Journal of Cellular Biochemistry 93:807–818 (2004)

Regulation of RANKL Promoter Activity Is Associated With Histone Remodeling in Murine Bone Stromal Cells

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Abstract Receptor activator of NFκ-B ligand (RANKL) is essential for osteoclast formation, function, and survival. Although RANKL mRNA and protein levels are modulated by $1,25(OH)_2D_3$ and other osteoactive factors, regulatory mechanisms remain unclear. In this study, we show that 2 kb or 2 kb plus exon 1 of a RANKL promoter sequence conferred neither $1,25(OH)_2D_3$ response nor tissue specificity. The histone deacetylase inhibitors trichostatin A (TSA) and sodium butyrate (SB), however, strongly increased RANKL promoter activity. A series of 5′-deleted RANKL promoter constructs from 2,020 to 110 bp showed fourfold increased activity after TSA treatment. TSA also dose dependently enhanced endogenous RANKL mRNA expression with 50 μM of TSA treatment causing equivalent RANKL expression to that seen with 1 nM $1,25(OH)_2D_3$. Using a chromatin immunoprecipitation (ChIP) assay we showed that TSA significantly enhanced association of both acetylated histone H3 and H4 on the RANKL promoter, with H4 > H3. A similar increase in acetylated histone H4 on the *RANKL* gene locus was seen after $1,25(OH)_2D_3$ treatment, but ChIP assay did not reveal localization of VDR/RXR heterodimers on the putative VDRE of the RANKL promoter. To explore the role of H4 acetylation of $1,25(OH)_2D_3$ stimulated RANKL, we added both TSA and $1,25(OH)_2D_3$ together. While the combination further increased acetylation of H4 on the RANKL locus, surprisingly, TSA inhibited $1,25(OH)_2D_3$-induced RANKL mRNA expression by 70% at all doses of $1,25(OH)_2D_3$ studied. These results suggest that TSA increases of endogenous expression of RANKL involve enhanced acetylation of histones on the proximal RANKL promoter. Preventing deacetylation, however, blocks $1,25(OH)_2D_3$ action on this gene. Chromatin remodeling is therefore involved in RANKL expression. J. Cell. Biochem. 93: 807–818, 2004.

Key words: histone H4; histone deacetylase inhibitors; ChIP assay; ST-2 cells

RANKL is the most potent osteoclastogenic molecule described. Its expression as a membrane anchored protein on the surface of osteoblasts and stromal cells in bone [Lacey et al., 1998; Yasuda et al., 1998], and as a secreted protein from T-lymphocytes [Weitzmann et al., 2001] and cancer cells [Zhang et al., 2001] serves to recruit and activate osteoclasts in bone initiating osteoclastogenic bone resorption. Despite the burgeoning and still growing literature regarding the role of RANKL in bone remodeling [Boyle et al., 2003], the regulation of RANKL expression has remained an insoluble problem.

RANKL is predominantly regulated at a transcriptional level. Many investigations have recorded the induction of RANKL expression by skeletally active agents including vitamin D [Yasuda et al., 1998], parathyroid hormone (PTH) [Lee and Lorenzo, 1999, 2002], nitric oxide [Fan et al., 2004] and IGF-I [Rubin et al., 2002a]. Mechanical stimulation, which represses osteoclastogenesis in vitro and in vivo [Kanematsu et al., 2002], also inhibits RANKL expression without changing RANKL mRNA stability [Rubin et al., 2002b]. Kitazawa et al. [1999], documented a weak regulation of the first 1,000 base pairs of the RANKL promoter sequence by osteotropic agents. Since that time, however, little convincing data has emerged identifying promoter elements that respond

Grant sponsor: NIAMS; Grant number: AR42360; Grant sponsor: VA Merit Review.

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Received 30 April 2004; Accepted 7 June 2004
DOI 10.1002/jcb.20217

robustly enough to underwrite the significant changes in mRNA steady state levels.

Indeed, evaluation of RANKL promoter activity and regulation, including sequence well beyond that first studied [Kitazawa et al., 1999] has not given up many secrets. Although RANKL expression is tissue-restricted, at least under normal physiological conditions, to bone cells and some cells of the immune system [Yasuda et al., 1998; Weitzmann et al., 2000], this specificity is not apparent in up to 7 kb of nucleotide sequence proximal to the transcriptional start site [O'Brien et al., 2002]. While regulatory promoter elements may be present at distances greater than 2 kb from the coding sequence for RANKL, it is clear that this exquisitely responsive gene, which is completely silent in most tissues, and silent until exposed to several resorptive hormones in bone cells, does not follow the usual patterns of regulation via promoter elements. The fact that the gene is generally repressed may suggest that chromatin remodeling is involved in its regulation.

In the study here, we present evidence that the RANKL promoter is sensitive to acetylation of histones. Trichostatin A (TSA), a histone deacetylase inhibitor, increases RANKL promoter activity as well as expression of the endogenous gene. Furthermore, $1,25(OH)_2D_3$, a potent stimulator of RANKL expression, also causes association of acetylated histone with the proximal RANKL promoter region. Surprisingly, TSA significantly inhibits $1,25(OH)_2D_3$-induced *RANKL* gene expression. These data suggest that modifications involving acetylation are critical to regulation of RANKL expression, causing increased activity at a weak proximal promoter site, and decreased activity at the $1,25(OH)_2D_3$ responsive site.

MATERIALS AND METHODS

Reagents

$1,25(OH)_2D_3$ (1,25 vitamin D3) was obtained from Biomol (Plymouth Meeting, PA). Histone deacetylase (HDAC) inhibitors, sodium butyrate (SB), and TSA were purchased from Sigma (St. Louis, MO). Anti-acetylated histone H3 and H4 antibodies and the chromatin immunoprecipitation (ChIP) assay kit were purchased from Upstate (Lake Placid, NY). Anti-VDR and RXR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All cell culture media were from Invitrogen (Carlsbad, CA). All restriction endonucleases were obtained from Promega (Madison, WI).

Cell Cultures

The ST-2 bone stromal cell line (Riken Cell Bank, Tsukuba Science City, Japan) was plated in α-MEM with 10% fetal bovine serum and antibiotics at 50,000 cells/6-well plates. For experiments using primary stromal cells, bone marrow was obtained from tibiae and femurs of 3–5 weeks old male C57BL/6 mice, and plated at 15 million cells per 6-well plates as previously described [Rubin et al., 2000]. After 1 h incubation to remove macrophages, non-adherent cells containing stromal cells were transferred to new 6-well plates. Twenty-four hours later, all non-adherent cells were discarded and the remaining stromal cells cultured for 1 week for experiments. The Institutional Animal Review Board approved all animal protocols.

