Histone Deacetylase 3 Interacts with Runx2 to Repress the Osteocalcin Promoter and Regulate Osteoblast Differentiation*

Tania M. Schroeder‡, Rachel A. Kahler§, Xiaodong Li¶, and Jennifer J. Westendorf**

From the ‡Graduate Program in Biochemistry, Molecular Biology and Biophysics, and the §Graduate Program in Microbiology, Immunology and Cancer Biology, ¶The Cancer Center, and the ||Department of Orthopaedic Surgery, the University of Minnesota, Minneapolis, Minnesota 55455

The runt domain transcription factor Runx2 (AML-3, and Cbfal) is essential for osteoblast development, differentiation, and bone formation. Runx2 positively or negatively regulates osteoblast gene expression by interacting with a variety of transcription cofactor complexes. In this study, we identified a trichostatin A-sensitive autonomous repression domain in the amino terminus of Runx2. Using a candidate approach, we found that histone deacetylase (HDAC) 3 interacts with the amino terminus of Runx2. In transient transfection assays, HDAC3 repressed Runx2-mediated activation of the osteocalcin promoter. HDAC inhibitors and HDAC3-specific short hairpin RNAs reversed this repression. In vivo, Runx2 and HDAC3 associated with the osteocalcin promoter. These data indicate that HDAC3 regulates Runx2-mediated transcription of osteoblast genes. Suppression of HDAC3 in MC3T3 preosteoblasts by RNA interference accelerated the expression of Runx2 target genes, osteocalcin, osteopontin, and bone sialoprotein but did not significantly alter Runx2 levels. Matrix mineralization also occurred earlier in HDAC3-suppressed cells, but alkaline phosphatase expression was not affected. Thus, HDAC3 regulates osteoblast differentiation and bone formation. Although HDAC3 is likely to affect the activity of multiple proteins in osteoblasts, our data show that it actively regulates the transcriptional activity of the osteoblast master protein, Runx2.

Runx2 is one of three mammalian homologues of the Droso phila protein Runt. Runx2 is required for osteoblast formation and chondrocyte differentiation and thus is an essential regulator of intramembranous and endochondral bone formation (1–3). Runx2 haploinsufficiency causes the autosomal dominant bone disorder cleidocranial dysplasia (4, 5), but elevated Runx2 levels cause osteopenia (6) and contribute to murine T cell lymphomagenesis (7). Furthermore, Runx2 polymorphisms are associated with altered bone mineral density and susceptibility to osteoporotic fractures (8). The sensitivity of osteoblasts to changes in wild-type Runx2 levels provides biological proof that its activity must be tightly controlled. At the molecular level, Runx2 is regulated by transcriptional and translational mechanisms, post-translational modifications, including phosphorylation, and by interactions with other transcription factors and chromatin-modifying enzymes (9–20). Because the Runx DNA-binding element is necessary but not sufficient to activate tissue-specific gene expression (21, 22), it was hypothesized over a decade ago that Runx proteins are promoter organizers (23). It is now apparent that Runx factors indeed interact with other transcription factors as well as recruit both co-activator and co-repressor complexes to promoters. Known co-activators of Runx2 include YAP, HES, and the histone acetyltransferases, p300, CBP, MOZ, and MORF (24–27). The list of Runx2-associated co-repressors includes just TLE, mSin3a, and histone deacetylase (HDAC)1 6 (18, 19, 28, 29); however, among all known transcriptional co-repressors, only a small fraction have been tested for interactions with Runx2. Because Runx2 is crucial for bone formation, we are interested in understanding how its transcriptional activity is affected by co-repressor complexes in osseous cells.

HDACs are enzymatic components of multiprotein complexes that are recruited by transcription factors to specific DNA regulatory sequences. They remove acetyl residues from nucleosomal histones and other substrates leading to chromatin condensation and gene repression (30–34). Eleven mammalian HDACs are classified into two groups on the basis of sequence similarities with yeast proteins. Class I HDACs (HDAC1–3, -8, and -11) are homologues of yeast RPD3 and are widely expressed in the nuclei of mammalian cells (35–37). Class II HDACs (HDAC4–7, -9, and -10) are similar to yeast Hda1/2 and appear to shuttle between nuclear and cytoplasmic compartments in specific cell types (18, 38–45). Major functions of HDACs are to regulate gene expression, transport ubiquitinated protein aggregates, and deacetylate tubulin (46–48). Global suppression of HDAC activity by chemical inhibitors commonly causes cell cycle arrest and may promote cell differentiation (49, 50).

HDAC3 is one of the better-studied HDACs. It associates with several transcription factors, including GATA1, peroxisome proliferator-activated receptor-γ, retinoblastoma protein, thyroid hormone receptor, TEL, and TFII-I (51–55). HDAC3 also interacts with the co-repressors N-CoR and SMRT (53, 56), which link HDAC3 to transcription factors and other co-repressors. HDAC3 is also the crucial catalytic component of several class II HDAC complexes (57, 58). Thus, HDAC3 appears to be a very important factor in multiple co-repressor complexes that

* This work was supported by National Institutes of Health Grants AR48147 and AR050938, The V Foundation for Cancer Research, and The Cancer Center at the University of Minnesota. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
** To whom correspondence should be addressed: The Cancer Cen ter, University of Minnesota, MMC 806, 420 Delaware St. SE, Minneapolis, MN 55455. Tel.: 612-626-3365; Fax: 612-626-4915; E-mail: weste047@umn.edu.

