Co-stimulation of the Bone-related Runx2 P1 Promoter in Mesenchymal Cells by SP1 and ETS Transcription Factors at Polymorphic Purine-rich DNA Sequences (Y-repeats)*

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Transcriptional control of Runx2 gene expression through two alternative promoters (P1 and P2) is critical for the execution of its function as an osteogenic cell fate determining factor. In all vertebrates examined to date, the bone related P1 promoter contains a purine-rich region (−303 to −128 bp in the rat) that separates two regulatory domains. The length of this region differs dramatically between species even within the same order. Using deletion analysis, we show that part of this purine-rich region (−200 to −128) containing a duplicated element (Y-repeat) positively regulates Runx2 P1 transcription. Electrophoretic mobility assays and chromatin immunoprecipitations reveal that Y-repeat binds at least two different classes of transcription factors related to GC box binding proteins (e.g. SP1 and SP7/Osterix) and ETS-like factors (e.g. ETS1 and ELK1). Forced expression of SP1 increases Runx2 P1 promoter activity through the Y-repeats, and small interfering RNA depletion of SP1 decreases Runx2 expression. Similarly, exogenous expression of wild type ELK1, but not a defective mutant that cannot be phosphorylated, enhances Runx2 gene expression. SP1 is most abundant in proliferating cells, and ELK1 is most abundant in postconfluent cells; during MC3T3-E1 osteoblast differentiation, both proteins are transiently co-expressed when Runx2 expression is enhanced. Taken together, our data suggest that basal Runx2 gene transcription is regulated by dynamic interactions between SP1 and ETS-like factors during progression of osteogenesis.

RUNX proteins (1) have divergent biological roles in mammalian development that are evident from their murine knock-out phenotypes. Runx1-, Runx2-, or Runx3-null mice have major defects in hematopoietic, osteoblastic, and neuronal development, respectively (2–9). Expression of RUNX proteins is tightly regulated at the transcriptional and post-transcriptional levels. Consequently, the proteins display distinct tissue- and development-specific expression, which contributes to their distinctive developmental roles. Several studies have explored the transcriptional mechanisms controlling the tissue-specific expression of Runx genes. An important conserved feature of the vertebrate Runx genes is their expression from two promoters (P1 and P2) that encode isoforms with distinct amino-terminal sequences (10–12). Functional analyses using transfection assays have demonstrated that the P1 and P2 promoters both contribute to the expression of Runx genes (13–24). However, our knowledge of the positive and negative elements that regulate basal and tissue-specific expression of Runx genes is still incomplete.

Transcriptional control of Runx2 during osteoblast differentiation is primarily mediated by the upstream P1 promoter that drives expression of the MASNS/p57 isoform (TIL1) (25, 26), which utilizes the most 5′ exon of the Runx2 gene. The P2 promoter regulates the expression of the MRIPV/p56 isoform (PEBP2aA), which is predominantly expressed in T-cells (27) but also in the mesenchymal lineage, including immature osteoblasts (28). Multiple elements in the Runx2 P1 promoter have been identified, which recruit transcriptional factors or respond to stimulation by developmental cell signaling pathways. The Runx2 gene is autoregulated by feedback through multiple RUNX motifs of the promoter (16, 24). Suppression of the Runx gene by a vitamin D3-responsive element provides regulatory coupling between tissue-specific and steroid hormone-dependent control of genes during bone formation (14). A T cell factor (TCF) regulatory element that is responsive to canonical WNT signaling stimulates Runx2 gene expression (29). In addition, several homeodomain (HD) proteins (e.g. MSX2, DLX3, DLX5, and HOXA10) function as a key series of molecular switches that regulate expression of Runx2 throughout bone formation (30, 31).

We previously reported that the regulatory sequences of the mouse, rat, and human Runx2 promoters are highly conserved, with the exception of a purine-rich region that separates two functional domains in the P1 promoter (24) and that differs in length between the species. In this study, we provide evidence that the rodent-specific extension of this polymorphic region
reflects duplication of a functional element (Y-repeat) that positively regulates Runx2 gene transcription. We show that each Y element interacts with SP1 and ETS factors that co-stimulate Runx2 gene transcription during osteoblast differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MC3T3-E1 cells were maintained in the α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). ROS17/2.8 cells were grown in F-12 medium supplemented with 5% fetal bovine serum. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. For differentiation studies, MC3T3-E1 cells were fed every second day at confluence with the above medium containing 10 mm β-glycerophosphate and 50 μg/ml ascorbic acid (32, 33).

**Preparation of Reporter Gene Constructs**—Rat Runx2 promoter deletion mutants (−351, −288, and −128) were generated by PCR using specific primers, and the gel-purified fragments were cloned into the XhoI/HindIII backbone of a firefly luciferase reporter gene (pGL3 basic), as we described previously (24). New rat Runx2 promoter deletion mutants (−240, −200, −174, −160, and −138) were constructed in a manner identical to that used for the −351 promoter, with forward primers starting at positions −240, −200, −174, −160, and −138, respectively.

