Gap Junctional Communication Modulates Gene Transcription by Altering the Recruitment of Sp1 and Sp3 to Connexin-response Elements in Osteoblast Promoters*

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Loss-of-function mutations of gap junction proteins, connexins, represent a mechanism of disease in a variety of tissues. We have shown that recessive (gene deletion) or dominant (connexin45 overexpression) disruption of connexin43 function results in osteoblast dysfunction and abnormal expression of osteoblast genes, including down-regulation of osteocalcin transcription. To elucidate the molecular mechanisms of gap junction-sensitive transcriptional regulation, we systematically analyzed the rat osteoblast promoter for sensitivity to gap junctional intercellular communication. We identified an Sp1/Sp3 containing complex that assembles on a minimal element in the −70 to −57 region of the osteocalcin promoter in a gap junction-dependent manner. This CT-rich connexin-response element is necessary and sufficient to confer gap junction sensitivity to the osteocalcin proximal promoter. Repression of osteocalcin transcription occurs as a result of displacement of the stimulatory Sp1 by the inhibitory Sp3 on the promoter when gap junctional communication is perturbed. Modulation of Sp1/Sp3 recruitment also occurs on the collagen Iα1 promoter and translates into gap junction-sensitive transcriptional control of collagen Iα1 gene expression. Thus, regulation of Sp1/Sp3 recruitment to the promoter may represent a potential general mechanism for transcriptional control of target genes by signals passing through gap junctions.

Gap junctions are transcellular channels formed by juxtaposition of two connexon hemichannels, hexameric structures present on adjacent cells, allowing direct intracellular communication via diffusion of ions, metabolites, and small signaling molecules. These hemichannels are composed of six protein subunits, connexins. Connexins are a family of gap junction proteins encoded by at least 17 different genes (1). Connexins can assemble as a homeric or heteromeric hemichannel, and the connexin isotypes that form the gap junction hemichannels dictate the molecular size and permeability of the resulting gap junction channel (2–6). In coupled cells, numerous gap junctions assemble between adjacent cells forming large gap junctional plaques.

Gap junctional communication plays a critical role in many cellular functions, as well as in development and embryogenesis (reviewed in Ref. 7). Accordingly, mutations of connexin genes have been implicated in several pathologic conditions, including Charcot-Marie-Tooth disease (8), non-syndromic sensorineural deafness (9), and visceroastral heterotaxia (10). Furthermore, loss of gap junctional communication accompanies the early stages of neoplasia (11, 12). Disruption of gap junctional communication in cell culture can generate a neoplastic phenotype (13, 14), and ectopic expression of connexins in neoplastic tissue can function as a tumor suppressor (15–17). However, the molecular mechanisms by which gap junctional coupling can modulate cell activities are not well understood. Identification of signaling events that are initiated and maintained at gap junctional plaques would give critical insights into how gap junctions modulate cellular function in health and disease.

In bone, ablation of the major gap junction protein expressed in osteoblasts, connexin43 (Cx43),† results in delayed intramembranous ossification in the skull, delayed endochondral ossification of the axial skeleton, and a generalized osteoblast dysfunction (25). Among the defects observed in osteoblasts isolated from Cx43 null mice is down-regulation of expression of several genes critical for bone formation, including osteocalcin, bone sialoprotein, and type I collagen (25). Likewise, disruption of gap junctional coupling in osteoblastic cells by treatment with gap junction inhibitors results in down-regulation of osteocalcin and bone sialoprotein (26, 27).

The rat osteosarcoma cell line, ROS17/2.8, is coupled exclusively by gap junctions formed by Cx43 (28). We have demonstrated previously that overexpression of connexin45 (Cx45), which forms gap junctions with a decreased size and opposite

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¶ The abbreviations used are: Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; CxRE, connexin-response element; ORF, open reading frame; RSV, Rous sarcoma virus; mut, mutant.
Gap Junction-sensitive Gene Transcription