RANKL Deletion Constructs

Using mouse genomic DNA (Genewalker Kit, BD Biosciences, Palo Alto, CA), we PCR-cloned 2,020 base pairs of the proximal RANKL promoter and subcloned these into the Bgl II and Hind III sites of the pGL3-basic vector (Promega). Deletion constructs of the RANKL promoter were made by digesting the pGL3-RANKL (−2,020) plasmid with the following enzymes and re-ligating: pGL3-RANKL (−1,550) (Nhe I + Nco I); pGL3-RANKL (−965) Bgl II; pGL3-RANKL (−725) (Nhe I + Afl II); pGL3-RANKL (−515) (Nhe I + Pvu II). Promoter deletion constructs pGL3-RANKL (−220), pGL3-RANKL (−150), and pGL3-RANKL (−110) were constructed by amplifying the small promoter segment and subcloning it into pGL3. The construct pGL3-RANKL (2K + exon1) was prepared by amplifying RANKL exon1 and cloning it into the Hind III site of pGL3-RANKL (−2,020).

Transient Transfection of Promoter-Reporter Constructs

ST-2 cells were seeded at a density of 150,000/well in 6-well plates. After 18–20 h, cells were co-transfected with 1.6 μg/well of RANKL promoter constructs with 0.1 μg of β-galactosidase (β-gal) vector and 8 μl of Lipofectamine (LPT, Invitrogen) in the absence of serum. Five hours later, an equal volume of medium with serum was added to bring the final concentration of serum to 10%. The next day, media was

changed and HDAC inhibitors were added to cultures as specified.

Luciferase Assay

The Promega Luciferase assay system was used to assess luciferase activity. Briefly, reporter lysis buffer (RLB) was added and incubated at room temperature for 15 min before centrifugation to remove cell debris. Twenty microliters of cell lysate was mixed with 100 μl of luciferase substrate and light emission measured with the LumiCount Luminometer (Packard, Downers Grove, IL). In each experiment, cell lysates were also measured for β-gal activity to allow for normalization of luciferase activity. To normalize pGL3-RANKL luciferase activity, ST-2 cells were co-transfected with a β-gal reporter construct. After 48 h of post-transfection, cell lysates were assayed. Twenty microliters of cell lysates with 100 μl of Galacton-Star substrate (Applied Biosystems, Bedford, MA) diluted 1:50 of the Reaction Buffer Diluent were added to each well and incubated for 40 min. Light emission was measured with the LumiCount Luminometer (Perkin-Elmer Life Sciences, Boston, MA).

Real-Time PCR for RANKL mRNA

Analysis of RANKL and 18S mRNA was performed using the iCycler (BioRad, Hercules, CA) as previously described [Fan et al., 2004]. Reverse transcription of 1 μg total RNA in 20 μl of reaction was performed with random decamers (Ambion, Austin, TX) and superscript II reverse transcriptase (Invitrogen). Amplification reactions were performed in 25 μl containing primers at 0.5 μM and dNTPs (0.2 mM each) in PCR buffer and 0.03 U Taq polymerase (Invitrogen) along with SYBR-green (Molecular Probes, Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 10–10,000 fold for 18S and 5–625 fold for RANKL to generate relative standard curves to which sample cDNA was compared. For RANKL, forward and reverse primers were 5′-CCA AGA TCT CTA ACA TGA CG-3′ and 5′-CAC CAT CAG CTG AAG ATA GT-3′, respectively creating a product of 140 bp. For 18S, an amplicon of 345 was generated with forward primer 5′-GAA CGT CTG CCC TAT CAA CT-3′and reverse 5′-CCA AGA TCC AAC TAC GAG CT-3′. Standards and samples were run in triplicate. Dilution curves showed that PCR efficiency was more than 95% for all species studied. RANKL was normalized for amount of 18S in the same RT sample, which was also standardized on a dilution curve from RT sample [Johnson et al., 2000].

ChIP Assay

ChIP assays were performed utilizing the Upstate Protocol with modifications. Briefly, ST-2 cells and primary bone stromal cells were fixed with 1% formaldehyde for 10 min at 37°C. Cells were washed and collected in ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 μg/μl aprotinin, and 1 μg/μl pepstatin A). Cell pellets were re-suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, with protease inhibitors as described above) with each 200 μl sample containing $\sim 10^6$ cells. Samples were incubated for 10 min on ice and sonicated at 15 W with 10 s bursts for three rounds. The supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 150 mM NaCl) containing protease inhibitors after removing cell debris. Twenty microliters aliquots of the diluted supernatant (the total chromatin fraction as INPUT) were incubated overnight at 65°C to reverse crosslinks. To reduce nonspecific background, the diluted soluble chromatin fractions were pre-cleared with Salmon Sperm DNA/Protein A Agarose-50% Slurry for 30 min at 4°C. The supernatant fraction was collected and transferred to a new tube with 5 μl of anti-acetyl-Histone H4 antibody or 10 μl of anti-acetyl-Histone H3 antibody overnight at 4°C. The immune complexes were added 60 μl of Salmon Sperm DNA/Protein A Agarose-50% Slurry for 1 h at 4°C and the supernatant was discarded. The beads were washed with the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The histone complexes were eluted in freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) and the histone-DNA crosslinks were reversed by overnight incubation at 65°C. The DNA from these samples was extracted through phenol/chloroform and ethanol-precipitated with 20 mg/ml glycogen. The DNA extracted was then

dissolved in 30 μl of H_2O. This same procedure was also followed for the input (in 100 μl H_2O). Minor modifications of this procedure, as described by Yamamoto et al. [2003] were utilized to immunoprecipitate the VDR and RXR DNA complexes.

PCR was utilized to analyze the DNA fragments from ChIP assays. For reaction, 3 μl of assayed DNA sample and 5 μl of input/start material were used in each 25 μl reaction. The PCR was run 30 s at 94, 55, and 72°C of each cycle for 28–30 cycles. Four sets of RANKL primer located in promoter region (distal set: upstream −1,834/−1,595 and proximal set: upstream −147/+1) and coding regions (exon 2 and 5) were used (Fig. 1). The four pairs of RANKL primer were: distal promoter pair: forward, 5′-GCA ACT GGC CTC ATT GGT GG-3′; reverse, 5′-CAC AAG GCA TTC ATT GGT CA-3′; proximal promoter pair: forward, 5′-GTC CCT GAG AGC GAA GAA GA-3′, reverse, 5′-GTA CCC CAG GCA GCC CTA-3′; exon 2 pair: forward, 5′-GAC TCC ATG AAA ACG CAG GT-3′, reverse, 5′-AGG CTT GTT TCA TCC TCC TG-3′; exon 5 pair: forward, 5′-ATC ATG AAA CAT CGG GAA GC-3′, reverse, 5′-AGT TTT TCG TGC TCC CTC CT-3′. Primer pairs in VDRE site for CYP24, osteopontin (OPN) and RANKL were as described in Yamamoto et al. [2003]. The PCR products were separated in 15% polyacrylamide or agarose gels and visualized by silver or ethidium bromide staining (BioRad). Some samples were also quantified with real-time PCR by using same primer sets shown in Figure 1.