Mouse and human Runx2 gene promoter fragments were generated by direct PCR amplification from mouse and human genomic DNA. We obtained 480-bp (mouse) and 317-bp (human) fragments spanning the promoter only (Fig. 1) that were each inserted into the XhoI/HindIII sites of pGL3-basic (Promega, Madison, WI). The amplification primers used for the generation of the two fragments were as follows: 480 bp for mouse forward XhoI primer (5′-aga ctc gag GCC TTA GCT ACA GAG TTC TGC T) and the reverse primer (5′-TGG CTG GTA GTG ACC TGC AGA GAT TA); 317 bp for human forward XhoI primer (5′-aga ctc gag CCC TTA ACT GCA GAG CTC TGC T) and the reverse primer (5′-TGG CTG GTA GTG ACC TGC GGA GAT TA).

Base substitution mutants were generated in −200, including EMut and ESMut, with the QuikChange II site-directed mutagenesis kit according to the manufacturer’s protocol. Oligonucleotides were designed to mutate each element as follows: Sp1 motif at −185 and −145, from 5′-AGGGAGG-3′ to 5′-AGTATTAGG-3′; Ets motif at −192 and −156, from 5′-GAGAAA-3′ to 5′-CACAAGA-3′.

All vectors used in transient transfections are cytomelagovirus-driven expression constructs encoding distinct transcription factors that are kindly provided by the indicated individuals: Sp1 and Sp3 (Dr. Guntram Suske, Philipps-University Marburg, Germany), Sp7/Osterix (Dr. Je-Yong Choi, Kyungpook National University, Daegu, Republic of Korea), Ets1 (Mark Perrella, Brigham and Women’s Hospital, Boston, MA), Ets2 (Angie Rizzino, University of Nebraska Medical Center, Omaha, NE), Elk1, Elk1D (Elk1 mutant in which the D domain is deleted), and Elk1M (Elk1 mutant in which all the putative phosphorylation sites are mutated) (Andrew Sharrocks, University of Manchester, Manchester, UK).

**Transient Transfections**—Cells in 6-well plates at 70% confluence were treated with 6 μl of FuGENE 6 transfection reagent (Roche Applied Science) in accordance with the manufacturer’s protocol. A series of deletions of the rat Runx2 P1 promoter (1, 24) fused to the firefly luciferase reporter were used in transfection assays. Luciferase reporters were co-transfected with vectors for transcription factors, including Sp1, Sp7, Ets1, Ets2, Elk1, Elk1D, and Elk1M, or the corresponding empty vector. Each well contained equal amounts of total DNA (1.0 μg) in co-transfection experiments.

**RNA Interference (siRNA)**—Mouse MC3T3-E1 osteoblastic cells at 30–50% confluence were transfected using a siGENOME silencing RNA smart pool that targets Sp1 (100 nm) and the siCON nontargeting smart pool (Dharmacon Research, Lafayette, CO) using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Opti-MEM I (a reduced serum medium from Invitrogen) was used to dilute the siRNA and Oligofectamine for transfection. After treating the cells with siRNA for 4 h, the transfected cells were supplemented with MEM containing 30% fetal bovine serum for a final concentration of 10% in the medium. The siRNA experiment was carried out for 48 h, at which time the cells were harvested for total RNA and protein to analyze the knockdown effect of siRNA on endogenous Sp1 by real time quantitative PCR and Western blot analysis.

**Luciferase Reporter Assays**—Firefly luciferase reporter plasmids, expression plasmids, and Renilla luciferase reference gene (pRL-null-Renilla) plasmid, as internal control, were co-transfected in each well. The total amount of DNA was maintained at a constant level (200 ng/well or 1 μg/well for co-transfections) by adding an appropriate amount of empty vector. After 24 h, the cells were harvested using 200 μl of passive lysis buffer (Promega, Madison, WI) per well. Cell lysates (20 μl) were evaluated for luciferase activity using the Dual-Luciferase reporter assay kit (Promega). Luciferase activity was measured according to the manufacturer’s instructions and normalized to values for Renilla luciferase.

