Sp1/Sp3 and PU.1 Differentially Regulate β5 Integrin Gene Expression in Macrophages and Osteoblasts*

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Mature osteoclast precursors and osteoblasts express the integrin αvβ5, the appearance of which on the cell surface is controlled by the β5, and not the αv, subunit. Here, we show that a 173-base pair proximal region of the β5 promoter mediates β5 basal transcription in macrophage (osteoclast precursor)-like and osteoblast-like cells. DNase I footprinting reveal four regions (FP1–FP4) within the 173-base pair region, protected by macrophage nuclear extracts. In contrast, osteoblast nuclear extracts protect only FP1, FP2, and FP3. FP1, FP2, and FP3 bind Sp1 and Sp3 from both macrophage and osteoblast nuclear extracts. FP4 does not bind osteoblast proteins but binds PU.1 from macrophages. Transfection studies show that FP1 and FP2 Sp1/Sp3 sites act as enhancers in both MC3T3-E1 (osteoblast-like) and J774 (macrophage-like) cell lines, whereas the FP3 Sp1/Sp3 site serves as a silencer. Mutation of the FP2 Sp1/Sp3 site totally abolishes promoter activity in J774 cells, with only partial reduction in MC3T3-E1 cells. Finally, we demonstrate that PU.1 acts as a β5 silencer in J774 cells but plays no role in MC3T3-E1 cells. Thus, three Sp1/Sp3 sites regulate β5 gene expression in macrophages and osteoblast-like cells, with each element exhibiting cell-type and/or activation-suppression specificity.

Recognition of bone matrix proteins by osteoblasts and osteoclast precursors profoundly affects their differentiation and function. Such interactions are mediated principally by integrins, which are heterodimeric transmembrane glycoproteins consisting of α and β chains (1–3).

The osteoclast is a physiological polykaryon derived by fusion of macrophages in a process apparently requiring attachment of the mononuclear precursors to bone matrix (4, 5). The integrin αvβ5 is expressed predominantly in mature osteoclasts and plays a critical role in osteoclastic bone resorption (6–10). In contrast, the integrin αvβ5, although structurally related to αvβ3 and sharing many of the same target ligands (11), is expressed on rodent osteoclast precursors but not on mature bone-resorbing polykaryons (11). Murine osteoclast precursors utilize integrin αvβ5 and not αvβ3 for attachment to matrix (12).

Given these facts, it is not surprising that αvβ5 and αvβ3 levels rise and fall, respectively, during osteoclast differentiation (12).

Osteoclasts express a variety of integrins (13–17) that interact with bone matrix proteins to prompt commitment to the bone synthesizing phenotype. Thus, human osteoclasts adhere to vitronectin in an Arg-Gly-Asp (RGD)-dependent manner (14) and an RGD peptide inhibits matrix mineralization in vitro (18, 19). Whereas osteoclasts at the bone surface express high levels of αvβ5, αvβ1, and αvβ14 (14, 20), adhesion of osteoclasts to vitronectin is mediated specifically by αvβ5.1

Sp1, a ubiquitously expressed transcription factor containing three zinc finger motifs, Cys2-His2, which bind the consensus sequence GGGCGGGGCG (21), regulates a various genes in a constitutive or an inducible manner (22, 23). Three Sp1-related proteins, Sp2, Sp3, and Sp4, have also been cloned (22). Although all four molecules have similar structural features, including highly conserved DNA binding domains, Sp1, Sp3, and Sp4 share closest homology (22). Although Sp3 is widely expressed and can activate or repress gene expression (24, 25), Sp4 is solely a transcriptional activator, found only in specific brain cells (26).

PU.1, a member of the E twenty-six family of transcription factors, is expressed in macrophages, B cells, mast cells, and neutrophils (27, 28). E twenty-six family proteins are characterized by a DNA binding domain that recognizes purine-rich sequences, typically containing a 5'-GGAA-3' core. PU.1 is necessary for both normal myelopoiesis (29, 30) and osteoclast differentiation (31).