RESULTS

Inhibition of HDAC Activity Induces RANKL mRNA Expression in ST-2 Cells

HDAC inhibitors can enhance the transcriptional activity of many genes [Maeda et al., 2000; Walker et al., 2001]. To determine the effect of HDAC inhibitors on RANKL expression in bone cell, we treated the murine bone stromal cell line ST-2 with TSA for 24 h. As shown in Figure 2, TSA caused a dose-dependent increase of RANKL mRNA expression reaching a plateau at 100 nM TSA with an increase of nearly 15-fold. This effect of 50 nM TSA was similar to the increase in RANKL observed after treatment with 1 nM $1,25(OH)_2D_3$.

RANKL Promoter Constructs Are Sensitive to HDAC Inhibitors but not to $1,25(OH)_2D_3$

To investigate whether the HDAC inhibitor TSA induced RANKL mRNA expression by directly altering promoter activity, we tested the effect of TSA on RANKL promoter constructs in ST-2 cells. ST-2 cells were transiently transfected with pGL3-Luc reporter constructs driven by either the RANKL 2,020 bp promoter or the 2,020 bp promoter with the addition of the exon 1 coding region (−2,020 to +384 bp). As shown in Figure 3A, the activity of both RANKL promoter constructs were significantly increased by treatment with 25 nM TSA. $1,25(OH)_2D_3$, known to be a strong stimulator for RANKL expression at both mRNA and protein levels [Yasuda et al., 1998] has been shown to exhibit a weak although positive effect on RANKL

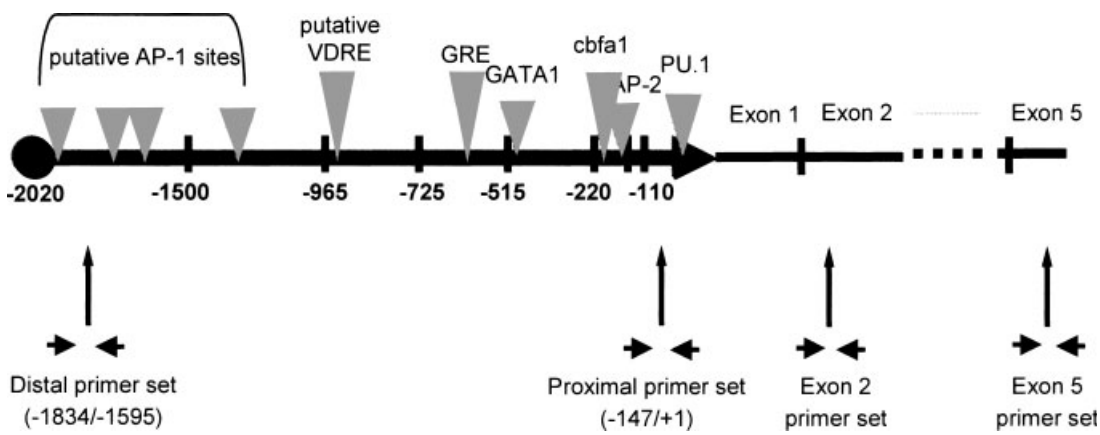

Fig. 1. Primer sets used for chromatin immunoprecipitation (ChIP) assay. PCR primer sets used for the detection of RANKL DNA fragments in ChIP assays are illustrated.

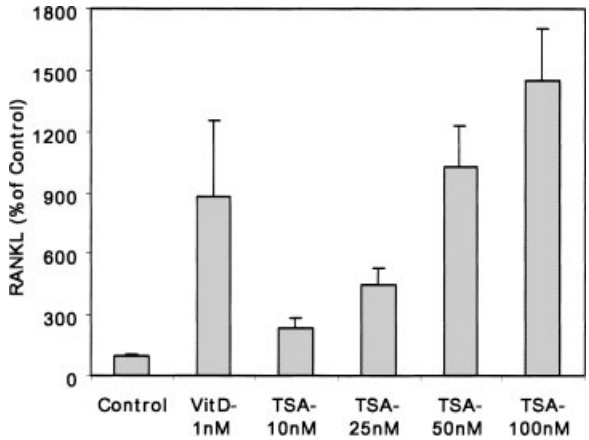

Fig. 2. Inhibition of histone deacetylase (HDAC) by trichostatin A (TSA) increases endogenous RANKL expression. ST-2 cells were treated with $1,25(OH)_2D_3$ for 48 h or TSA for the last 24 h of culture. Total RNA was isolated and assessed for RANKL and 18S mRNA by RT-PCR. Basal expression of RANKL mRNA/18S (control) is shown as 100%. Results represent compiled data from seven experiments.