**RNA isolation and Analysis**—RNA was isolated from cultures of MC3T3-E1 cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. After purification, 5 μg of total RNA was DNase-treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). RNA (1 μg) was then reverse transcribed using random hexamers as primers and the SuperScript 1st Strand Synthesis kit (Invitrogen) according to the manufacturer’s protocol. The relative expression of a panel of genes was assessed by quantitative real time PCR. Primer Express software was used to predict optimum reverse transcription-PCR primer sets (Table 1), except for GAPDH1 primers (Applied Biosystems). Quantitative PCR was performed using SYBR Green 2× master mixture (Applied Biosciences, Foster City, CA) and a two-step cycling protocol (anneal and elongate at 60 °C, denature at 94 °C). Specificity of

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2 The abbreviations used are: siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; UTR, untranslated region; nt, nucleotide(s); ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.
Primers for quantitative PCR

| Primer name | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|-------------|------------------------|-----------------------|
| Runx2       | GGCCCTCTCTCCTGAGACTCTT | TGGCCCTCTCCTGAGCTCTTA |
| Osteocalcin  | CGTACAAGACGGCTTGTGTTCCA | GCCTGCTCTGCCTGTCTACA |
| Alkaline phosphatase | TTGGCAAGGAAAGAGGAGGAG | GTTTCAAGGCTATTTTTCAAGG |
| Osteopontin  | ACTCCAACTCCCTCCTAGCTCG | TGGAGGTTCTGGTCTCTATG |
| Collagen 1 A1 | CCAGAGAGGAGAGCACTGCTC | AGTGTACGTTAGCAAGCAATC |
| Sp1         | ATGGGGTCTCTCGGATACCA   | GAGATGTCTGAGCTGCTTCT |
| Sp3         | GCTTACCTTTGTTTCCTC     | TCTGGTTCAGCGGTTTPCT |
| Ets1        | CCGGGGTTCTCTCCTTAGAGC  | GAGTCTGATTGCCTGCCTACT |
| Ets2        | CCTGTCCGCAACAGTCTTTC   | GCTGATGCAGAGGTTACAGA |
| Elk1        | ACGGCGGAGATTTGTGTTCA   | GCTATGCGAGGCTGAGTCAG |
| Pu-1        | ATGGTACAGGCTGCTAAATG   | GTGTCGCTCTGGTTCTCCCA |

Oligonucleotides for electrophoretic mobility shift assays

Wild type and mutant oligonucleotides were used in gel shift assays. The YR oligonucleotide containing one Y-repeat region (underlined) from the Runx2 P1 promoter was used as the probe for the electrophoretic mobility shift assay. Other oligonucleotides were used as competitors.

| Oligonucleotide | Sequence (5′-3′) |
|----------------|-----------------|
| YR             | CAGAAGGAAAGAGGAGGAGGAGGAG |
| YR-M1          | CAGAAGGAAAGAGGAGGAGGAGGAG |
| YR-M2          | CAGAAGGAAAGAGGAGGAGGAGGAG |
| YR-M3          | CAGAAGGAAAGAGGAGGAGGAGGAG |
| Sp1-con        | ATCGTCCGCGGCCGCGGCAGCC |
| Sp1-Mut        | ATGCGTCCGCGGCCGCGAGCC |
| Ets1-con       | GATCTTCCGCGAGGAAATTGCA |
| Ets1-Mut       | GATCTTCCGCGAGGAAATTGCA |

Electrophoretic Mobility Shift Analysis—MC3T3-E1 and NIH3T3 cells were lysed in 50 µl of lysis buffer (2% SDS, 10 mM dithiothreitol, 10% glycerol, 12% urea, 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, 25 µM MG132) and boiled for 5 min. Equal amounts of total protein were analyzed by SDS-PAGE. Antibodies used in this study were purchased from Santa Cruz Biotechnology except for a RUNX2 mouse monoclonal antibody (generous gift from Drs. Yoshiaki Ito and Kosei Ito, National University, Singapore). Primary antibodies were detected with secondary antibody conjugated to horseradish peroxidase. Secondary antibodies were detected using Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences).

RESULTS

A Polymorphic Purine-rich Repeat Region (Y-repeat) Is Responsible for Differences in Basal Runx2 P1 Promoter Activity among Mammalian Species—The Runx2 P1 promoter in mammals is composed of two highly conserved regulatory domains, a proximal region (nt −124 to −1 in the rat) and distal region (nt −458 to −303 in the rat), that are linked by a polymorphic purine-rich sequence (nt −303/−128). Comparison of the rat, mouse, and human Runx2 promoters shows that the proximal and distal promoter regions of each species have a high degree of similarity (>95%) (24). However, the three species differ in...
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The Polymorphic Purine-rich Region Contains Tandemly Arranged Transcriptional Elements (Y-repeat)—We performed deletion analysis of the rat Runx2 promoter to investigate the role of the purine-rich region in transcriptional control. Progressively deleted promoters were transfected into three cell lines, including mouse MC3T3-E1 osteoblasts, rat ROS17/2.8 osteosarcoma cells, and human U2OS osteosarcoma cells. It has been reported that Runx2 promoter activity is not reduced by deletion of sequences distal from the Y-repeat. We compared the Y-repeat activity in all three cell lines, whereas only about 20% of promoter activity was maintained in the −128 promoter, in which the entire purine-rich region 1 was deleted (Fig. 2). This result suggests that the region between −200 and −128 contains major transcriptional determinants.