Received for publication, April 2, 2004, and in revised form, July 22, 2004
Printed in U.S.A.
HDAC3-deficient animals have not been described; however, HDAC3 suppression in HeLa cells by RNA interference blocked cell proliferation and transcriptional repression by thyroid hormone receptor (59, 60).

In this study, we identified HDAC3 as a transcriptional co-repressor of Runx2 using a candidate gene approach. HDAC3 interacted with the amino terminus of Runx2, which acts as an autonomous repression domain on a heterologous promoter. HDAC3 repressed Runx2-induced activation of the osteocalcin promoter and was found associated with the osteocalcin promoter in osteoblasts. Stable suppression of HDAC3 by RNA interference accelerated osteoblast mineralization and the expression of osteoblast differentiation genes. These data identify HDAC3 as a regulator of Runx2 transcriptional activity in osseous cells and suggest that its expression regulates bone formation.

EXPERIMENTAL PROCEDURES

Cell Culture—COS and C2C12 cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 50 units/ml of penicillin and 50 μg/ml of streptomycin. CH31T1/2, MC3T3-E1, ROS17/2.8, and UMR-106 were maintained in minimal essential medium (MEM) supplemented with 10% FBS, 1% nonessential amino acids, 200 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. To induce differentiation, confluent MC3T3-E1 cells were cultured in α-MEM containing 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10 mM β-glycerolphosphate. The differentiation medium was replaced every 3 days.

Plasmids—The GAL-TK-luciferase (Luc) reporter plasmid and the GAL-Runx2 fusion proteins were previously described (18). Human and mouse HDAC3 mammalian expression vectors were kindly provided by Dr. Edward Seto (Lee Moffit Cancer Center, Tampa, FL) (61). The pSHAG and pSHAG-Firefly luciferase (Fl) vectors were obtained from Dr. Gregory Hannon (Cold Spring Harbor Laboratories) (62). The pSHAG-HDAC3 short-hairpin RNA vectors were constructed by anneal-
Runx2 Interacts with HDAC3

A. HDAC3 interacts with the amino terminus of Runx2. A, HDAC3 interacts with the first 227 amino acids of Runx2 (MRIPV isoform) in vivo. COS cells were transfected with GAL-Runx2-(1–227) (5 μg) and the indicated CMV-FLAG-HDAC construct (5 μg). Lysates were collected at 48 h post-transfection and immunoprecipitated with antibodies recognizing GAL (top) and FLAG (bottom) epitopes. The asterisk is adjacent to HDAC3, which is partially hidden by the Runx2-(1–227) protein in lane 4. B, HDAC3 interacts with the Runx2 amino terminus in vitro. Upper panel, GST-Runx2 fusion proteins were incubated with 32P-labeled HDAC3. Proteins complexes were washed and separated by SDS-PAGE. HDAC3 was detected by autoradiography. The GST-(50–179) and GST-(50–179, L148D) fusion proteins contain sequences from the runt domain of Runx1 (AML-1A). Lower panel, Coomassie staining of GST fusion proteins used in the upper panel. Asterisks indicate the appropriate full-length protein.

B. Runx2 and HDAC3 interact in osteoblasts. A, osteoblast cell lines express HDAC3. Whole cell lysates (100 μg) were prepared from MC3T3, C2C12, ROS17/2.8, and UMR-106 cells and immunoblotted with the indicated antibodies. B, Runx2 and HDAC3 interact in osteosarcoma cell lines. Whole cell lysate prepared from ROS17/2.8 cells, divided into five tubes (–4 μg/tube), and immunoprecipitated with no antibody (–), nonspecific (Control), HDAC3, or Runx2 (C19, S19, and AML3) antibodies. The whole cell lysate (100 μg) and immunoprecipitates were immunoblotted with anti-HDAC3.
Novabiochem Corp.) at 4 °C and high speed centrifugation. Lysates were then incubated with nonspecific rabbit IgG (Rockland), HDAC3 (Cell Signaling), or Runx2 antibodies (C19, S19, and AML3) as indicated for 16 h at 4 °C. Immune complexes were collected with Protein A-Sepharose beads (Sigma-Aldrich) for 30 min at 4 °C. The beads were washed three times with a buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 25 μg/ml H11032, and protease inhibitors (Mini Complete protease inhibitor mixture tabs). The lysates were sonicated on ice and cleared by centrifugation. GST proteins were purified by incubating E. coli protein A-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. HDAC3 proteins were in vitro transcribed and translated in rabbit reticulocyte lysates (Promega) with 5°S-labeled amino acids (Trans-32P-label, ICN Pharmaceuticals, Inc., Irvine, CA) from pBlue-script-HDAC3 according to the manufacturer’s instructions. GST proteins and radiolaabeled murine HDAC3 were mixed and incubated for 1 h at 4 °C, washed three times with lysis buffer, resolved by SDS-12% PAGE, and visualized by autoradiography.

