gerstenfeld L, Lian JB, Stein GS. 2001. *runt* homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol 21:2891–2905.

Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TL, Centrella M. 1998. CBFa(AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69:353–363.

Jimenez MJ, Balbin M, Lopez JM, Alvarez J, Komori T, Lopez-Otin C. 1999. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol 19:4431–4442.

Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T. 1990. The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299.

Kim IS, Otto F, Zabel B, Mundlos S. 1999. Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 80:159–170.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Leboy P, Grasso-Knight G, D'Angelo M, Volk SW, Lian JV, Drissi H, Stein GS, Adams SL. 2001. Smad-Runx interactions during chondrocyte maturation. J Bone Joint Surg Am 83-A Suppl 1:S15–S22.

Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Ryoo HM. 1999. Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor β 1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73: 114–125.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792.

Lian JB, Stein GS. 2001. Osteoblast biology. In: Marcus R, Feldman D, Kelsey J, editors. Osteoporosis. San Diego: Academic Press. pp. 21–71.

Mengshol JA, Vincenti MP, Brinckerhoff CE. 2001. IL-1 induces collagenase-3 (MMP-13) promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation by p38 MAPK and JNK pathways. Nucleic Acids Res 29:4361–4372.

Merriman HL, van Wijnen AJ, Hiebert S, Bidwell JP, Fey E, Lian J, Stein J, Stein GS. 1995. The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: interactions with the osteocalcin gene promoter. Biochemistry 34:13125–13132.

Meyers S, Downing JR, Hiebert SW. 1993. Identification of AML-1 and the t(8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins; the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol 13:6336–6345.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddington RSP, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Prince M, Banerjee C, Javed A, Green J, Lian JB, Stein GS, Bodine PV, Komm BS. 2001. Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. J Cell Biochem 80:424–440.

Staal A, van Wijnen AJ, Birkenhäger JC, Pols HAP, Prahl J, DeLuca H, Gaub M-P, Lian JB, Stein GS, van Leeuwen

JPTM, Stein JL. 1996a. Distinct conformations of VDR/RXRα heterodimers are specified by dinucleotide differences in the vitamin D responsive elements of the osteocalcin and osteopontin genes. Mol Endocrinol 10:1444–1456.

Staal A, van Wijnen AJ, Desai RK, Pols HA, Birkenhager JC, DeLuca HF, Denhardt DT, Stein JL, Van Leeuwen JP, Stein GS, Lian JB. 1996b. Antagonistic effects of transforming growth factor-beta on vitamin D3 enhancement of osteocalcin and osteopontin transcription: reduced interactions of vitamin D receptor/retinoid X receptor complexes with vitamin E response elements. Endocrinology 137:2001–2011.

Tsuji K, Ito Y, Noda M. 1998. Expression of the PEBP2α A/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone 22:87–92.

Yamaguchi A, Kahn AJ. 1991. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tissue Int 49:221–225.

Yamaguchi A, Katagiri T, Ikeda T, Wozney J, Rosen V, Wang E, Kahn A, Suda T, Yoshiki S. 1991. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.

Yamaguchi A, Komori T, Suda T. 2000. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev 21:393–411.