Deletion analysis of the rat Runx2 promoter revealed that the Y-repeat contributes to maximal promoter activity. Luciferase activity was normalized to Renilla luciferase activity and represents mean ± S.D. of triplicate wells from at least three independent experiments.

The Y-repeat consists of a tandem repeat of Y repeats, which are 18-bp segments of the Runx2 promoter. The Y-repeat is a tandemly arranged transcriptional element that contributes to basal Runx2 P1 promoter activity. The Y-repeats are composite transcriptional elements containing Sp1-and ETS-related recognition motifs. We examined the Y-repeat for the presence of motifs for sequence-specific transcription factors and detected an atypical Sp1 binding site and a potential Ets motif. To validate the identity of these sequence motifs, we performed in vitro protein-DNA interaction experiments. Electrophoretic mobility shift assays revealed specific DNA-protein complexes that were competed by Sp1 and Ets-specific oligonucleotides.

The Y-repeat motif was also detected in other Runx2 promoters, including mouse and human Runx2 promoters. Sequence conservation of the Y-repeat motif was high across species, suggesting that this motif is conserved and contributes to basal Runx2 promoter activity.

FIGURE 2. Y-repeat contributes to Runx2 promoter activity. A series of deletion mutants spanning the rat Runx2 promoter were transfected in ROS17/2.8 (white bars), MC3T3-E1 (gray bars), and U2OS cells (black bars). The graphs show the activities of the promoters relative to the rat −351 promoter. Each copy (open arrow) of the Y-repeat contributes to maximal Runx2 promoter activity. Luciferase activity was normalized to Renilla luciferase activity and represents mean ± S.D. of triplicate wells from at least three independent experiments.
show that the Y-repeat contains both Sp1 and Ets-related protein-DNA complexes.

To provide further evidence that Sp1-related complexes bind to the Y-repeat, we performed competition assays with Sp1 and Ets consensus motifs. When unlabeled Sp1 consensus oligonucleotide was added, the upper Sp1-related bands disappeared, but these complexes remained in the presence of a mutant Sp1 consensus oligonucleotide (Fig. 3C). Similarly, ETS1 protein binding is corroborated by competition assays with wild type and mutant oligonucleotides spanning an Ets1 consensus motif (Fig. 3C). The identity of the proteins mediating the Sp1- and ETS-related complexes was determined with supershift assays using antibodies against Sp1 and ETS1. The addition of the Sp1 antibody generated a supershifted band, whereas the ETS1 antibody blocked formation of the Ets-1-related complex (Fig. 3C, right). We note that there was more Sp1 binding than Ets1 binding to Y-repeat regions in proliferating MC3T3-E1 cells that did not express mature bone-phenotypic markers (e.g. osteocalcin) (Fig. 3C). However, in ROS17/2.8 osteosarcoma cells that are known to express osteocalcin, ETS1 appears to be the major protein binding to the Y-repeat region (data not shown). Taken together, these results show that ETS1 and Sp1 proteins form protein-DNA complexes at the Y-repeats in proliferating cells and that each Y-repeat represents a modular unit containing Sp1 and ETS elements.

**SP1 and ETS Proteins Co-stimulate Runx2 Promoter Activity**—Because the Y-repeat regions are important for Runx2 promoter activity and mediate interactions with Sp1 and Ets1-like factors, we further examined the regulatory activities of Sp1 and Ets1 on Runx2 transcription using co-transfection experiments. Expression vectors for Sp1 and Ets1, respectively, were co-transfected in MC3T3-E1 cells with constructs in which the luciferase reporter was fused to serial deletions of the Runx2 promoter. The data show that both Sp1 and Ets1 can increase the activity of promoter segments spanning the Y-repeat regions by about 2–2.5-fold (e.g. −351 or −200 promoters) (Figs. 4, A and B). However, deletions of the Y-repeat regions (e.g. −174 or −138 promoters) reduced or blocked activation by Sp1 and Ets1. Thus, our data suggest that Sp1 and Ets1 specifically activate the Runx2 promoter through the Y-repeats.

Additionally, we assessed possible contributions of several other members of the SP1/KLF (specificity protein/Krüppel-like factor) family, including SP3 and SP7/Osterix. Forced expression of SP3 or SP7 did not have appreciable effects on Runx2 promoter activity (Fig. S1A). Overexpression of either ETS2 or Pu.1 also did not affect promoter activation (Fig. S1B). Forced expression of ELK1 (ETS-like transcription factor) repressed the Runx2/luciferase promoter construct as well as a promoterless luciferase construct, thus preventing us from assessing the specific role of ELK1 in Runx2 gene transcription (data not shown).