Chromatin Immunoprecipitation Assays—The chromatin immunoprecipitation (ChIP) assays were performed in ROS17/2.8 cells as previously reported (66). Briefly, cross-linked lysates were immunoprecipitated without any antibody, anti-HA (12CA5), Runx2 (S19 and C19), or HDAC3 antibodies. An additional control reaction (mock) contained no chromatin or antibody. Nested PCR was performed with primer pairs generated to detect bases encompassing the proximal Runx2 binding site in the rat osteocalcin promoter. External primer pairs for PCR were: 5’-GGATTTAGTAGGTTTTTCC-3’ and 5’-CZAAGAGGCATGCT-GTGGTTG-3’. The internal primer pairs for the nested PCR were: 5’-GAGGGTTTCTTCTTCAAGTT-3’ and 5’-TGGGAAAGCGCTGG-AAGG-3’.

cDNA Synthesis and Reverse Transcription PCR—Total RNA from MC3T3 cells stably expressing Fli and HDAC3 shRNAs was isolated with TRizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed to cDNA with the Invitrogen Superscript kit according to its instructions. PCR was performed on the cDNA using primers specific for HDAC3 (5’-CGGAGGAGGGATTAGAATCTGAACTCAGC-3’ and 5’-TACACCAAGGAGGATAGTAAACTCTTGTAG-3’), Runx2 (5’-ATG-GATGACATAGACTGACATGGCC-3’ and 5’-TAGATTTCTAAAGGATGGGAAAC-3’), and actin (5’-GACTCTGATAGAGATGGCC-3’ and 5’-CAATGTCAGGATGTTGAC-3’).

Quantitative Real-time PCR—cDNA was prepared as described above. Real-time PCR was carried out in a Lightcycler (Roche Diagnostics) using the following primer sets: HDAC3 (5’-CCGCGTCTCACAATCGACATCCAC-3’ and 5’-TACCGGAGAGGATAGTAAACTCTTGTAG-3’), osteocalcin (5’-CTGTGTGGTGCTGTCACTGAC-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’), osteopontin (5’-AAGG-3’), bone sialoprotein (5’-GAAAGGTTTTCAGTCAGCAG-3’ and 5’-CTGTGTGGTGCTGTAC-3’), and actin (5’-AAGGAGGAGGATAGTAAACTCTTGTAG-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’).

Chromatin Immunoprecipitation Assays—The chromatin immunoprecipitation (ChIP) assays were performed in ROS17/2.8 cells as previously reported (66). Briefly, cross-linked lysates were immunoprecipitated with no antibody, anti-HA (12CA5), Runx2 (S19 and C19), or HDAC3 antibodies. An additional control reaction (mock) contained no chromatin or antibody. Nested PCR was performed with primer pairs generated to detect bases encompassing the proximal Runx2 binding site in the rat osteocalcin promoter. External primer pairs for PCR were: 5’-GGATTTAGTAGGTTTTTCC-3’ and 5’-CZAAGAGGCATGCT-GTGGTTG-3’. The internal primer pairs for the nested PCR were: 5’-GAGGGTTTCTTCTTCAAGTT-3’ and 5’-TGGGAAAGCGCTGG-AAGG-3’.

cDNA Synthesis and Reverse Transcription PCR—Total RNA from MC3T3 cells stably expressing Fli and HDAC3 shRNAs was isolated with TRizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed to cDNA with the Invitrogen Superscript kit according to its instructions. PCR was performed on the cDNA using primers specific for HDAC3 (5’-CGGAGGAGGGATTAGAATCTGAACTCAGC-3’ and 5’-TACACCAAGGAGGATAGTAAACTCTTGTAG-3’), Runx2 (5’-ATG-GATGACATAGACTGACATGGCC-3’ and 5’-TAGATTTCTAAAGGATGGGAAAC-3’), and actin (5’-GACTCTGATAGAGATGGCC-3’ and 5’-CAATGTCAGGATGTTGAC-3’).

Quantitative Real-time PCR—cDNA was prepared as described above. Real-time PCR was carried out in a Lightcycler (Roche Diagnostics) using the following primer sets: HDAC3 (5’-CCGCGTCTCACAATCGACATCCAC-3’ and 5’-TACCGGAGAGGATAGTAAACTCTTGTAG-3’), osteocalcin (5’-CTGTGTGGTGCTGTCACTGAC-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’), osteopontin (5’-AAGG-3’), bone sialoprotein (5’-GAAAGGTTTTCAGTCAGCAG-3’ and 5’-CTGTGTGGTGCTGTAC-3’), and actin (5’-AAGGAGGAGGATAGTAAACTCTTGTAG-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’).

Chromatin Immunoprecipitation Assays—The chromatin immunoprecipitation (ChIP) assays were performed in ROS17/2.8 cells as previously reported (66). Briefly, cross-linked lysates were immunoprecipitated with no antibody, anti-HA (12CA5), Runx2 (S19 and C19), or HDAC3 antibodies. An additional control reaction (mock) contained no chromatin or antibody. Nested PCR was performed with primer pairs generated to detect bases encompassing the proximal Runx2 binding site in the rat osteocalcin promoter. External primer pairs for PCR were: 5’-GGATTTAGTAGGTTTTTCC-3’ and 5’-CZAAGAGGCATGCT-GTGGTTG-3’. The internal primer pairs for the nested PCR were: 5’-GAGGGTTTCTTCTTCAAGTT-3’ and 5’-TGGGAAAGCGCTGG-AAGG-3’.

cDNA Synthesis and Reverse Transcription PCR—Total RNA from MC3T3 cells stably expressing Fli and HDAC3 shRNAs was isolated with TRizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed to cDNA with the Invitrogen Superscript kit according to its instructions. PCR was performed on the cDNA using primers specific for HDAC3 (5’-CGGAGGAGGGATTAGAATCTGAACTCAGC-3’ and 5’-TACACCAAGGAGGATAGTAAACTCTTGTAG-3’), Runx2 (5’-ATG-GATGACATAGACTGACATGGCC-3’ and 5’-TAGATTTCTAAAGGATGGGAAAC-3’), and actin (5’-GACTCTGATAGAGATGGCC-3’ and 5’-CAATGTCAGGATGTTGAC-3’).