Given that the β5 integrin subunit is expressed in both osteoclast precursors and osteoblasts, we turned to the molecular mechanism by which the gene is regulated in these two important cell types. Taking advantage of our recently cloned murine integrin β5 gene promoter (32), we identified three Sp1/Sp3 sites in a 173-bp β5 proximal promoter region and established that they mediate basal transcription in osteoblasts. Each of the Sp1/Sp3 sites plays a different role in this process. The first Sp1/Sp3 site (−53 to −48) acts as weak enhancer, the second site (−26 to −17) serves as a strong enhancer, and the third (−29 to +36) represses transcription. The same three Sp1/Sp3 regulate the β5 promoter in macrophages in a manner similar to that in osteoblasts. In contrast to its typical enhancer function, a downstream PU.1 site (+73 to +84), which is functional only in osteoclast precursors, serves as a silencer of the β5 promoter.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pGL3-basic plasmid, a promoterless luciferase construct, was purchased from Promega (Madison, WI). A 1-kb β5
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promoter-luciferase construct (pGL3-1kb(+) and its deletion mutants (pGL3–796), pGL3–633), pGL3–483), pGL3–340), pGL3–172), and pGL3–63) were prepared as in our previous study (32). Briefly, in pGL3–1kb(+), a nearly 1-kb proximal region (from –875 to +110) of the β5 promoter containing the transcriptional start site (which was designated as the 5′ end) in the luciferase reporter plasmid was cloned into the pGL3-basic plasmid. All mutants were made by deleting the 5′ end of the 1-kb promoter fragment in the pGL3–1kb(+) in a progressive fashion (32). Therefore, these constructs contain promoter fragments with the same 3′ ends (+110) and different 5′ ends (the locations of these different 5′ ends are indicated by the numbers in the parenthesis of the mutant constructs). In the latter study, three reporter constructs (Mu-1, Mu-2, Mu-3, Mu-12, Mu-13, Mu-23, Mu-123, and Mu-4) as detailed below, under “Site-directed Mutagenesis.”

Cell Culture and Transfection—The mouse macrophage cell line J774 (J774A.1) and a mouse pre-osteoblast cell line, MC3T3-E1, were cultured in minimum essential medium α modification (Sigma) containing 10% heat-inactivated fetal bovine serum from Life Technologies, Inc. Cells were transfected with LipofectAMINE Plus™ reagent (Life Technologies, Inc.) as follows. One day prior to transfection, J774 cells were scraped off tissue culture dishes with Cell Lifters (Costar, Corning, NY), whereas MC3T3-E1 cells were lifted by trypsinization. The cells were counted and replated in six-well plates (5 × 105 cells/well). The next day, every well was treated with a mixture prepared as follows. 1 μg of reporter plasmid and 1 μg of cytomegalovirus β-galactosidase (β-gal) plasmid was diluted with 30 μl of Opti-MEM (Life Technologies, Inc.) and mixed with the Plus reagent to form a pre-complex at room temperature for 15 min. The pre-complex was then mixed and incubated with the LipofectAMINE reagent (diluted with Opti-MEM) at room temperature for 15 min to form DNA Plus LipofectAMINE, which was added to each well. The complex was mixed gently into the medium and incubated at 37 °C at 5% CO2 for 4 h. Then, the medium was replaced with minimum essential medium α modification containing 10% fetal bovine serum and incubated at 37 °C at 5% CO2. 24 h later, cells were washed with phosphate-buffered saline twice, lysates were prepared, and luciferase activity was measured using the luciferase assay kits from Promega (Madison, WI), with normalizations to β-gal activity measured separately.

Nuclear Extract Preparation—Nuclear extracts used for both DNase I footprinting assays and gel shift assays were prepared as follows. Both cell types cultured until they reached confluence were washed three times with cold phosphate-buffered saline and incubated with 20 ml of phosphate-buffered saline containing 5 ml EDTA and 5 ml EGTA for 30 min on ice. Cells from two plates were scraped off the dishes with a rubber policeman, pooled, spun down, resuspended in 1.5 ml of cold buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 2% polyvinyl alcohol) on ice for 60 min. For the bovine serum albumin control, 2.5 μg of bovine serum albumin was used instead of 10 μg of nuclear extract in binding reaction containing 60 μM instead of 50 μM KCl. Three binding reactions for the nuclear sample and three control reactions were set up to allow for three different concentrations.

DNase I (10 unit/ml) was purchased from Roche Molecular Biochemicals. After a 60-min incubation on ice, the experimental binding reactions were treated with 1 μl of 1/10, 1/20, or 1/40 dilution of the stock for 2 min, and the bovine serum albumin controls were treated with 1 μl of 1/400, 1/800, and 1/1600 dilution of the stock for 2 min. DNase I-treated binding reactions were digested with proteinase K, followed by extraction with phenol. The DNA fragments were precipitated with ethanol and then separated by 6% sequencing gel. An unrelated sequencing reaction was used as a size standard.