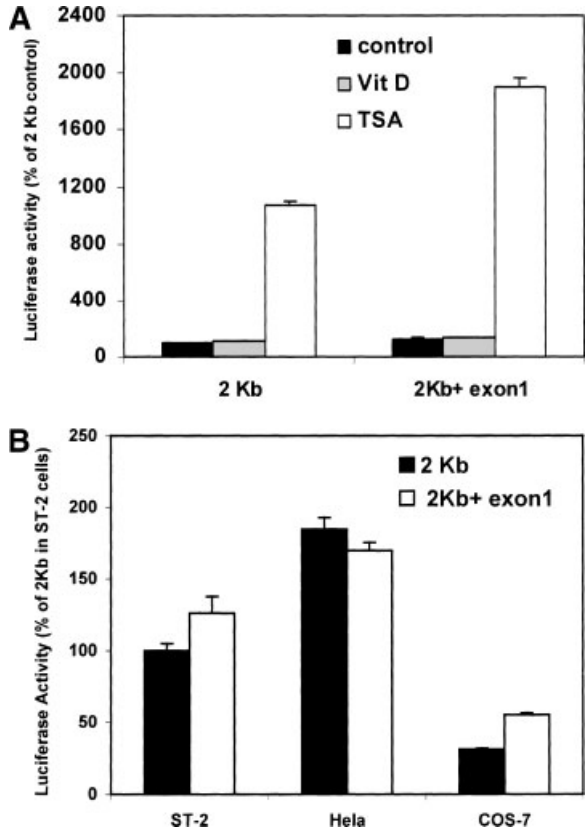

Fig. 3. Transiently transfected RANKL promoter constructs are neither hormone-responsive nor tissue specific. **A**: Transiently transfected RANKL promoter construct activity does not respond to $1,25(OH)_2D_3$ but is stimulated by TSA. ST-2 cells were transiently transfected with pGL3-RANKL (∼−2 kb) or pGL3-RANKL (−2 kb + exon 1) as well as a β-galactosidase (β-gal) reporter construct and treated with $1,25(OH)_2D_3$ for 48 h or TSA for 24 h. Forty-eight hours post-transfection, luciferase and β-gal assays were performed. Results represent compiled data from two experiments. **B**: RANKL promoter construct activity is not bone specific. pGL3-RANKL (∼−2 kb) or pGL3-RANKL (−2 kb + exon 1) were transiently transfected into ST-2, HeLa or COS-7 cells. Luciferase activities, with the 2 kb RANKL/ β-gal basal activity as 100%, are shown. Results represent compiled data from two experiments.

promoter activity [Kitazawa et al., 1999, 2003]. We found no response of the 2,020 bp RANKL promoter construct to $1,25(OH)_2D_3$ treatment in ST-2 cells even with inclusion of the exon 1 coding region despite the exquisite responsiveness of endogenous RANKL to $1,25(OH)_2D_3$ (Fig. 3A). Furthermore, neither of the two RANKL promoter constructs were tissue specific: as shown in Figure 3B these constructs were expressed in HeLa cervical carcinoma cells as well as in COS-7 primate kidney cells. As the data is shown as luciferase normalized with concurrent transfected β-gal activities, it is apparent that the transcriptional activity of the RANKL promoter is at least as active in HeLa cells as in bone cells, with substantial expression as well in COS-7 cells. These data confirm previous observations demonstrating that the RANKL promoter containing 7 kb of upstream coding sequence was also not repressed in COS-7 cells [O'Brien et al., 2002].

We next mapped the positive TSA response in the upstream 2,020 bp RANKL promoter by examining the activity of serial 5′-deletion constructs. As shown in Figure 4A, basal promoter activity (compared to the 2,020 bp RANKL promoter construct) was retained until sequence was deleted below −220 base pairs from transcription start site. The promoter construct containing −965 bp upstream of the transcription start site manifested the highest activity. Importantly, TSA increased luciferase activity by 7–12 fold in constructs containing −515 bp of upstream promoter sequence or more. Those RANKL constructs containing less than −515 bp upstream from the transcription start site also showed response to TSA: TSA treatment increased promoter activity by four-fold promoter in the −110 bp RANKL promoter, not significantly different from TSA induction of the −150, −220, or −965 bp promoter constructs (Fig. 4B). Since the TSA response was conserved in the −110 bp promoter construct, we examined TSA dependent responses in three short

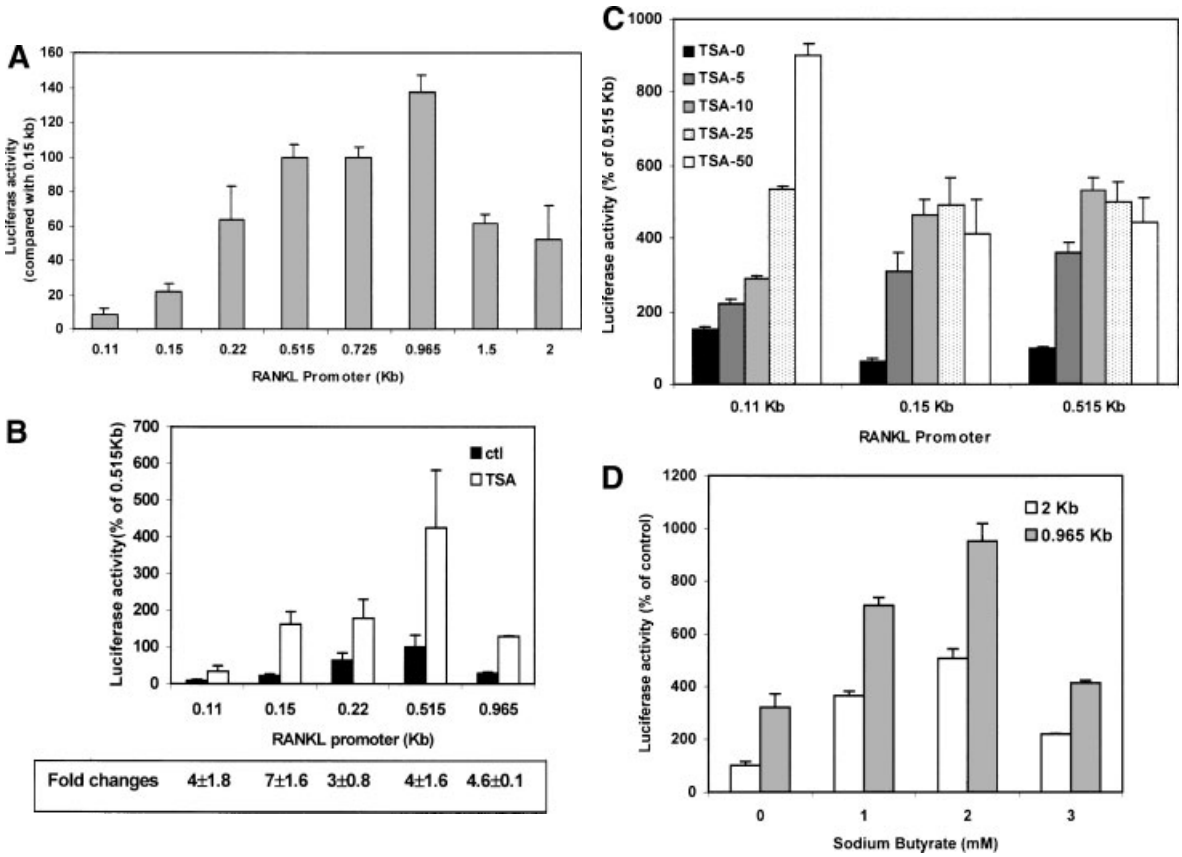

Fig. 4. TSA stimulates activity of 5′-deleted RANKL promoter constructs. A series of RANKL promoter constructs were prepared from the original construct containing −2,020 bp of sequence upstream from the transcription start site. Deletion constructs were transiently transfected into ST-2 cells and luciferase assay performed 48 h later. All experiments have been replicated at least three times. **A**: Basal activities of RANKL promoter deletion constructs compared to the basal activity of the −515 bp RANKL promoter at 100%. **B**: TSA (25 nM) treatment increased luciferase activities of RANKL promoter deletion constructs. The highest basal activity in this representative experiment was seen in the −515 bp RANKL promoter, here shown as 100%; basal activities of other constructs are compared to this as shown in the black bars. The addition of TSA, shown in the open bars, increased the activity of all RANKL promoter constructs equivalently. Fold induction due to TSA is shown below the graph. **C**: TSA induction of RANKL promoter activity was dose-dependent in three 5′-deleted RANKL promoter constructs shown in the figure. **D**: Sodium butyrate (SB) mimicked TSA-induced RANKL promoter activity in a dose dependent fashion. The open bars show activity of the −965 bp RANKL promoter construct and the gray bars that of the −2,020 bp construct. All activities are compared to the −2,020 bp construct (100%) in the absence of TSA.