Quantitative Real-time PCR—cDNA was prepared as described above. Real-time PCR was carried out in a Lightcycler (Roche Diagnostics) using the following primer sets: HDAC3 (5’-CCGCGTCTCACAATCGACATCCAC-3’ and 5’-TACCGGAGAGGATAGTAAACTCTTGTAG-3’), osteocalcin (5’-CTGTGTGGTGCTGTCACTGAC-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’), osteopontin (5’-AAGG-3’), bone sialoprotein (5’-GAAAGGTTTTCAGTCAGCAG-3’ and 5’-CTGTGTGGTGCTGTAC-3’), and actin (5’-AAGGAGGAGGATAGTAAACTCTTGTAG-3’ and 5’-GAGGAGGAGGATAGTAAACTCTTGTAG-3’).

Chromatin Immunoprecipitation Assays—The chromatin immunoprecipitation (ChIP) assays were performed in ROS17/2.8 cells as previously reported (66). Briefly, cross-linked lysates were immunoprecipitated with no antibody, anti-HA (12CA5), Runx2 (S19 and C19), or HDAC3 antibodies. An additional control reaction (mock) contained no chromatin or antibody. Nested PCR was performed with primer pairs generated to detect bases encompassing the proximal Runx2 binding site in the rat osteocalcin promoter. External primer pairs for PCR were: 5’-GGATTTAGTAGGTTTTTCC-3’ and 5’-CZAAGAGGCATGCT-GTGGTTG-3’. The internal primer pairs for the nested PCR were: 5’-GAGGGTTTCTTCTTCAAGTT-3’ and 5’-TGGGAAAGCGCTGG-AAGG-3’. 
same as described above. For all reactions, the temperature change rate was 20°C/s for denaturation, annealing, and elongation. Fluorescence was measured at channel F1 at the end of each elongation cycle. Relative quantification of gene expression was determined by using the 2^{-ΔΔCT} method where fold change in gene expression is relative to Day 0 F1 shRNA samples (67). All samples were normalized to actin.

**RESULTS**

The Amino Terminus of Runx2 Acts as an Autonomous Repression Domain That Is Sensitive to the HDAC Inhibitor, TSA—We previously showed that Runx2 contains multiple repression domains (18). That report focused on a potent repression domain in the Runx2 carboxy terminus (encompassing amino acids 383–513 of the MRIPV isoform), which interacts with TLE and HDAC6. We also showed that a truncated Runx2 protein (residues 1–383) lacking these repression domains suppressed transcription, albeit at lower levels. To identify the repression domain(s) within the first 383 residues of Runx2, we fused various Runx2 sequences to the GAL4 DNA binding domain (GAL) (Fig. 1B). NIH3T3 cells were transiently transfected with the GAL–Runx2 constructs and a reporter, GAL-TK-Luc, containing four GAL binding sites (Fig. 1A). Similar to our previously published results, GAL–Runx2-(1–513) repressed the promoter 6.2-fold, and GAL–Runx2-(1–383) repressed it 13.7-fold (Fig. 1C). Three Runx2 proteins ((1–327), (1–250), and (1–227)) lacking the activation domain also repressed transcription by 10.6-, 3.8-, and 3.3-fold, respectively. A GAL–Runx2 protein (321–383) containing just the Runx2 transactivation domain did not repress transcription (data not shown). These data demonstrate that Runx2 contains an autonomous repression domain within its amino terminus.

To determine if HDAC activity is involved in the repression by the amino terminus of Runx2, the HDAC inhibitor, trichostatin A (TSA), was added to the transcription assays (Fig. 1D). TSA partially inhibited the repression of Runx2-(1–383) and Runx2-(1–327), but completely reversed repression by Runx2-(1–250). These results suggest that an HDAC complex interacts with the first 250 residues of the Runx2 and contributes to Runx2-mediated transcriptional repression; however, HDAC-independent mechanisms also contribute to Runx2-mediated repression.

Runx2 Interacts with HDAC3—We used a candidate approach to identify HDACs that may interact with the amino terminus of Runx2. COS cells were co-transfected with pCMV5-GAL–Runx2-(1–227) and pCMV-FLAG-HDAC1, 2, 3, 4, 5, or 6. GAL–Runx2-(1–227) was used because it repressed as well as GAL–Runx2-(1–250) in repression assays (Fig. 1C) and was more stable than GAL–Runx2-(1–250) (Fig. 1B). Whole cell extracts were immunoprecipitated with anti-FLAG-conjugated agarose beads. The immunoprecipitates and a fraction of the whole cell extracts were separated by SDS-PAGE and immunoblotted with HDAC3 or β-catenin antibodies (loading control). GAL–Runx2-(1–227) protein (residues 1–227) interacted with HDAC3 complexes than with HDAC4 complexes, it is important to note that there is significantly less GAL–Runx2-(1–227) protein in the lysates of cells expressing HDAC4. In fact, all the HDACs tested, with the exception of HDAC3, blocked the expression of the GAL–Runx2 fusion protein. This likely occurs because the HDACs repress transcrip-