Gal Shift Assays—Oligonucleotides (oligos) used for gel shift assays were synthesized by Life Technologies, Inc. and end-labeled with 32P by T4 polynucleotide kinase (Life Technologies, Inc.). 1 × 106 cpm probe was incubated with 2 μg of nuclear extracts (prepared as described above) in a 20-μl volume of binding reaction (10 μl Tris-Cl, pH 7.5, 100 mM NaCl, 10% glycerol, 50 μg/ml poly(dIdC)) on ice for 20 min. In competition experiments, a 20 × 100 μM excess amount of unlabeled competitors was premixed with 1 × 106 cpm of labeled probe before being added to the binding mixture. The binding reaction was then allowed to proceed for 20 min on ice. In supershift experiments, a 1 × 106 cpm probe was incubated with 2 μg of nuclear extracts in a 20-μl volume of binding reaction for 20 min on ice, at which time 2 μl of specific antibodies (2 μg/ml) was added, followed by incubation on ice for an additional 30 min. All binding mixtures were separated, using 0.5× TBE buffer as the running buffer, at 4 °C at 100 V for 3.5 h by 4–20% gradient TBE gels (Novex, San Diego, CA) in a Novex XCell™ II mini cell electrophoresis system. The gels were transferred to 3M blotting paper, dried, and exposed to film overnight at –80°C. Antibodies (anti-Sp1, anti-Sp3, and anti-Pu.1) and the nonimmune serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Site-directed Mutagenesis—Point mutations were introduced in the context of pGL3(–63), which contains 173-bp proximal β5 promoter fragment (from –63 to +110), using a QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligos used to mutate the promoter were designed to be compatible with the pGL3(–63) vector (Stratagene). The vector was digested with HinI and purified using Quick SpinTM Sephadex Column G-50 (Roche Molecular Biochemicals). The purified large fragment BssHI–HindIII(+) was cut with PstI and HindIII to obtain the fragment PstI–HindIII (see Fig. 2A). The fragment (PstI–HindIII) was labeled with 32P, redigested with BssHI, and purified using Quick Spin™ Sephadex Column G-50 as described above. The resulting fragment BssHI–HindIII(+), labeled only at its HindIII end, was cut with HindIII and labeled with Klenow (Life Technologies, Inc.). The double-strand fragments (Mu-1, Mu-2, Mu-3, and Mu-123) were synthesized by Life Technologies, Inc. and purified by polyacrylamide gel electrophoresis. PCRs were performed in a 50-μl volume with Pfu polymerase (Stratagene, La Jolla, CA), 10 ng of DNA template, and 125 ng of each oligo using the following conditions: 95°C for 30 s, 1 cycle; 95°C for 30 s, 55°C for 1 min and 68°C for 12 min, 16 cycles; and 4°C. The PCR was treated with DpnI (10 units) for 60 min at 37°C. XLI-Blue supercompetent cells were transformed with the DpnI-treated PCR mixture as described in the instruction manual and plated on ampicillin plates. Plasmids were prepared from individual colonies and sequenced to confirm the correctness of the introduced mutations. All double-end-labeled probes were synthesized using pGL3–63 as template, the respective pair of oligos, and the PCR condition described above. The double-site mutants (Mu-12, Mu-13, and Mu-23) were made by performing a second round of PCR using the single-site mutants as template and the appropriate pairs of oligos. The generation of the triple-site mutant (Mu-123) was just an extension of the same approach, using a third set of oligos to perform another round of PCR.

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Sequence Analysis—Sequence analysis was performed using the Genetic Computer Group (Madison, WI) sequence analysis software.

**RESULTS**

Identification of Transcription Factor Binding Sites in the Proximal Region of the β5 Promoter—Osteoclast precursors and osteoblasts express the integrin αvβ5 (11, 12, 14, 20). We previously cloned the integrin β5 promoter and observed that a 173-bp proximal β5 promoter fragment, pGL3(–63) (–63 to +110), is capable of mediating β5 gene transcription in the myeloid cell line FDCP-Mac11(32), suggesting that this region contains basal transcription factor binding sites. To pursue this observation, we turned to the macrophage cell line, J774, which more easily transfected and in which pGL3(–63) also conferred basal promoter activity (Fig. 1A). Similar results were obtained with the murine osteoblast cell line MC3T3-E1 (Fig. 1B).