To assess the precise contributions of the Sp1 and Ets1 motifs to Runx2 promoter activity, we generated specific mutations in the −200 promoter. The point mutations were based on the mutant oligonucleotides we tested in gel shift assays (see Fig. 3B). Mutation of the Ets motif (EMut) reduced the activity of the −200 promoter, whereas mutation of both the Sp1 and Ets1 motifs (EMMut) further diminished promoter activity (Fig. 4C). These results suggest that Sp1 and ETS co-stimulate Runx2

![Figure 3. Sp1 and Ets1 interact with the Y-repeat region in vitro.](image-url)

Electrophoretic mobility shift assays were performed with probes spanning the Y-repeat (Probe YR) and mutant competitor oligonucleotide (A) with point mutations in putative Sp1 or Ets motifs (YR-M1 and YR-M2, respectively) or a mutation that does not affect binding events (YR-M3). Binding reactions were complemented with nuclear extracts prepared from proliferating MC3T3-E1 cells (B and C). Complexes mediated by Sp1-related (closed arrowheads) or Ets-related factors (open arrowhead) were identified by the absence of specific competition with the YR-M1 or YR-M2 oligonucleotides. Gel shift immunoassays (C, right) were performed by incubating binding reactions with the indicated antibodies. Normal IgG was used as negative control. The Sp1 antibody causes a supershift (Sp1 + α), whereas the Ets antibody causes a block shift (Ets1 + α), indicating that these two proteins are the most prominent DNA binding activities in actively proliferating MC3T3-E1 cells.
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| A | B | C | D |
|---|---|---|---|
| Ets-motifs | Sp1-motifs | -351 | -200 | -174 | -138 |
| EV | Sp1 | EV | Sp1 | EV | Sp1 | EV | Sp1 |
| -351 | -200 | -174 | -138 |
| Fold induction | Fold reduction |

**FIGURE 4.** Sp1 and Ets1 activate the Runx2 promoter through the Y-repeats. A and B, forced expression of Sp1 or Ets1 stimulates Runx2 promoter activity. Sp1 (A) or Ets1 (B) or their respective vectors (EV) were co-transfected with a series of deletion mutants of the Runx2 promoter fused to a luciferase reporter in MC3T3-E1 cells. Luciferase activity was detected after 24 h, and the graphs show fold induction relative to the rat P1 promoter. C, mutation of the Sp1 or Ets motifs reduces Runx2 promoter activity. Transfection assays were performed using reporters with mutations in the -200 promoter within the Ets motif (EMut) or the overlap between the Ets and Sp1 motifs, and promoter activities were measured by luciferase assays 24 h after transfection. D, Sp1 and Ets1 co-stimulate Runx2 promoter activity. Expression vectors for Sp1 and Ets1 were co-transfected alone (500 ng of Sp1 or Ets1) or together (250 ng of Sp1 and 250 ng of Ets1) with the wild type -200 promoter, EMut, or ESMut in MC3T3-E1 cells. Luciferase activity was normalized to Renilla luciferase activity and represents the mean ± S.D. of triplicate wells from at least three independent experiments.

transcription. Forced expression of both Sp1 and Ets1 resulted in higher promoter activity than with either protein alone (Fig. 4D). As negative controls, we co-transfected Ets1 and Sp1 with the corresponding mutant promoters (Fig. 4D). Mutation of Ets1 motifs only decreased the contribution to promoter activation by Ets1 and had no effect on the activity by Sp1. Neither Sp1 nor Ets1 was able to activate the ESMut promoter, which abolishes the interactions of both Sp1 and ETS in the Y-repeat regions. Taken together, the transcription assays indicate that Sp1 and ETS1 co-stimulate Runx2 transcription through their cognate motifs in the Y-repeats.

The Genomic Runx2 Promoter Interacts with Endogenous Sp1 and ETS Proteins in Vivo—To confirm that Sp1 and ETS1 indeed bind to Y-repeat regions of the Runx2 P1 promoter in vivo, ChIP assays were performed in MC3T3-E1 and ROS17/2.8 cells. The primers (Rx2-3'-UTR) were optimized to detect interactions to the Y-repeat regions (-210/-92). Control primer pairs (Rx2-3'-UTR) from 3'-untranslated regions of the gene were used to verify specific binding of DNA fragments. Anti-SP1 and anti-ETS1 antibodies as well as an antibody against RNA polymerase II (positive control) and nonspecific IgG (negative control) were used for ChIP assays. The results reveal that RNA polymerase II was specifically associated with the Runx2 promoter, which is expected, because the gene was actively transcribed (Fig. 5). Sp1 and ETS1 interacted with Y-repeats of the Runx2 promoter but not the 3'-UTR region of the promoter (Fig. 5). Moreover, there was more Sp1 binding in MC3T3-E1 cells than in ROS17/2.8 cells, whereas more Ets1 binding to the Y-repeat region was present in ROS17/2.8 cells, consistent with gel shift results (data not shown). These findings suggest that Sp1 and ETS factors contribute to control of Runx2 expression through the Y-repeat regions in MC3T3-E1 osteoblasts and ROS17/2.8 osteosarcoma cells.