![Fig. 5. Suppression of HDAC3 by RNA interference prevents HDAC3-mediated repression of Runx2 activity. A, HDAC3 shRNA #1 suppresses HDAC3 expression. COS cells were transiently transfected with pCMV-HDAC3 and pSHAG-HDAC3 shRNA vectors #1 or #2. Lysates were collected, separated by SDS-PAGE, and immunoblotted with HDAC3 or β-catenin antibodies (loading control). B, HDAC3 suppression relieves HDAC3 suppression of Runx2 activity. These assays were performed as described in Fig. 4A except pSHAG-HDAC3 (1 μg) vectors were added. Cells were transfected with no HDAC3 shRNA (top panel), shRNA#1 (middle), or shRNA#2 (bottom).](image-url)
To gain a better understanding of the roles that HDAC3 and HDAC4 may play in regulating Runx2 in osteoblasts, we performed immunoblotting on protein extracts from preosteoblastic MC3T3 cells, the myogenic/osteoblast progenitor cell line, C2C12, and two rat osteosarcoma cell lines, ROS17/2.8 and UMR-106. HDAC3 was detected in each of the cell lines, but HDAC4 was detectable only in UMR-106 osteosarcoma cells (Fig. 3A). All of these cells express Runx2 (data not shown). Osteocalcin mRNA was detectable in all 5 cell lines in vitro (Fig. 3B). HDAC3 was present in high levels in the ROS17/2.8 cells, but not in the UMR-106 or C2C12 cells (data not shown).

Because HDAC3 was more broadly expressed than HDAC4 in this panel of cells, we decided to focus on its role in regulating Runx2 more closely for the rest of this study. Using immunoprecipitation from the rat osteosarcoma cell line ROS17/2.8, we ascertained that full-length Runx2 interacts with HDAC3 in vivo (Fig. 3B). HDAC3 was present in three Runx2 immune complexes that were collected with antibodies recognizing distinct epitopes of Runx2 (Fig. 3B), as well as in the HDAC3 immune complexes. HDAC3 was not present in the control immunoprecipitates. Together, these data demonstrate that HDAC3 interacts with Runx2 in vivo.

We further mapped the interaction domain of Runx2 with HDAC3 with in vitro pull-down experiments. Approximately equal amounts of recombinant GST-Runx fusion proteins (Fig. 2B, lower panel) or GST alone were incubated with in vitro transcribed and translated HDAC3. HDAC3 interacted with the GST-Runx2 proteins containing the amino-terminal residues (Fig. 2B, upper panel). These included GST-Runx2(1–117), GST-Runx2(1–227), and GST-Runx2(1–327). In contrast, HDAC3 did not interact with GST, GST-Runx2(94–927), or GST-Runx2(252–315). These data indicate that the extreme amino terminus (resides 1–117) of Runx2 is necessary and sufficient for the interaction with HDAC3. The runt domain (RunTD) of Runx1 is 93% identical to the RunTD of Runx1. Durst et al. (68) recently demonstrated that HDAC3 interacts with Runx1. To determine if HDAC3 interacts with this highly conserved domain, GST-Runx1(50–179) or GST-Runx1(50–179, L148D) were incubated with HDAC3-expressing reticulocyte lysates. The L148D mutation disrupts DNA binding (69). Neither interacted with HDAC3 (Fig. 2B); thus, RunTD does not contribute significantly to the interaction between Runx proteins and HDAC3.

**HDAC3 Represses Runx2-dependent Activation of the Osteocalcin Promoter**—The functional significance of the Runx2-HDAC3 interaction was determined by testing the effects of HDAC3 on Runx2 transcriptional activity. The osteocalcin promoter is a well-characterized target of Runx proteins and contains three binding sites for Runx2 (70, 71). COS cells were transiently transfected with an osteocalcin reporter plasmid (mOG2-luc) (72) and mammalian vectors expressing Runx2 and HDAC3. Cell lysates were collected, and luciferase assays were performed. Consistent with previous results from our laboratory and others, Runx2 (MRIPV isoform) activated the bone-specific osteocalcin promoter by 7.5-fold (Fig. 4A) (1, 73, 74). Similarly, the longer Runx2 isoform (MASNSL) activated the osteocalcin promoter by 11.5-fold (Fig. 4B, lane 6). HDAC3 did not affect the basal activity of the osteocalcin promoter but inhibited Runx2-dependent activation of this promoter in a concentration-dependent manner (Fig. 4, A and B). The repression was sensitive to the HDAC inhibitors, TSA and CHAP31 (Fig. 4B). These inhibitors modestly increased the basal activity of the promoter (lanes 1–5) and Runx2-dependent activation (lanes 6–8). Importantly, both inhibitors completely reversed HDAC3-mediated repression of Runx2 activity (lanes 12–15); although, high concentrations of CHAP31 (100 nM) were slightly toxic. These data demonstrate that HDAC activity is required for HDAC3-mediated repression of Runx2.

To confirm that HDAC3 is required for the repression, shRNAs that suppress HDAC3 expression by RNA interference were added to the transcription assays. We created four shRNAs for the murine HDAC3 cDNA and subcloned them into the pSHAG vector. In transient expression assays, only one shRNA (denoted here as #1) suppressed HDAC3 levels in COS7 cells (Fig. 5A). A second shRNA (#2) that did not affect HDAC3 levels was used as a control for these experiments. In the absence of any HDAC3 shRNAs, Runx2 (MRIPV isoform) activated the osteocalcin promoter 8-fold, and HDAC3 blocked this activation as previously shown (Fig. 5B, top). Addition of HDAC3 shRNA #1 prevented HDAC3-mediated repression of Runx2 (Fig. 5B, middle). In contrast, HDAC3 shRNA #2, which does not suppress HDAC3 expression, did not reverse HDAC3-mediated repression (Fig. 5B, bottom). Together, these data demonstrate that HDAC3 represses Runx2-dependent activation of the osteocalcin promoter.