To identify potential transcription factor binding sites by which the 173-bp proximal promoter region controls β5 transcription, we performed in vitro DNase I footprinting assays with nuclear extracts of J774 or MC3T3-E1 cells. Four restriction sites (PstI, BssHII, BglII, and HindIII) flanking the 173-bp region were used to generate suitable probes for footprinting assays. A, in vitro footprinting assays, using probe BssHII to HindIII and J774 or MC3T3-E1 nuclear extracts. The protected regions from this experiment are indicated by bars above the sequence in A.

**FIG. 1.** A 173-bp proximal β5 promoter region mediates basal transcription activity in both J774 and MC3T3-E1 cells. A, J774 cells were cotransfected with a series of 5′-deletion mutants of the murine β5 integrin promoter linked to luciferase, described previously (32), and cytomegalovirus-β-gal plasmid. Luciferase activity was normalized to levels of β-gal. The experiment was repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± S.D. The smallest deletion mutant, pGL3(–63), which contains a promoter fragment from –63 to +110, continues to mediate basal promoter activity. B, the experiment shown in A was repeated with MC3T3-E1 cells.

**FIG. 2.** In vitro footprinting assays reveal four regions within the 173-bp β5 promoter protected by J774 nuclear proteins and three regions protected by MC3T3-E1 nuclear proteins. A, nucleotide sequence of a proximal β5 promoter (–215 to +110). This sequence is derived from pGL3–1kb(+) construct in which a 1-kb β5 promoter fragment was subcloned, in the sense orientation to luciferase coding sequence, into pGL3-basic vector (32). The 35-bp pGL3 sequence is detailed in lowercase. The transcription start site (designated +1) is indicated by an arrow. Two restriction sites (PstI and BssHII) located about 200 bp upstream of the start site, and two (BglII and HindIII) in the pGL3 vector were used to generate suitable probes for footprinting assays. B, in vitro footprinting assays, using probe BssHII to HindIII and J774 or MC3T3-E1 nuclear extracts. The protected regions are shown by bars under the sequence in A. C, a similar experiment using probe BssHII to BglII and J774 or MC3T3-E1 nuclear extracts. The protected regions from this experiment are indicated by bars above the sequence in A.
proteins, whereas MC3T3 extracts protected only three sites (FP1–FP3). To confirm this result, we repeated the footprinting assays with the probe BseHI*-BglII (Fig. 2C). Similar results were obtained with nuclear extracts prepared from mouse primary bone marrow macrophages or osteoblasts (data not shown).

To determine whether these protected genomic sequences bind nuclear proteins, we synthesized four oligonucleotides (FP1–FP4), each containing one of the protected sequences (Fig. 3A). In gel shift assays (Fig. 3B), oligos FP1, FP2, and FP3 gave rise to slowly (A) and quickly (B) migrating major bands with both J774 and MC3T3-E1 nuclear extracts. As demonstrated by subsequent studies (Figs. 4–6), A comprises two or three bands, depending on which oligo was used for binding. Oligo FP2 also yielded a number of minor, high mobility bands (band C) from J774 nuclear extracts and one (band D) from MC3T3 nuclear extracts. Oligo FP3 generated a minor band (band E) with high mobility only from MC3T3 nuclear extracts. Oligo FP4 also yielded a number of minor, high mobility bands, depending on which oligo was used for binding. As seen in Fig. 4A, 32P-labeled FP1 oligos give rise to four bands (a–d, lane 1), each being eliminated by excess unlabeled probe (lanes 2 and 3). A 22-bp oligo containing an Sp1 consensus sequence abolished bands a, b, and c (lanes 4 and 5). When mutated, the consensus Sp1 oligo had no effect on bands a, b, or c (lanes 6 and 7), suggesting that they are Sp1-related proteins. Given that band d was not affected by excess unlabeled wild type Sp1 oligo, it probably represents a nuclear protein that is not a member of the Sp1 family. Gel shift analysis using 32P-labeled FP2 oligo yielded three major bands, a, b, and c, as well as several minor bands, all eliminated by excess unlabeled FP2, (Fig. 4B, lanes 1 and 2). Wild type, but not mutated, Sp1 oligo abrogated all bands. As seen in Fig. 4C, FP3 also specifically bound Sp1 related proteins. FP4 probe, incubated with J774 nuclear extract, yielded one band (Fig. 4D, lane 1, c), diminished by unlabeled FP4 (lanes 2 and 3). Wild type (lanes 4 and 5) but not mutated (lanes 6 and 7) PU.1 oligo also abolished the band.