Expression Profiles of Sp1 and ETS Factors during Differentiation of MC3T3-E1 Cells—Because Sp1 and ETS1 proteins modulate Runx2 promoter activity and RUNX2 protein controls osteoblast differentiation, Sp1 and Ets mRNA and protein levels were assayed during osteoblastic differentiation of MC3T3-E1 cells using quantitative PCR and Western blotting. The mRNA levels of Sp1 and Ets1 were assessed in relation to established stage-specific markers of differentiation, including alkaline phosphatase, osteopontin, and osteocalcin. Under our experimental conditions, alkaline phosphatase increased after day 4, when cells had become confluent and differentiation commenced, and osteopontin expression was high at all stages but highest on day 24, whereas osteocalcin expression steadily increased during the differentiation time course with the highest expression at day 24 (Fig. 6A). The mRNA level of Sp1 steadily declined during osteoblast differentiation in culture, consistent with its prominent expression and housekeeping function in proliferating cell types. When we tested four different Ets factors (Ets1, Ets2, Elk1, and Pu.1), we observed that Ets2 mRNA was more prominently expressed than Ets1 during differentiation, whereas the hematopoiesis-related Ets factor Pu.1 was expressed at barely detectable levels (Fig. S2). More interestingly, Elk1 mRNA was consistently expressed at higher levels than the other three Ets factors we examined (i.e. Ets1, Ets2, and Pu.1) and was up-regulated by 5-fold from confluence (day 0) to early differentiation (day 8) (Fig. 6B). Thus, Elk1 represents...
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![Figure 5](image)

**FIGURE 5. In vivo interactions of the Runx2 promoter with Sp1 and Ets1.**
Chromatin immunoprecipitation experiments (ChIP assays) were performed with MC3T3-E1 and ROS17/2.8 cells and primers that amplify the promoter region spanning the Y-repeat (open arrows) (−210/−92) in the rat Runx2 promoter. The horizontal arrows indicate the positions of the forward and reverse primers. Primers spanning the 3′-UTR of the Runx2 gene were used as a negative control. Input represents 1% of each chromatin fraction that was used for immunoprecipitation. The ChIP data presented are representative of three experiments.

The most prominently expressed Ets transcription factor in differentiating MC3T3 osteoblasts.

Based on these results, we focused our follow-up studies on Sp1 and Elk1 factors during osteoblast differentiation. To investigate whether changes in Sp1 and Elk1 mRNA expression are reflected by commensurate changes in protein levels, we performed Western blot analysis. Consistent with the changes in mRNA levels, SP1 protein levels were dramatically decreased after day 4, whereas the expression of ELK1 was clearly up-regulated. Meanwhile, phosphorylation of ELK1 was enhanced as osteoblast differentiation progressed and reached a peak at day 16, when RUNX2 protein was expressed at the highest level (Fig. 6C). Our results suggest that SP1 may be important for stimulating basal Runx2 P1 promoter activity in proliferating osteoblasts and early differentiation of osteoblast (before day 4) and that up-regulation of ELK1 sustains basal level transcription of the Runx2 gene during later stages of development (after day 4).

**Functional Interactions of SP1 and ELK1 with the Y-repeat Region of the Runx2 P1 Promoter during Osteoblast Differentiation in Vivo—**We performed ChIP assays to detect whether the regulation of Sp1 and ETS factors at the mRNA and protein levels during osteoblast differentiation is reflected by a corresponding change in their ability to genomic occupancy at the endogenous Runx2 locus in vivo (Fig. 7).

We used antibodies for SP1, ELK1, and ETS2, as well as nonspecific IgG (negative control); ETS1 was not tested, because its expression is very low in differentiated cells. The results show that SP1 associates with the Runx2 promoter region spanning the Y-repeats (−210/−92) but the binding decreased from day 0 to day 16. In contrast, ELK1 binding is detected by day 8 and remains associated with the promoter during differentiation (Fig. 7). No binding of either SP1 or ELK1 was observed at the Runx2 3′-UTR. Although ETS2 has been reported to be abundant during differentiation of MC3T3-E1 cells (42), we did not observe ETS2 binding to the Y-repeats of Runx2 (Fig. S3); this result is consistent with our finding that forced expression of ETS2 does not affect Runx2 promoter activity (Fig. S1B). Taken together, our data indicate that SP1 is a major interacting protein at the Y-repeats of the Runx2 promoter in proliferating cells and early differentiated cells, whereas ELK1 is recruited to the Y-repeats at later stages of MC3T3-E1 cell differentiation.