promoters (−110, −150, and −515 kb) as a function of the TSA concentration. As shown in Figure 4C, the induction of luciferase activity by TSA increased in a dose-dependent manner up to 50 nM for all three constructs. Indeed, the −110 bp promoter exhibited the strongest dose response to TSA stimulation.

We also examined the effects of SB, another HDAC inhibitor. SB has been reported to up-regulate the activity of the transiently transfected IGFBP3 promoter [Walker et al., 2001; Tsubaki et al., 2002]. Results in Figure 4D show that SB also induces the −965 and −2,020 RANKL promoter constructs in a dose dependent manner, falling back at 3 mM. Thus, these two HDAC inhibitors are able to strongly stimulate the activity of RANKL promoter constructs in bone cells.

TSA Induced RANKL Gene Expression Causes Increased Acetylation of Histone H3 and H4 on the RANKL Promoter Sequence

We next investigated whether changes in acetylation of histone modulate *RANKL* gene expression: ChIP was performed with ST-2 cells using antibodies against acetylated histone H3 and H4. ChIP results were quantitated by real-time PCR using three pairs of primers spanning

the *RANKL* gene as indicated in Figure 1 (−1,834 to −1,595 bp, −147 to +1 and exon 2). First we examined the association of acetylated histone with the portions of the distal promoter (−1,834/−1,595) and proximal RANKL promoter (−147/+1). As shown in Figure 5, antibodies against acetylated histone H3 and H4 precipitated fragments from the RANKL promoter region from both control and TSA treated cells. However, the level of histone acetylation on the RANKL promoter was increased when cells were treated with TSA. Levels of RANKL promoter fragment associated with acetylated histone H4 were significantly higher than their association with acetylated histone H3 throughout the RANKL promoter region (Fig. 5A). The RANKL (−147/+1)/INPUT ratio assayed by real-time PCR is summarized in Figure 5B. Quantitatively, the addition of 25 nM TSA significantly increased acetylated H4∼RANKL promoter association. The increase in acetylation of H3 on the RANKL promoter was significantly less than that of H4-AC when assayed quantitatively: TSA increased the acetylation of H4 by 294-fold and H3 by 12.6-fold compared to cells not treated with TSA and immunoprecipitated with the same antibodies. Signals were weak or absent when precipitations were carried out in the absence of antibody.

To assess levels of TSA enhanced histone acetylation across the length of the *RANKL* gene, we carried out ChIP analyses with four PCR primers using antibody against acetylated histone H4 since acetylated histone H4 (H4-AC) showed a stronger association with the *RANKL* gene than did acetylated histone H3 (H3-AC). Here again, the RANKL promoter sequence was associated with acetylated histone H4 in TSA treated cells when the primers amplified −1,834 to −1,595 and −147 to +1 (Fig. 5C). There was also a TSA induced increased in acetylation of histone H4 associated with coding sequence representing both exon 2 and exon 5, suggesting that a large portion of the *RANKL* gene, including both promoter and coding sequence, is wrapped along chromatin.

TSA induction of acetylation of histone H4 on the RANKL promoter was also dose dependent. Figure 6A shows an acrylamide gel with samples treated with TSA (0–100 nM). Amplicons of RANKL promoter sequences revealed increased association with the immunoprecipitated H4-AC in response to increasing doses of TSA. This response was quantitated with PCR