These experiments suggest, but do not prove, that FP1, FP2,
and FP3 recognize Sp1-related transcription factors (Fig. 4, A–C, bands a, b, and c) and that FP4 contains a PU.1 response element. Once again, to assess whether the mutant FP4 oligos bind nuclear proteins other than PU.1, we performed gel shift assays with each mutant probe. Whereas M1–M3 each recognized a molecule not seen by the wild type probe, M4–M6 did not bind J774 nuclear proteins (data not shown).

FP4 contains two PU.1-like consensus sequences (Fig. 3A), from which we generated six mutants (M1–M6; Fig. 6C). M1 and M2 were mutated in the canonical putative PU.1 site partially protected by J774 nuclear extracts, and M3–M6 were mutated in the noncanonical sequence present within the fully protected region. M1 and M2 competed with FP4 for PU.1 binding, whereas M3–M6 failed to do so (Fig. 6D). Given that the nucleotides mutated in M1 and M2 retained the ability to compete for PU.1 binding, we conclude that the canonical PU.1 site located partially in the protected region is not functional. In contrast, M3–M6 failed to compete, indicating that their mutated nucleotides are critical for binding. Thus, the atypical site within the protected region of FP4 is a candidate PU.1 response element. Once again, to assess whether the mutant FP4 oligos bind nuclear proteins other than PU.1, we performed gel shift assays with each mutant probe. Whereas M1–M3 each recognized a molecule not seen by the wild type probe, M4–M6 did not bind J774 nuclear proteins (data not shown).

Wild Type, but Not Mutated, FP1, FP2, and FP3 Bind Sp1/Sp3 from Osteoblast Nuclear Extracts—To determine whether osteoblast nuclear proteins recognizing FP1, FP2, and FP3, like those derived from J774 cells, are also Sp1 and Sp3, we performed gel shift/competition assays with MC3T3-E1 nuclear extracts. As shown in Fig. 7A, the FP1 probe gave rise to three bands (a, b, and c, lane 1) all of which were abolished by unlabelled FP1 and Sp1 (lanes 2–5) but not mutant (m) Sp1 or FP1 oligos (lanes 6–9). FP2, but not mFP2, also specifically bound Sp1-related nuclear proteins, and once again wild type, but not mutant, Sp1 oligos competed for binding (Fig. 7B). Finally, similar to our studies with J774 cells, five MC3T3-E1 derived bands appeared in gel shift assays with FP3 probe (Fig. 7C, lane 1, bands a–e) each being disrupted by unlabelled oligo FP3 (lanes 2 and 3). Unlabelled Sp1 oligos competed for binding nuclear proteins of bands a, b, and c, but less effectively for the minor bands (lanes 4 and 5). Neither mutant Sp1 nor FP3 oligos affected these bands (lanes 6–9). We also found that mFP1, mFP2, and mFP3, although they lost their capacity to bind Sp1-related proteins, did not associate with other MC3T3-E1 nuclear proteins (not shown). Supershift assays revealed that the nuclear proteins associating with FP1 and FP2 were indeed Sp1 and Sp3 and that FP3 binds Sp1, Sp3, and other unknown proteins (Fig. 8). Furthermore, these experiments revealed that unlike our findings with J774 nuclear extracts, a third slowly migrating band (Fig. 4A, d) was not generated with oligonucleotide FP1.

Sp1/Sp3 Sites in FP1, FP2, and FP3 Differentially Regulate the β5 Gene in J774 and MC3T3 Cells—To determine whether the three Sp1/Sp3 sites regulate β5 basal transcription in
macrophages and osteoblasts, we introduced the point mutations, individually and in combination, which block Sp1/Sp3 binding in gel shift assays, into the parental pGL3(–63) reporter construct (Fig. 9A). When transfected into J774 macrophage-like cells, the triple mutant (Mu-123) exhibited only background luciferase activity (Fig. 9B). Thus, one or more of the three Sp1/Sp3 sites are critical for b5 basal promoter activity. Because the activity of Mu-1 was reduced compared with wild type construct (p \(< 0.0001\)) whereas Mu-23 was more active than Mu-123 (p \(< 0.0001\)), we conclude FP1 contains a weak enhancer element. Additionally, the Sp1 site in FP2 was essential for basal transcription because Mu-2 exhibited only background activity and Mu-13 was more active than Mu-23 (p \(< 0.0002\)). Finally, the FP3 Sp1/Sp3 site represses b5 transcription, because its sole mutation (Mu-3) prompted increased reporter activity (p \(< 0.0001\)). Thus, the three Sp1 sites differentially mediate b5 promoter activity in J774 cells. The regulatory activity of the same three sites in MC3T3-E1 cells mirrors that in J774 cells other than the fact that the Sp1 site in FP2 is not critical in osteoblasts, because its mutation did not result in total loss of promoter activity (Fig. 9C).