**Knockdown of Sp1 by RNA Interference Reduces RUNX2 mRNA and Protein Levels in MC3T3-E1 Cells—**Because our data show that SP1 and ETS factors physically interact with the Y-repeat regions of the Runx2 promoter, we further investigated whether these two factors directly regulate endogenous expression of Runx2. Because SP1 protein is abundant in proliferating MC3T3-E1 cells, we used RNA interference to detect the effects of Sp1 depletion on Runx2 expression at the mRNA and protein levels. Sp1 siRNA (siSp1) reduces Sp1 mRNA levels by 70% at 48 h post-transfection but has no effect on the expression of the Sp1-related transcription factor Sp3 (Fig. 8A). We observed a complete knockdown of Sp1 protein expression,
indicating that our RNA interference strategy is effective. Strikingly, depletion of Sp1 mRNA and protein levels decreased RUNX2 mRNA levels by 50% and protein levels by 90% (Fig. 8, A and B). The Sp1 siRNA also reduced alkaline phosphatase mRNA levels by 30% but did not affect collagen IA1. Unexpectedly, the Ets1 mRNA level was also down-regulated by 50% due to knockdown of Sp1 (Fig. 8A). There were also no changes in the protein levels of the cell cycle markers cyclin E and CDK2, suggesting that down-regulation of either SP1 or RUNX2 by Sp1 siRNA does not cause an acute cell cycle arrest (Fig. 8B). Hence, our results establish that endogenous Runx2 gene expression is responsive to modulations in SP1 protein levels and corroborate our reporter gene data as well as electrophoretic mobility shift analysis and ChIP results indicating that the Runx2 gene is a direct target for Sp1.

**Up-regulation of Runx2 Expression by Exogenous Elk1 in Osteoblasts and Fibroblasts**—Because ELK1 (unlike SP1) is expressed at low levels in proliferating MC3T3-E1 osteoblasts, a forced expression strategy was carried out to examine whether ELK1 regulates Runx2 expression. The results show that wild type ELK1 increases RUNX2 mRNA levels by about 3-fold in MC3T3-E1 osteoblasts and 2-fold in NIH3T3 fibroblasts (Fig. 9A). We also tested two Elk1 mutants that contained either a deletion of the D domain (Elk1D mutant) or a mutation of phosphorylation sites (Elk1M mutant). Removal of the D domain in the Elk1D mutant prevents phosphorylation by ERK and JNK, but not p38 MAPK, because it removes a protein module that recruits ERK and JNK MAPKs to support phosphorylation of ELK1. Forced expression of the Elk1D mutant, which apparently remains partially phosphorylated by p38 MAPK, still enhanced Runx2 expression by 1.5-fold (Fig. 9, A and B). However, the Elk1M mutant in which all putative phosphorylated sites are mutated did not alter Runx2 gene expression in either MC3T3-E1 or NIH3T3 cells (Fig. 9A). Our experimental evidence shows that the exogenous ElkM mutant protein indeed is unphosphorylated, whereas phosphorylation of endogenous ELK1 remains detectable (Fig. 9B). Consistent with the regulation of Runx2 mRNA levels by ELK1, wild type ELK1 significantly enhanced RUNX2 protein levels in both MC3T3-E1 cells and NIH3T3 cells but not ElkM (Fig. 9B). Thus, our results demonstrate that Runx2 is an ELK1-responsive gene and a direct target of ELK. Furthermore, the differences in Runx2 regulation by distinct ELK1 mutants suggest that p38 MAPK regulation of ELK1 contributes to control of endogenous Runx2 gene expression.

**DISCUSSION**

In this study, we provide several lines of evidence that show that SP1 and ETS-related factors (i.e. ETS1 and ELK1) function as key molecular switches that regulate the basal expression of
Runx2 in osteoblasts. Both sets of factors interact in partially overlapping arrangement with a common element (Y-repeat) located in a purine-rich region in the Runx2 bone-related P1 promoter. SP1 and ETS factors are capable of stimulating Runx2 transcription independently but can also co-stimulate the Runx2 promoter. Our data are consistent with previous studies indicating that SP1 and ETS family members are transcriptional activators that can stimulate transcription through cooperative interactions to enhance expression of a number of genes (e.g. Fas ligand, platelet-derived growth factor A, and β-1,4-GalT V) (35–37). In proliferating osteoblastic cells, SP1 levels predominate over the levels of Ets-related factors (e.g. Ets1 and Elk1), whereas during osteoblast maturation, ELK1 becomes the major regulator of Runx2 transcription through the Y-repeat due to decreased expression of SP1 and ETS1. During early stages of differentiation, co-existence of SP1 and Elk1 may result in co-stimulation of basal Runx2 transcription. Thus, the spatial organization of their recognition motifs generates a functional switch in which transcription is stimulated by Sp1, Ets factors, or both, depending on the physiological concentration of these factors in the cell (see Fig. 10).