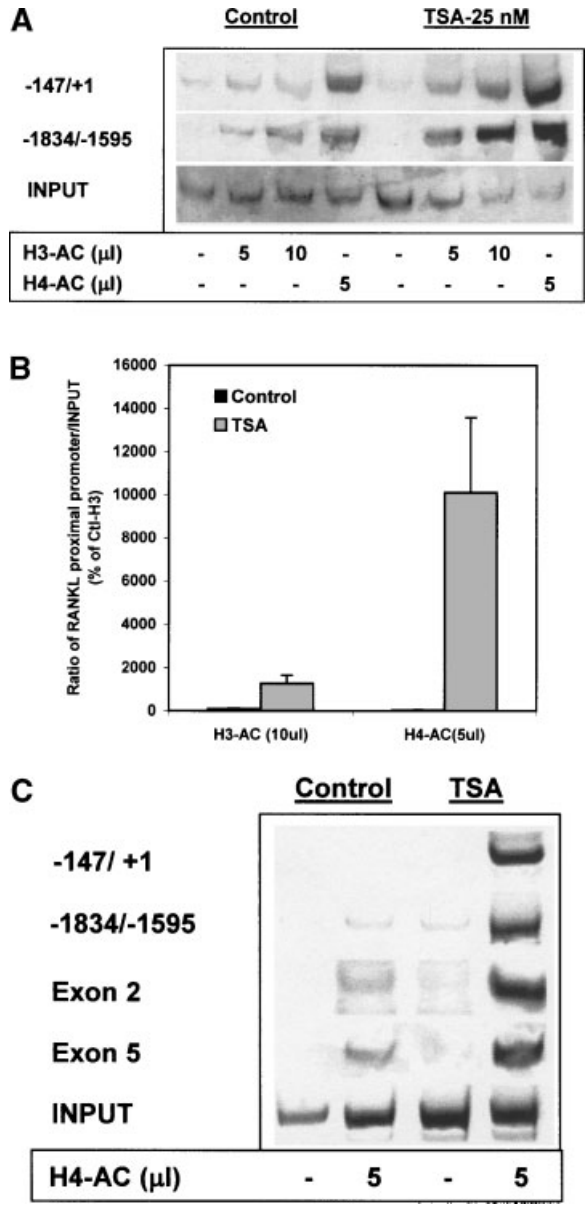

Fig. 5. ChIP analysis of acetylation of histones H3 and H4 on the RANKL promoter. ST-2 cells were treated with 25 nM of TSA for 6 h. ChIP assay was performed utilizing anti-acetylated histone H3 and H4 antibodies. **A**: ST-2 cell lysates were immunoprecipitated with antibodies to acetylated histone H3 and H4. DNA fragments from ChIP assays were PCR'd using RANKL promoter primer sets. TSA increased acetylation of both histone H3 (H3-AC) and H4 (H4-AC) on the RANKL promoter. Input for each reaction is shown in the bottom silver stain gel to assess sample loading. This experiment was confirmed two times. **B**: Precipitated DNA was quantitatively assessed by real-time PCR using the primer set reading −147 to +1 of the RANKL promoter. Data are shown as percentage increase compared to non-TSA treated cells immunoprecipitated with the H3-AC or H4-AC antibody. TSA increased the acetylation of H4 on the RANKL promoter more strongly than that of H3. **C**: Primers for coding regions in both exon 2 and exon 5 were also studied after ChIP with the antibody to H4-AC. The figure shows that TSA increases acetylation of H4 along the RANKL locus.

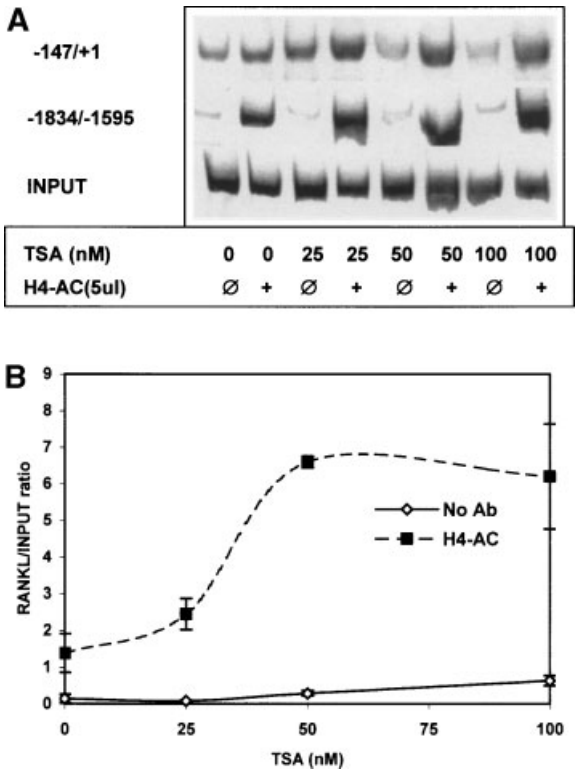

Fig. 6. ChIP analysis shows a dose dependent effect of TSA to increase acetylation of histone H4 on the RANKL promoter. ST-2 cells were treated with different doses of TSA for 6 h. ChIP was performed with anti-acetylated histone H4 antibody. **A**: The figure shows a dose dependent increase in acetylation of H4 on promoter region of the *RANKL* gene. Input is shown in the bottom silver stained row. **B**: Real-time PCR was performed after ChIP using distal RANKL promoter primers (−1,834/−1,595). TSA dose-dependently increased the acetylation of H4 on the RANKL promoter.

as shown in Figure 6B using primers for the −1,834 to −1,595 region. Here, the effect of TSA was clearly shown to be dose dependent, increasing from 25 to a plateau at 50 nM. These data indicate that agents that decrease HDAC activity promote increased acetylation of histone H4 within the RANKL promoter region.

$1,25(OH)_2D_3$ Mimics TSA Effect on the Proximal Promoter Association With H4, but has no Effect on the Putative $1,25(OH)_2D_3$ Response Element

We next explored the relationship between vitamin D and a putative $1,25(OH)_2D_3$ response element (VDRE) in the RANKL promoter (upstream of −937 to −922 bp) with a ChIP assay that used an antibody against vitamin D receptor (VDR) as bait. Previous studies indicate that RANKL is regulated by $1,25(OH)_2D_3$ and indeed our studies (Fig. 2) support this view. $1,25(OH)_2D_3$ failed to induce transfected promoter constructs, however, suggesting a surprising complexity. In order to explore the effects of $1,25(OH)_2D_3$, we used ChIP assays to assess the effects of $1,25(OH)_2D_3$ or VDR/RXR localization and chromatin histone modification. Interestingly, ChIP analysis using antibodies to the VDR or RXR did not reveal localization of the heterodimer to the region at −935 of the RANKL promoter proposed to mediate $1,25(OH)_2D_3$ action. This lack of VDR and RXR binding was in contrast to the strong localization of the two proteins to VDRE-containing regions of both the *Cyp24* and *OPN* genes promoters described previously [Yamamoto et al., 2003] (Fig. 7A).

In view of the above, we next examined the effects of $1,25(OH)_2D_3$ on histone acetylation.

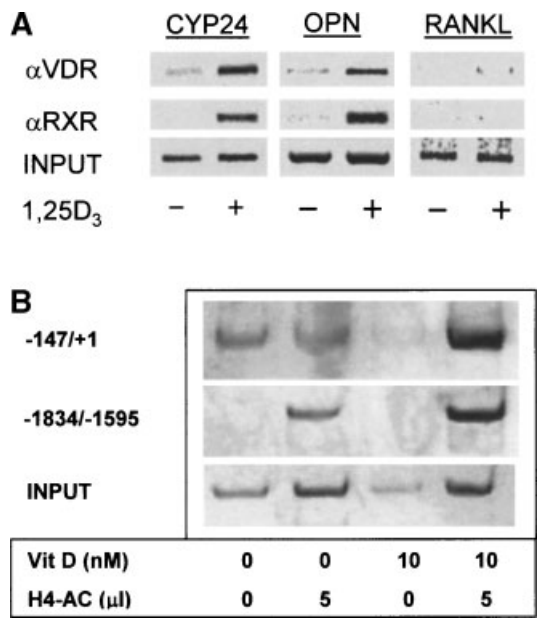

Fig. 7. Vitamin D-stimulated RANKL expression involves chromatin remodeling. ST-2 cells were treated with 10 nM $1,25(OH)_2D_3$ for 24 h and subjected to ChIP analyses. **A**: ChIP analysis of $1,25(OH)_2D_3$ stimulated ST-2 cells does not reveal localization of VDR/RXR heterodimers on the putative VDRE of the RANKL promoter. Chromatin was immunoprecipitated with antibodies to VDR or RXR and the precipitated DNA analyzed primers containing the VDRE site(s) of CYP24 and OPN or the putative VDRE site in the RANKL promoter. $1,25(OH)_2D_3$ stimulated the binding of VDR/RXR heterodimers to both VDRE-containing regions of the CYP24 and OPN promoters but not to the putative VDRE in the RANKL promoter. **B**: Cell lysates were subjected to ChIP analysis using antibodies against acetylated H4. $1,25(OH)_2D_3$-induced RANKL increased acetylation of histone H4 on the RANKL promoter.