**DISCUSSION**

The integrin α5β5 mediates specific activities of a wide range of cells following binding to its various ligands, each of which contains the amino acid sequence RGD. Thus, internalization of vitronectin from extracellular matrix and its subsequent
intracellular degradation by fibroblasts are blocked by an antibody to $\alpha_\beta_5$, but not by an antibody to $\alpha_\beta_3$ (35). Similarly, although both $\alpha_\beta_3$ and $\alpha_\beta_5$ are present on mesoendothelial cells, antibodies to the latter integrin, but not the former, inhibit internalization of asbestos fibers in vitro. Coating of the fibers with vitronectin, a ligand for $\alpha_\beta_5$, enhances further the uptake capacity. Attesting to biological significance, $\alpha_\beta_5$ but not $\alpha_\beta_3$ shows immunological colocalization with the fibers in vivo (36). Furthermore, adenovirus-induced permeabilization is mediated by attachment of cells to the RGD-containing penton base domain of the virus, through $\alpha_\beta_5$ (37). Whereas attachment of smooth muscle cells is regulated by $\alpha_\beta_3$, migration is dependent solely on $\alpha_\beta_5$ (38).

Of greater relevance to the present studies, $\alpha_\beta_5$ is important for the function of both macrophages and osteoblasts. We demonstrate that murine osteoclast precursors, in the form of immature bone marrow macrophages, express $\alpha_\beta_5$ and use this receptor to attach to matrix proteins, an early and obligate step in osteoclastogenesis (12). Furthermore, phagocytosis of apoptotic cells and cross-presentation of antigen to cytotoxic T lymphocytes by macrophage-derived dendritic cells involves $\alpha_\beta_5$, working in conjunction with CD36 (39). Finally, an antibody to $\alpha_\beta_5$ inhibits attachment of human osteoblasts to vitronectin, whereas a function-blocking antibody to $\alpha_\beta_3$ is without effect.1

Given that $\alpha_\beta_5$ plays a central role in the activity of osteoblasts and osteoclast precursors, we turned to the mechanisms regulating expression of this integrin. Our earlier work (12, 40, 41), and that of others (42) reveals that appearance of $\alpha_\beta_5$-associated integrin on the surface of a cell is controlled by expression of the appropriate $\beta$, and not the $\alpha$, subunit. When first isolated, both osteoblasts and osteoclast precursors express $\alpha_\beta_5$, whose levels are regulated by TGF $\beta$ (osteoblasts),1 granulocyte-macrophage CSF (osteoclast precursors (12)), or the osteoclastogenic cytokine OPG (osteoclast precursors).4 In this regard, we isolated the murine $\beta_5$ integrin promoter and identified a novel response element that mediates a granulocytemacrophage CSF-dependent decrease in transcription (32). We have also delineated the TGF $\beta$-responsive region in osteoblasts.5

In the course of the experiments defining cytokine-responsive components of the $\beta_5$ promoter, we noted that basal expression is supported by a region included in –63 to +110. Using footprinting analysis, we identified regions of this sequence protected by nuclear extracts from both osteoblasts and osteoclast precursors. Our data reveal the presence of four protected sites, three of which represent potential binding domains for SP family proteins, whereas the fourth region has two potential binding sites for the lineage-specific transcription factor PU.1. In competition assays using both consensus oligonucleotides and antibodies to the three transcription factors, we confirmed that the three SP sites bind a combination of Sp1 and Sp3, whereas only one of the two PU.1 sites is active. The functional PU.1 site we identified in the $\beta_5$ promoter is 5'-AAAAAGGAAGG-3', which, although it differs significantly from the core PU.1 sequence, 5'-GAGGAA-3', is similar to a previously reported noncanonical PU.1 binding domain (34). Our finding is not without precedent (several PU.1 responsive genes lack a consensus sequence (43–45)) and supports the hypothesis that sequence alone is not sufficient to determine function of a putative transcription factor binding site.