**FIG. 1.** Transcription from the rat osteocalcin proximal promoter is gap junction-sensitive. A, total RNA isolated from ROS17/2.8 cells and ROS/Cx45 cells was collected, DNase I-treated, and reverse-transcribed. Real time PCR was performed using osteocalcin-specific primers and normalized to levels of GAPDH. Osteocalcin expression was markedly reduced in cells expressing Cx43. B, ROS17/2.8 cells were transfected with the −637 to +32, −199 to +32, or −92 to +32 rat osteocalcin promoter, respectively, upstream of a luciferase reporter. Cells were cotransfected with either pcDNA3 or pSSPV-Cx45 expression plasmid. 72 h post-transfection, the cells were lysed in 1× passive lysis buffer. Lysates were analyzed for luciferase activity. Values were normalized to β-galactosidase activity. Reporter activity of all the constructs was markedly reduced in cells overexpressing Cx45, indicating that the connexin-sensitive element is located in the −92 to +32 region of the osteocalcin promoter. Data are from representative experiments and are presented as means ± S.D.

charge selectivity to that of Cx43 channels, in ROS17/2.8 cells (ROS/Cx45) results in a drastic decrease in chemical and electrical coupling of the resulting gap junctions, indicating that in a mixed Cx43/Cx45 environment the biophysical properties of Cx45 prevail. Thus, Cx45 acts as a partial dominant negative connexin for Cx43 (2, 3, 29). By using this model of Cx45 overexpression to inhibit gap junctional communication, we and others (29, 30) have observed close similarities between ROS/Cx43 and osteoblasts derived from mice in which the Cx43 gene has been ablated, indicating transcriptional down-regulation of osteocalcin and bone sialoprotein and up-regulation of osteopontin transcription.

In this study, we have further exploited the partial dominant negative action of Cx45 on Cx43 to elucidate the molecular mechanisms by which gap junctional communication modulates gene transcription. By using the osteocalcin promoter as a primary readout, we have identified a minimal element in the proximal promoter of this gene that is sensitive to gap junctional communication. We also identified the complex binding this element as containing the ubiquitous transcription factors Sp1 and Sp3, and we demonstrate that Sp1 can activate transcription from this element, whereas Sp3 represses transcription. Finally, we demonstrate that gap junctional communication regulates transcription by altering the recruitment of Sp1 to the promoter, an action that is common to the collagen Iα1 (collIα1) gene as well. Thus, modulation of Sp1 and Sp3 recruitment is one mechanism by which intercellular signals propagated through gap junctions alter gene transcription.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Standard molecular biology reagents were purchased from Promega (Madison, WI). NOVEX 4–20% TBE gels and LipofectAMINE PLUS reagent were from Invitrogen. Antibodies against Cx43, Sp1, and Sp3 and protein A/G plus were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine and phosphothreonine antibodies are from Zymed Laboratories Inc. (San Francisco, CA). Anti-actetyllysinine antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). SYBR Green PCR master mix was from Applied Biosystems (Foster City, CA). QIAquick PCR purification kit was from Qiagen (Valencia, CA). Oligonucleotides were synthesized by Washington University Protein and Nucleic Acid Chemistry Laboratory (St. Louis, MO). Radionucleotides were purchased from ICN (Costa Mesa, CA). Rabbit anti-Cx43 antibodies and pSSPV-Cx45 plasmids were provided by Dr. Thomas Steinberg (Washington University, St. Louis, MO). Unless stated otherwise all chemicals were from Sigma.

**Cell Culture—**The rat osteosarcoma cell line, ROS17/2.8, was provided by Dr. Gideon Rodan (Merck). ROS17/2.8 cells stably transfected with chick Cx43 (ROS/Cx45) have been described previously (3). Cells were cultured in α-minimum Eagle's medium containing 10% fetal bovine serum and antibiotics and used at passage numbers between 3 and 14. Drosophila SL2 cells were obtained from Dr. Ross Cagan (Washington University, St. Louis, MO) and cultured in Schneider cell medium + 10% fetal bovine serum and antibiotics.