**HDAC3 Associates with the Osteocalcin Promoter**—Having determined that HDAC3 is expressed in osteoblasts, binds to Runx2, and represses Runx2-dependent activation of the osteocalcin promoter, we next asked if HDAC3 is associated with the osteocalcin promoter in vivo. To examine this, chromatin immunoprecipitation (ChIP) experiments were performed in ROS17/2.8 cells. Cross-linked and fragmented DNA-protein complexes were immunoprecipitated with either no antibody, anti-HA antibodies, Runx2 antibodies (S19 or C19), or anti-HDAC3 antibodies. Nested PCR analysis on purified DNA was performed with primers that span the proximal Runx2 binding site in the osteocalcin promoter. As shown in Fig. 6, osteocalcin promoter DNA was amplified in samples collected with Runx2 or HDAC3 antibodies (Fig. 6). There was little or no amplification in the mock sample (no chromatin input) and samples collected with beads alone (no antibody) or the anti-HA antibodies (nonspecific control). These data demonstrate that HDAC3 can interact with the region of the osteocalcin promoter containing a Runx2 binding site in the osteocalcin promoter. As shown in Fig. 6, osteocalcin promoter DNA was amplified in samples collected with Runx2 or HDAC3 antibodies (Fig. 6). This allowed us to determine the functional significance of HDAC3 on osteoblast differentiation. HDAC3 expression was suppressed in MC3T3 cells using RNA interference. The HDAC3 shRNAs...
were placed into a pSIN-MSCV-Puro vector and stably introduced into MC3T3 cells by retroviral transduction. HDAC3 protein levels in MC3T3 cells expressing shRNA #1 were less than 50% of those in cells expressing Ffl shRNA (Fig. 7A). HDAC3 mRNA levels were down-regulated by -5-fold in undifferentiated MC3T3 cells (Fig. 7, B and E) and remained low in differentiating populations. Runx2 mRNA levels were unchanged (Fig. 7B). We did not observe any drastic changes in cell proliferation or growth as a result of this level of HDAC3 suppression (data not shown). We also did not observe any changes in the levels of a double strand RNA response gene (oligoadenylate synthetase 1) as a result of shRNA production (data not shown). When the MC3T3 cells expressing shRNAs for HDAC3 or Ffl were placed in an in vitro differentiation assay, no gross changes in the rate of alkaline phosphatase production, which is a relatively early marker of osteoblast differentiation, were seen (Fig. 7C). In contrast, matrix mineralization and nodule formation occurred 2–3 days earlier in HDAC3-suppressed cells as compared with control cells (Fig. 7D). To determine whether genes that are both regulated by Runx2 and involved in mineralization were also expressed earlier in HDAC3-suppressed cells, we collected RNA at time points up to the onset of mineralization (day 6) and performed quantitative PCR. HDAC3 expression in MC3T3 cells containing shRNA #1 was down-regulated at day 0 and remained suppressed at days 4 and 6. Consistent with our ChIP and functional assays, osteocalcin mRNA was increased at day 4 in HDAC3-suppressed cells relative to MC3T3 cells expressing Ffl shRNA (Fig. 7E). Low levels of osteocalcin transcripts were detected in undifferentiated cells. By day 6, osteocalcin transcripts were expressed at equal levels in the control and HDAC3-suppressed cells. Relatively similar results were seen with osteopontin and bone sialoprotein, although bone sialoprotein transcripts were not detected in undifferentiated cells (75, 76). Thus, HDAC3 suppression accelerates osteoblast mineralization and the expression of various genes that are up-regulated during osteoblast differentiation. These data suggest that HDAC3 plays a role in regulating the timing of osteoblast differentiation, perhaps by directly regulating Runx2 activity.

**DISCUSSION**

In this report, we demonstrate that HDAC3 regulates Runx2 trans-activity and contributes to in vitro osteoblast mineralization. Our studies show that HDAC3 interacts with the amino terminus of Runx2 to regulate osteoblast-specific gene expression. A physical interaction between Runx2 and HDAC3 was demonstrated in vivo using co-immunoprecipitation. Functional interactions between these proteins were also observed in in vitro transcription assays. Additional studies indicated that Runx2 and HDAC3 co-localize to similar subnuclear structures (data not shown). We mapped the interaction to the amino-terminal 117 residues of Runx2, which contains an autonomous repression domain. To date, HDAC3 is the first protein shown to interact with the amino terminus of Runx2. Our biochemical studies were performed with the Runx2 isoform beginning with amino acids MRIPV; however, functional studies demonstrated that HDAC3 represses this isoform as well as the alternative isoform starting with residues MASNSL (Fig. 4B). Thus HDAC3 can regulate both major Runx2 isoforms. The region of the Runx2 amino terminus that is shared by both isoforms contains a glutamine-alanine (QA) repeat region (residues 35–80). It is possible that the QA domain contributes to interactions with HDAC3. However, Runx1 also interacts with HDAC3 but does not contain a QA-rich region (68). Further structural and functional examinations of this domain will be important, because it is altered in a small percentage of cleidocranial dysplasia patients and in individuals with varying bone densities (5, 8).