To determine the function of the various Sp and PU.1 binding sites, we generated a series of mutants, all in the context of

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1 S., Wei S. L. Teitelbaum, and F. P. Ross, unpublished data.
2 S.-L. Cheng, C.-F. Lai, A. Fausto, M. Chellaiiah, X. Feng, K. McHugh, S. L. Teitelbaum, R. Civitelli, K. A. Hruska, F. P. Ross, and L. V. Avioli, manuscript in preparation.
the –63 to +110 genomic fragment, which supports basal transcription in both osteoblasts and macrophage-like cells. Transfection of the individual mutants, alone or in combination, into both cell types, reveals each binding site plays a separate role in controlling β5 gene expression.

As expected, the region of the β5 promoter that binds PU.1 is active in myeloid-derived, osteoclast-related cells but is without effect in osteoblastic cells, which are mesenchymal in origin. PU.1 is found primarily in pre-B and B cells, macrophages, and osteoclasts, which are produced as a result of macrophage differentiation and fusion (46). Consistent with the critical role of PU.1 in macrophage formation, our group has demonstrated that PU.1 is essential for osteoclast differentiation and that levels of this transcription factor are increased during this event (31). In most circumstances, PU.1 acts as a transcriptional enhancer (47). Because PU.1 binds TBP in vitro (48), an attractive hypothesis holds that PU.1 assembles the basal transcription machinery on myeloid promoters, nearly all of which lack a TATA box. A recent report indicates that PU.1 regulates lineage-specific commitment of pluripotent hematopoietic precursors by decreasing expression of a master gene for nonmyeloid commitment, such as GATA-1, while at the same time increasing expression of the macrophage CSF, granulocyte-macrophage CSF, and granulocyte CSF receptors (49), each of which plays a role in expansion of a specific myeloid sublineage.

The experiment was repeated three times and a representative result is shown. Each bar is the mean of three replicates ± S.D. C, the transfection experiment shown in B was repeated with MC3T3-E1 cells.

**FIG. 9.** The three Sp1/Sp3 binding sites contribute differently to basal β5 promoter activity in J774 and MC3T3-E1 cells. A, schematic diagram of a series of pGL3(–63) constructs containing mutations in different Sp1/Sp3 binding sites. Each contains a point mutation(s) shown previously to abrogate binding in gel shift assays (Figs. 6 and 7). B, basal β5 promoter activity in J774 cells transfected with the mutants shown in A. Cells were cotransfected with pGL3(–63), the series of mutants, or pGL3-basic as negative control and cytomegalovirus-β-gal plasmid. Luciferase activity normalized to the level of β-gal.

**FIG. 10.** PU.1 negatively regulates basal β5 promoter activity in J774 cells. A, schematic presentation of a pGL3(–63) reporter containing a mutation in the functional PU.1 binding site. The mutant (Mu4) was generated by introducing the point mutations shown to abrogate the binding capacity in gel shift assays above (Fig. 6) into pGL3(–63). B, basal β5 promoter activity in J774 and MC3T3-E1 transfected with parental pGL3(–63) or Mu4 mutant. J774 and MC3T3-E1 cells were transfected and assayed as described in Fig. 9, B and C. The experiment was repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± S.D.
We find that mutation of the active PU.1 site in the promoter of the β5 integrin gene leads to increased basal transcription, revealing that PU.1 is a suppressor of constitutive β5 promoter activity. Although, as discussed above, PU.1 is usually a transcriptional activator, it can also inhibit gene expression. For example, the 3′ enhancer region of the murine Ig κ locus contains a PU.1 binding region required for tissue B cells/T cells-specific Vκ-Jκ joining (50), which also acts as a negative element in pre-B cells. Attempts to detect PU.1-binding proteins, which might alter its transcriptional function, were unsuccessful (51). Additionally, transcription of the murine MHC-II gene I-Ak fails when PU.1 is transiently co-expressed, leading to the proposal that PU.1 inhibits gene expression, by displacing NF-Y, a constitutive transcriptional activator of this gene (52). Finally, binding of PU.1 to the transcriptional start site suppresses expression of the CD11c gene (53). In summary, the proposed mechanisms by which PU.1 inhibits gene expression require either (α) binding close to the transcriptional start site or (β) interaction with other proteins acting as transcriptional co-repressors. Because the PU.1 binding site of the β5 promoter is not close to the transcriptional start site of the gene and we detected only a single band binding to an oligonucleotide containing the PU.1 sequence, it is unlikely that either displacement of the basal transcriptional complex or recruitment of a transcriptional co-repressor represents the mechanism by which PU.1 suppresses β5 gene expression. Thus, our findings suggest a potentially novel mechanism by which PU.1 can regulate gene expression.