It has been reported that histone acetylation at the osteocalcin locus is functionally linked to $1,25(OH)_2D_3$ [Shen et al., 2002]. We therefore explored whether $1,25(OH)_2D_3$ also caused an increase in acetylated histone H4 in the RANKL promoter, in a fashion similar to that of TSA. ST-2 cells were treated with $1,25(OH)_2D_3$ for 24 h and cell lysates subjected to ChIP using antibody to acetylated histone H4. $1,25(OH)_2D_3$ caused an increase in acetylated histone H4 on the proximal RANKL promoter (Fig. 7B).

TSA Inhibits $1,25(OH)_2D_3$-Induced *RANKL* Gene Expression

In an effort to learn whether $1,25(OH)_2D_3$ induction of RANKL mRNA expression involved increased histone H4 acetylation of the proximal promoter, we asked whether the effects of TSA and $1,25(OH)_2D_3$ on endogenous RANKL mRNA expression were additive. Both agents were administered simultaneously with the concentration of $1,25(OH)_2D_3$ at increasing levels. As shown in Figure 8A, RANKL expression increased in response to $1,25(OH)_2D_3$ in a dose dependent manner. The addition of TSA, however, had an unexpected effect: TSA inhibited $1,25(OH)_2D_3$-induced RANKL expression at all concentrations examined.

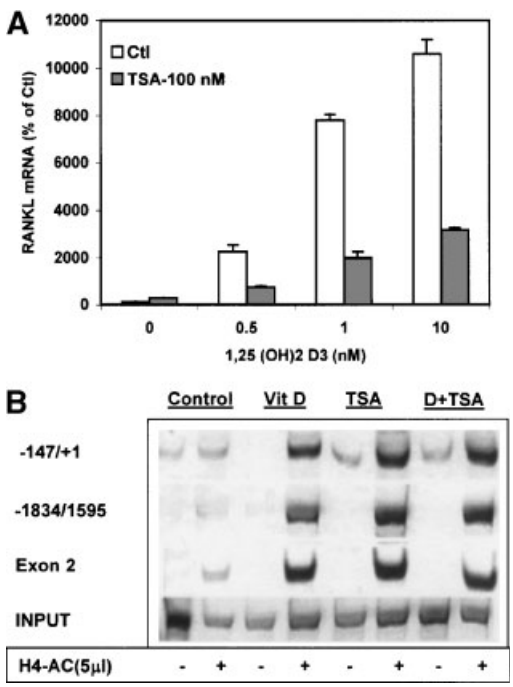

Fig. 8. TSA inhibits $1,25(OH)_2D_3$-induced RANKL expression. ST-2 cells were treated with $1,25(OH)_2D_3$ alone ± TSA for 24 h. **A**: $1,25(OH)_2D_3$ dose dependently increased RANKL mRNA expression. However, $1,25(OH)_2D_3$-induced RANKL mRNA was reduced by ~70% in the presence of 100 nM TSA at all doses of $1,25(OH)_2D_3$ studied. **B**: Cell lysates were subjected to ChIP assay. The association of acetylated histone H4 with RANKL promoter was strongly enhanced when ST-2 cells were treated with TSA and $1,25(OH)_2D_3$ alone or with the combination of both.

We therefore asked whether the acetylation of histone H4 in the RANKL promoter was altered in ST-2 cells treated with both agents using ChIP analyses. As shown in Figure 8B, $1,25(OH)_2D_3$ and TSA exerted additive effects to increase acetylated histone H4 association on the RANKL promoter and coding region fragments. These data suggest that the site through which $1,25(OH)_2D_3$ interacts at the RANKL promoter is distant from the region that is acetylated at H4.

DISCUSSION

The expression of Receptor Activator of NF-κB ligand (RANKL) on stromal/osteoblastic cells is essential for osteoclast formation and bone remodeling. RANKL in osteoblastic cells is up-regulated by various hormones and cytokines such as 1,25-dihydroxyvitamin D_3, parathyroid hormone (PTH), glucocorticoids, interleukins, prostaglandin E2, TNF-α, TGF-β, and IGF-I [Lacey et al., 1998; Kanematsu et al., 2000; Fu et al., 2002; Ishida et al., 2002; Lee and Lorenzo, 2002; Rubin et al., 2002a]. Inhibitors of RANKL expression have also been described, including nitric oxide [Fan et al., 2003, 2004] and mechanical force, as would be consistent with positive, rather than negative, bone remodeling [Rubin et al., 2000; Kanematsu et al., 2002]. Kitazawa et al. [1999] cloned the 5′-flanking basic promoter region of mouse *RANKL* gene and explored its activity and sensitivity to several of these factors. The basic RANKL promoter (upstream −1,000) was thought to be composed of an inverted TATA-box (−28), an inverted CAAT-box (−60), three putative Runx2 binding sites and direct repeats of the putative VDRE and GRE half-sites (−935 and −640, respectively). They reported that $1,25(OH)_2D_3$ or dexamethasone increased RANKL promoter driven luciferase activity by about twofold in ST-2 cells. This level of regulation was inconsistent with $1,25(OH)_2D_3$'s ability to strongly increase *RANKL* gene expression. It should be noted, however, that the putative RANKL VDRE diverged from the classical VDRE since the two half-sites were separated by four base pairs rather than three base pairs, which

would be more typical of a thyroid response element.

Despite the above, induction of the RANKL promoter by $1,25(OH)_2D_3$ has not been reproduced. We show here that $1,25(OH)_2D_3$ treatment, which is accompanied by large increases in RANKL expression, has no effect on the activity of 2 kb of RANKL promoter sequence. Indeed, localization of the VDR/RXR heterodimer to the RANKL promoter was also not observed while a strong association of VDR/RXR proteins with known regions containing the VDREs of the CYP24 and osteopontin genes was concurrently demonstrated. The 2,020 bp RANKL promoter was also not tissue specific, exhibiting strong basal activity in both HeLa and COS-7 cells. These findings confirm those of O'Brien et al. [2002], who failed to show a significant regulation by $1,25(OH)_2D_3$ of the RANKL promoter containing up to 7,000 of upstream sequence. Since the putative VDRE did not bind the VDR as shown in Figure 7A, these data would explain the lack of response of the −2,020 bp constructs to $1,25(OH)_2D_3$ (Fig. 3A). Clearly the proximal promoter region contains neither the vitamin D responsive region nor the region responsible for tissue specificity.