HDAC3 suppressed Runx2-dependent activation of the osteocalcin promoter in transiently transfected cells. This repression was sensitive to the HDAC inhibitors, TSA and CHAP31. In addition, the activity was specific to HDAC3, because shRNAs directed against HDAC3 eliminated the repression. Previous studies implicated histone acetyltransferases in activation of the osteocalcin promoter (26) and described the amino terminus, including the QA-rich region, as an activation domain in the context of the entire protein but not autonomously (77). This is the first study to identify a HDAC as a co-repressor of the osteocalcin promoter. Our results may indicate that the activity of this region depends on interactions with specific cofactors. Moreover, these studies support the hypothesis that chromatin remodeling is essential for the regulation of the osteocalcin promoter (78, 79). The one or more signals that switch Runx2 from an activator to a repressor remain to be identified. An interesting possibility is that cellular signaling cascades targeting Runx2 (80) will affect its interactions with co-repressors. Runx2 is a phosphoprotein (80), and phosphorylation of specific residues may dictate interactions with cofactor complexes. Phosphorylation of JNK and c-Jun relieve interactions between c-Jun- and HDAC3-containing repression complexes (81). Because the AP-1 complex cooperates with Runx2 to activate osteoblast genes (20, 82), it is also a strong candidate to indirectly regulate HDAC3 interactions with Runx2. Of interest, Imai and colleagues (83) recently demonstrated that interactions between mSin3A and Runx1 (AML1) are relieved by Runx1 phosphorylation. The serine residues that are modified to control mSin3a interactions are conserved in Runx2. Current studies in our laboratory are focusing on their roles in regulating the interactions between Runx2 and co-repressors, including HDAC3.

Although mammalian Runt domain proteins are best known for their ability to activate gene expression, their *Drosophila* homologue Runt was originally described as a transcriptional repressor (84). Within the last 3 years, the underappreciated repression capabilities of mammalian Runt domain proteins have been identified and numerous mechanisms of repression reported. Runt domain proteins interact with a variety of transcriptional co-repressors to regulate gene expression (18, 28). Each family member interacts with the Groucho/TLE and mSin3A proteins (28). Runx1 interacts with at least six HDACs (68). We have shown that Runx2 interacts with HDAC3,

---

Fig. 7. HDAC3 accelerates later stages of osteoblast differentiation. A, stable suppression of HDAC3 by RNA interference in MC3T3 cells. Whole cell lysates (75 μg) were prepared from undifferentiated MC3T3 cells stably expressing Ffl or HDAC3 shRNAs and immunoblotted with HDAC3 and β-catenin antibodies. B, HDAC3 suppression does not alter Runx2 expression in stable MC3T3 cell lines. Reverse transcription-PCR was performed on RNA collected from undifferentiated MC3T3 cells stably expressing Ffl or HDAC3 shRNAs. PCR was performed using primers recognizing the indicated cDNA. C, HDAC3 suppression does not alter alkaline phosphatase expression. MC3T3 cells stably expressing Ffl and HDAC3 shRNAs were differentiated in the presence of ascorbic acid and β-glycerol phosphate. Alkaline phosphatase staining was performed on the indicated days. D, HDAC3 suppression accelerates Ca2+ accumulation in the matrix. Alizarin red staining was performed on differentiating MC3T3 cells on the indicated days. E, HDAC3 suppression accelerates osteocalcin, osteopontin, and bone sialoprotein expression. Real-time PCR was performed on cDNA from differentiated MC3T3 cells stably expressing either Ffl or shRNA #1 on the indicated days. Gray bars represent Ffl shRNA-expressing cells, and black bars represent HDAC3 shRNA-expressing cells, the asterisk indicates a p value of <0.05, between Ffl and HDAC3 shRNA cells at that time point, as determined by unpaired t-testing.
HDAC6, and possibly HDAC4 (this report and Ref. 18). It remains to be conclusively determined if these co-repressor complexes interact with Runx factors as part of unique or shared co-repressor complexes. It is appreciated that HDACs are the enzymatic components of large multiprotein complexes that interact with many transcription factors. HDAC3 complexes contain nuclear receptor co-repressors, SMRT, and N-CoR (53, 56). It was also reported that HDAC3 is the required enzymatic component of complexes containing Class II HDACs, HDAC4, -5, and -7 (57, 58). Interactions between HDAC3 and Class II HDACs (e.g. HDAC6) were not addressed in these studies. Our data also indicate that their expression patterns are different in that HDAC3 is strictly nuclear and expressed throughout osteoblast differentiation (data not shown), whereas HDAC6 shuttles from cytoplasm to the nucleus and is up-regulated during late stages of differentiation (18). Therefore, we currently favor the hypothesis that HDAC3 and HDAC6 regulate Runx2 activities in different contexts. Although it is almost certain that Runx2 co-repressor complexes contain many co-repressors, more work needs to be done to define the spatial and temporal organization of these complex components during osteoblast differentiation. The physical and functional relationships between Runx2 and HDAC3 may be important for regulating bone formation. HDAC3 is expressed in C2C12 cells, which have osteogenic potential upon BMP2 stimulation, MC3T3 preosteoblasts, and two osteosarcoma cell lines. Suppression of HDAC3 by RNA interference in MC3T3 cells caused early expression of osteocalcin, osteopontin, and bone sialoprotein, and consequently accelerated a late stage event in osteoblast differentiation, namely calcium deposition in the mineralizing matrix. An earlier marker of osteoblast maturation, alkaline phosphatase production, was not affected by HDAC3 suppression. Interestingly, the dissociation of HDAC3 from peroxisome proliferator-activated receptor-γ-retinoblastoma protein complexes by retinoblastoma protein phosphorylation in adipocytes stimulated their differentiation (52). Thus, HDAC3 may be a broad regulator of cellular maturation and terminal differentiation. Of note, general suppression of HDAC activity in tumor cells facilitates their differentiation and several small molecule HDAC inhibitors are in phase I or II clinical trials (49, 50). Our data showing that HDAC3 binds the osteocalcin promoter and directly regulates the activity of Runx2 suggests that HDAC3 may play a crucial role in regulating osteoblast differentiation. These results suggest that suppressing HDAC3 activity may be a mechanism of increasing bone formation.