Our identification of SP1 and ETS factors as regulators of the Runx2 P1 promoter complements previous studies on transcriptional mechanisms that mediate the developmental, tissue-specific, and steroid hormone-responsive expression of Runx2. For example, our group and others have previously demonstrated that the Runx2 P1 promoter contains both positive and negative elements, including multiple autoregulatory Runx2 motifs (24), a vitamin D3-responsive element (17), WNT signaling-responsive T cell factor/LEF (lymphoid-enhancer factor) elements (29), and BMP2-responsive homeodomain motifs (29), as well as binding sites for AP1 (17), NF1 (22), and HLH-related factors (38). Because SP1 is proliferation-specific and ELK1 integrates signals from MAPK pathway (e.g. p42/p44 ERK, JNK, and p38) in postconfluent cells, we propose that the Y-repeat supports transcription of the Runx2 gene at multiple developmental stages in osteogenic cells.

One important ramification of our finding that SP1 and ELK-1 control Runx2 transcription is that these factors and their associated regulatory pathways and cell signaling cascades may contribute to osteoblast differentiation and skeletal development. The SP/KLF family has been reported to support progression of osteoblast differentiation, as reflected by the regulatory roles of SP1 and SP3 in the regulation of bone-related genes like β3-integrin (39), osteocalcin (40), or α2(XI) collagen (COL11A2) (41). In addition, SP7/Osterix is a bone-related transcription factor that functions genetically downstream of Runx2 (42, 43). However, our data indicate that SP1 and not SP3 or SP7 is rate-limiting for Runx2 gene transcription through the Y-repeat. Knockdown of SP1 down-regulates Runx2 expression by both direct and indirect mechanisms. Decreased Ets1 gene expression by SP1 deletion may further strengthen the repression of Runx2 expression, since our data show that both factors activate Runx2 transcription through the Y-repeats.

Our results obtained with forced expression approaches suggest that ELK1 is a major physiological regulator of endogenous Runx2 gene expression. Other studies have proposed that the Ets members ETS1 and ETS2 regulate osteoblast growth and differentiation based on their temporal expression profiles (44). Our data reveal that ELK1 is much more prominently expressed than either ETS1 or ETS2 and that of these latter two, only...
ETS1 increases Runx2 promoter activity. Therefore, we suggest that Elk1 is a biological regulator of Runx2 promoter activity during osteoblast maturation.

Because only wild type Elk1 but not a nonphosphorylatable mutant induces Runx2 expression, our findings indicate that Elk1 may transduce signals from the MAPK cascade to stimulate Runx2 gene expression. Consistent with this concept, we show that increased expression and phosphorylation of Elk1 temporally correlates with enhanced Runx2 expression during osteogenic differentiation. Elk1 is a major nuclear target of three distinct classes of mitogen-activated protein kinases, including ERK, JNK, and p38 (45). Several reports have provided evidence that MAPK pathways are involved in osteoblast differentiation as well as the induction of Runx2 mRNA by TGF-B1 and BMP-2 (46–49). The possibility arises that Elk1 interactions with the Y-repeat together with other SMAD-responsive interactions elsewhere on the Runx2 promoter may mediate convergence of MAPK signaling on the Runx2 promoter to regulate osteoblast differentiation.

The Y-repeat we identified in our study is a component of a polymorphic purine-rich region that separates two distinct functional domains in the Runx2 P1 promoter. Our finding that the Y-repeat represents a duplicated Sp1/Ets bipartite element permits the interpretation that the rodent-specific extension of the purine-rich region may accommodate a requirement for increased basal transcription of the Runx2 gene. RUNX proteins are highly conserved in all metazoan species, and one general question remains how the biological functions of these proteins can evolve during diversification of species. In vertebrates, RUNX2 protein contains a polymorphic region within the coding sequence (Q/A stretch), and natural variation of this Q/A stretch may alter protein function (50). In our study, we find the rat and mouse Runx2 promoters that contain an extended purine-rich region have much higher promoter activity than the corresponding human Runx2 promoter, which has a much shorter purine-rich region. Furthermore, experimental shortening of this polypurine region in the Runx2 promoter significantly reduces basal promoter activity (i.e. due to loss of Sp1 and Ets-related binding sites). Therefore, we propose that polymorphic regions in both promoter and coding sequences may support skeletal adaptations during evolution of the vertebrate lineage.

In conclusion, our findings suggest specific mechanisms for regulating Runx2 gene transcription by the combined activities of Sp1 and Ets factors on the Runx2 promoter during osteoblastic proliferation and differentiation. We show that these factors operate via a Y-repeat in a polymorphic purine-rich region of the Runx2 promoter that is essential for maximal Runx2 gene transcription. The interesting idea arises that duplication of the Y-repeat is a beneficial stochastic event that occurred during evolution of the rodent lineage to increase basal Runx2 gene expression.

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