Kitazawa et al. have suggested that CpG methylation around the transcription start site of the mouse *RANKL* gene might be involved in silencing gene expression [Kitazawa et al., 1999; Kitazawa and Kitazawa, 2002], providing an indirect mechanism by which RANKL expression could be controlled. CpG methylation-dependent repression occurs when alterations in chromatin structure are mediated by interaction with methyl-CpG-binding proteins (MeCP) [Bhakat and Mitra, 2003]. Nan et al. [1998] found that the MeCP associates with a co-repressor complex containing repressor mSin3A and HDACs and mediates transcriptional repression. TSA relieves the transcriptional repression by inhibiting the action of HDACs, indicating that the deacetylation of histones is an essential component of repression mechanisms involving MeCPs [Nan et al., 1998]. Thus, we wondered whether chromatin remodeling might offer clues to the regulation of the RANKL promoter.

Chromatin structure plays an important role in regulating many DNA-mediated processes, including transcription, replication, and recombination [Shen et al., 2002; Soutoglou and Talianidis, 2002; Khanna-Gupta et al., 2003]. In its natural state, DNA is packaged around a set of highly conserved histone proteins (H1, H2A, H2B, H3, and H4). The packaging of DNA with histones and other non-histone proteins results in a condensed chromatin state that is inaccessible to the transcriptional machinery [Sterner and Berger, 2000]. Posttranslational modification of these packaging proteins, including acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation on the "tail" domains of histones is now known to affect gene transcription—essentially converting transcriptionally inactive chromatin to transcriptionally competent chromatin. In general, increases in histone acetylation have been associated with an open chromatin conformation and enhanced gene expression, e.g., the chromatin histone regulating expression of mouse *Igf2r* gene [Yang et al., 2003]. Recent studies have shown that acetylation of histones H3 and H4 is functionally coupled to the histone remodeling events that mediate the developmental induction of osteocalcin gene transcription by $1,25(OH)_2D_3$ in rat osteoblast-like cells [Shen et al., 2003]. Our data suggest that histone acetylation is likely involved in modulating RANKL expression.

In our study, the HDAC inhibitor TSA dose-dependently induced RANKL mRNA expression in bone stromal cells to levels comparable to those induced by 1 nM $1,25(OH)_2D_3$. Both TSA and SB, a second HDAC inhibitor, also significantly increased RANKL promoter activity, although the transfected promoter construct was insensitive to $1,25(OH)_2D_3$. Promoter deletion studies revealed that the proximal −110 base pairs of the promoter region were sufficient for TSA responsivity. To further clarify the link between RANKL promoter regulation and chromatin structure, ChIP assays were employed to assess the effect of TSA on RANKL histone acetylation. Four sets of primers spanning *RANKL* gene: an upstream pair (−1.9 kb), a pair close to the transcription start site (−147 to +1) and two primer pairs recognizing sequences in exon 2 and 5 were used to localize the acetylation of H4 on the RANKL promoter. Our data revealed that TSA dose-dependently increased RANKL promoter acetylation at histone H3 and H4. Indeed, H4 acetylation was much more profound that that observed for H3 along the *RANKL* gene locus. Differential association of gene promoters with specific histones has been shown previously: H4 was more susceptible to

TSA modulation than histone H3 in the differentially methylated region (DMR2) of *Igf2r* gene while histone H3 is more susceptible than histone H4 in DMR1 promoter region of *Igf2r* gene [Yang et al., 2003]. Our data suggest that TSA strongly increases the acetylation of histones on the RANKL promoter, thus increasing transcriptional competency of the *RANKL* gene.

Shen et al. [2002] showed that acetylated histone H3 and H4 were associated with the osteocalcin promoter only when the osteocalcin gene was active, suggesting that acetylation of histone H3 and H4 is functionally coupled to chromatin remodeling and essential for $1,25(OH)_2D_3$-induced osteocalcin gene expression in bone cells. Acetylated histone H4 rather than acetylated histone H3 was uniformly associated with broad regions of the osteocalcin locus and was correlated with $1,25(OH)_2D_3$ upregulation of this gene [Shen et al., 2002, 2003]. We also found that $1,25(OH)_2D_3$ alone could increase acetylation of histone H4 in the RANKL promoter. Indeed, ChIP analysis revealed that $1,25(OH)_2D_3$, TSA or the combination had similar effects to enhance this modification of histone H4 in the RANKL promoter region.

However, the induction of RANKL by $1,25(OH)_2D_3$ diverges from those histone associations seen in the osteocalcin promoter. Accordingly TSA, while inducing RANKL mRNA expression alone, strongly inhibited $1,25(OH)_2D_3$ induction of RANKL expression at all doses of the hormone studied. Since the H4 acetylation on the proximal RANKL promoter was further enhanced by both TSA and $1,25(OH)_2D_3$, these data indicate that $1,25(OH)_2D_3$'s effect is exerted at an independent site. Indeed, the fact that TSA inhibits $1,25(OH)_2D_3$ activity by 70% could be interpreted to indicate that $1,25(OH)_2D_3$ activity requires a deacetylation rather than an acetylation step. This interpretation varies from accepted dogma since histone deacetylation has been traditionally associated with the repression of gene expression. Recently, however, De Nadal et al. [2004] investigated transcription of genes induced by osmolar stress through the Hog1 mitogen-activated protein kinase in yeast. High osmolarity rapidly activates Hog1, causing the upregulation of several genes required to withstand the environmental stress. The gene activation involves recruitment of a specific histone deacetylase complex (Rpd3-Sin3), which leads to histone deacetylation and entry of RNA polymerase II [De Nadal et al., 2004]. While the $1,25(OH)_2D_3$ responsive region in the RANKL promoter has not yet been identified, it is possible that histone deacetylation may be required to activate the *RANKL* gene. Thus, the $1,25(OH)_2D_3$ effect on chromatin remodeling in the *RANKL* gene will require further investigation.

In summary, the HDAC inhibitor TSA has a significant effect on the endogenous expression of RANKL that appears to involve enhanced acetylation of histones on the proximal RANKL promoter sequence. In the $1,25(OH)_2D_3$ stimulated state, however, TSA mediated inhibition of HDAC represses the $1,25(OH)_2D_3$ effect. Thus, histone acetylation has weak effects on the proximal RANKL promoter both endogenously and on a transfected reporter construct. In the presence of $1,25(OH)_2D_3$, however, the major effect of preventing histone deacetylation is to inhibit RANKL expression. Our data suggests that $1,25(OH)_2D_3$ has as yet unexplored effects that may involve activation of histone deacetylases.

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