Acknowledgments—We thank Drs. Andre van Wijnen and Soraya Gutierrez Gallegos for helpful discussions and technical assistance.

REFERENCES
1. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Cell 89, 747–754.
2. Enomoto, H., Enomoto-Iwamoto, M., Iwamoto, M., Nomura, S., Hiemeno, M., Komori, T., Yagi, R., Chen, L. F., Shigesda, K., Murakami, Y., and Ito, Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11590–11595
3. Narlikar, G., Fan, J. H., and Kingston, R. E. (2002) Cell 108, 475–487.
4. Hwang, C. A., and Schreiber, S. L. (1997) Curr. Opin. Chem. Biol. 1, 300–308.
5. Gronstein, M. (1997) Nature 389, 349–352.
6. Hon, L., Schroth, G. P., Matthews, H. R., Yau, P., and Bradbury, E. M. (1993) J. Biol. Chem. 268, 365–371.
7. Gray, S. G., and Eckstrom, T. J. (2001) Exp. Cell Res. 262, 75–83.
8. Lues, G., Lu, C., Li, S., and Partridge, N. C. (2002) J. Biol. Chem. 277, 15254–15264.
Verdin, E. (2002) Mol. Cell 9, 45–57
59. Glaser, K., Li, B. J., Slavter, M. J., Wei, R.Q., Albert, D. H., and Davidsen, S. K. (2003) Biochem. Biophys. Res. Commun. 310, 529–536
60. Ishizuka, T., and Lazar, M. A. (2003) Mol. Cell. Biol. 23, 5122–5131
61. Yang, W. M., Yao, Y.-L., Sun, J.-M., Davis, J. R., and Seto, E. (1997) J. Biol. Chem. 272, 28001–28007
62. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002) Genes Dev. 16, 948–958
63. Hemann, M. T., Fridman, J. S., Hernando, E., Paddison, P. J., Cordon-Cardo, C., Hannon, G. J., and Lowe, S. W. (2003) Nature 33, 396–400
64. Koka, S., Neudauer, C. L., Li, X., Lewis, R. E., McCarthy, J. B., and Westendorf, J. J., (2003) J. Cell Sci. 116, 1745–1755
65. Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988) Gene (Amst.) 66, 1–10
66. Shen, J., Montecino, M., Lian, J. B., Stein, G. S., van Wijnen, A. J., and Stein, J. L. (2002) J. Biol. Chem. 277, 20284–20292
67. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
68. Durst, K., Lutterbach, L. B., Kummalue, T., Friedman, A. D., and Hiebert, S. W. (2003) Mol. Cell. Biol. 23, 607–619
69. Lenex, N., Meyers, S., and Hiebert, S. W. (1995) Oncogene 11, 1761–1769
70. Geoffroy, V., Ducy, P., and Karsenty, G. (1995) J. Biol. Chem. 270, 30973–30979
71. Merriman, H. L., van Wijnen, A. J., Hiebert, S., Bidwell, J. P., Fey, E., Lian, J., Stein, J., and Stein, G. S. (1995) Biochemistry 34, 13125–13132
72. Ducy, P., and Karsenty, G. (1995) Mol. Cell. Biol. 15, 1858–1869
73. Banerjee, C., Hiebert, S. W., Stein, J. L., Lian, J. B., and Stein, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4968–4973
74. Kahler, R. A., and Westendorf, J. J. (2008) J. Biol. Chem. 278, 11937–11944
75. Sad, M., Murri, E., Komori, T., Kawahata, H., Sugimoto, M., Terui, K., Shimizu, H., Yasui, T., Ogihara, H., Yasui, N., Ochi, T., Kitamura, Y., Ito, Y., and Nomura, S. (1998) Oncogene 17, 1517–1525
76. Javed, A., Barnes, G. L., Jasanyu, B. O., Stein, J. L., Gerstenfeld, L., Lian, J. B., and Stein, G. S. (2001) Mol. Cell. Biol. 21, 2891–2905
77. Thirunavukkarasu, K., Mahajan, M., McLaren, K. W., Stifani, S., and Karsenty, G. (1998) Mol. Cell. Biol. 18, 4197–4208
78. Montecino, M., Lian, J., Stein, G., and Stein, J. (1998) Biochemistry 35, 5093–5102
79. Gutierrez, J., Sierra, J., Medina, R., Puchi, M., Imashchenetsky, M., van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. (2002) Biochem. 39, 13565–13574
80. Franceschi, R. T., and Xiao, G. (2003) J. Cell Biol. 88, 446–454
81. Weiss, C., Schneider, S., Wagner, E. P., Zhang, X., Seto, E., and Bohmann, D. (2003) EMBO J. 22, 3686–3695
82. Hess, J., Porte, D., Munz, C., and Angel, P. (2001) J. Biol. Chem. 276, 20025–20036
83. Inai, Y., Kurokawa, M., Yamaguchi, Y., Izutsu, K., Nitta, E., Mitani, K., Satake, M., Noda, T., Ito, Y., and Hirai, H. (2004) Mol. Cell. Biol. 24, 1033–1043
84. Manoukian, A. S., and Krause, H. M. (1993) Development 118, 785–796