In addition to the PU.1 binding site, the β5 proximal promoter region contains three domains recognizing Sp1 and Sp3, each differentially regulating basal transcription in J774 and MC3T3 cells. In both lines, a mutation approximately 30 bp downstream of the +1 residue results in a 4- and 2-fold transcriptional enhancement, respectively, suggesting that binding of the Sp1/Sp3 complex to this genomic region is suppressive. This hypothesis is confirmed by the fact that β5 gene expression is blocked in the double mutant Mu-12, in which only the putatively inhibitory upstream site is active.

With a single exception (see below) the available data demonstrate that Sp1 invariably activates transcription (22). Sp3, on the other hand can either enhance or suppress transcription, with the outcome dependent on the context of the element within the intact promoter and the number of Sp binding sites (22, 25, 54). Similar to our findings, basal expression of the human glucagon-like peptide-1 receptor gene is regulated by three Sp1/Sp3 sites in the proximal promoter (55). Although the two more proximal sites act to enhance expression, the third, located at −344 to −339, is repressive. This inhibitory activity is dependent on Sp3, confirming the capacity of this protein to dampen promoters containing multiple Sp1 binding sites. Similarly, the human adenine nucleotide translocase gene is either activated or repressed, depending on which of the three Sp1 sites is mutated (56).

Our electrophoretic mobility shift assay and supershift data demonstrate that in addition to Sp1 and Sp3, the FP3 region binds a rapidly migrating protein(s) in MC3T3 but not J774 nuclear extracts. We propose these proteins may play a role in β5 gene repression in osteoblasts. Although these molecules are presently uncharacterized, one candidate is p74, a protein identified only in metabolic labeling experiments and documented to inhibit Sp1 gene transcription in vivo (57). Because Sp1 and Sp3 are the only proteins in macrophage nuclear extracts that bind to the FP3 oligonucleotide, the mechanism by which this region of the promoter decreases β5 transcription is speculative. Because Sp proteins are known to bind DNA (58), an event that can modulate gene activation (59), it is possible that upon binding, the Sp1/Sp3 heterodimer causes chromatin bending, thereby limiting access of the basal transcriptional machinery to the initiation site.

Although the downstream Sp binding site in the β5 gene is inhibitory, the two distal GC-rich elements act as transcriptional enhancers in both MC3T3 and J774 cells. On the other hand, although mutation of FP2 completely arrests transcription in macrophase-like cells, the event is only partially blunted in osteoblasts. Thus, as is the case with the TATA-less carbamoyl-phosphate synthase gene promoter (60), the central Sp1/Sp3 binding site acts as a basal promoter in macrophages. Of interest, nuclear extracts from both cell types, probed with the FP2 oligonucleotide, yield a rapidly migrating band. Attesting to specificity the band is competed by wild type, but not mutated, FP2. Although the identity of this protein remains unknown, it is a candidate for mediating that component of basal transcription that is Sp1/Sp3-independent. The fact that only FP2, and not FP1 or FP3, supports the binding of this molecule is not surprising, because the flanking sequences of all three Sp sites are distinct.

Sp1 and/or Sp3, in many instances, interact with a wide range of transcription factors, each binding to its own response element (61–64). We found that transcriptional activity of constructs encompassing an 800-bp fragment of the β5 promoter immediately 5′ of the region mediating basal activity is greater than that of the basal promoter alone (Fig. 1). Because this larger genomic region contains putative binding sites for a number of transcription factors (32), Sp1 and Sp3, in addition to regulating basal transcription, may interact with one or proteins bound to the upstream regions and thus regulate overall expression of the β5 gene in the bone cells.

Sp1 and its related protein Sp3 are implicated in the transcriptional regulation of many genes during different stages of development (22, 23). Of particular relevance, Sp1 regulates a number of integrin subunits, including CD18, the β chain of the leukocyte integrins (65); CD11c which forms a heterodimeric complex with CD18 (66, 67); and the α2 (68, 69), α6 (70), αQ (71), and α chains (72). Moreover, Sp3 activates the leukocyte integrin genes CD11c and CD11b in myelomonocytic cells (73). Our findings that Sp1 and Sp3 modulate expression of the rate-limiting β subunit during cytokine-induced differentiation of both major bone cell types reinforces the role of the two transcription factors in integrin biology.

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