**Reverse Transcription and Real Time PCR—**Total RNA was isolated from confluent cultures of ROS17/2.8 and ROS/Cx45 cells. RNA (2 µg) was DNase I-treated and then reverse-transcribed using Superscript II reverse transcriptase and oligo(dt) primers. One-fortieth of this reaction was used for real time PCR analysis of gene expression, using SYBR Green I dye chemistry. PCR product accumulation was monitored using a GeneAmp 5700 sequence detection system (PerkinElmer
Fig. 3. **Protein-DNA complexes assembling on the osteocalcin CT element are sensitive to gap junctions.** A, EMSAs were performed using radiolabeled CT (lanes 1–3), −120 to −100 (lanes 4–6), or −146 to −125 (lanes 7–9) oligonucleotides. Comparison of nuclear extracts prepared from ROS17/2.8 cells (lanes 2) and nuclear extracts prepared from ROS/Cx45 cells (clone D; lane 3) revealed differential DNA binding activities on the CT element. The slow migrating complex (arrowhead) assembled on the CT element is more abundant in ROS/Cx45 extracts compared with ROS17/2.8 extracts. No difference in binding activities between the same extracts was observed using other probes (lanes 5, 6, 8, and 9). Quantitation of the intensity of the indicated slow migrating complex (arrowhead) is shown in the bar graph below. Lanes 1, 4, and 7 contain no extract. B, EMSAs were performed using radiolabeled CT or AP1 cognate oligonucleotides. Nuclear extracts were prepared from ROS17/2.8 and additional clonal lines of connexin45 stably transfected cells, ROS/Cx45 C and E. As observed for ROS/Cx45 clone D, the binding of the indicated complex (arrowhead) to the CT element was increased in both the E and C extracts (lanes 2 and 6, respectively), as compared with ROS17/2.8 extract (lanes 1 and 5). In contrast, binding of the same nuclear extract to an AP1 cognate was not affected by overexpression of Cx45 (lanes 3 versus 4 and lanes 7 versus 8). Quantitation of the intensity of the indicated slow migrating complex (arrowhead) is shown in the bar graph below. Quantitation of binding activity to the AP1 and −146 to −125 oligonucleotides is based on densitometric analysis of the most abundant (i.e. slowest migrating) complex.

Life Sciences). The mean cycle threshold value (Ct) from triplicate samples was used to calculate gene expression. PCR products were normalized to levels of GAPDH. Relative gene expression levels were determined as described in User’s Bulletin (PN 4303859) from Applied Biosystems. The gene-specific primers used are as follows: OC-F, CCAAGCACTCTGAGTCTGACAA, and OC-R, CCGGAGTCTATTCACCACGGC. Rodent GAPDH primers were purchased from Applied Biosystems.

**Nuclear Extracts and Electrophoretic Mobility Shift Assays—** Nuclear extracts were prepared according to the methods of Dignam et al. (31) with the addition of 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin, 1 mM NaVO4, and 1 mM phenylmethylsulfonyl fluoride. Extracts were dialyzed against Dignam buffer D (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol) and stored in 50-μl aliquots at −70 °C. Electrophoretic mobility shift assays (EMSA) were performed as described previously (32). Briefly, 10 pmol of gel-purified oligonucleotides was labeled with Klenow fragment and [32P]dCTP. Binding reactions were performed in the presence of 0.1 pmol of labeled oligonucleotide, 10 μg of acetylated bovine serum albumin, 1 μg of double-stranded poly(dI-dC), and 2 μg of nuclear extract. Total reaction volume was adjusted to 15 μl with Dignam buffer D. For supershift experiments, 2 μg of antibody was added to the binding reaction. Binding reactions were carried out for 20 min at 25 °C. The samples were electrophoresed on NOVEX 4–20% polyacrylamide gels in 0.375× TBE. Gels were dried, and bands were visualized by autoradiography. The following oligonucleotides were used in this study (numeration is relative to the transcription start site of the rat osteocalcin promoter): CT, GGTCTCCTGCCCCCTCTGCT, Sp1, ATTCGATCGGGGCGGGGAGA. The amplified product was gel-purified, the ends blunted using Klenow fragment, phosphorylated, and ligated into the KpnI site of a minimal RSVLUC promoter (33). The −92mtCT-OCLUC construct was generated by sequenci-
Values were normalized to transfection, the cells receiving gap junction inhibitors were treated for oleic acid. ROS17/2.8 cells treated with the gap junction inhibitor, RSV-LUC construct. The cells were cotransfected with either pcDNA3 or treated with oleamide. Lysates were analyzed for luciferase activity. Values were normalized to β-galactosidase activity. Reporter activity was markedly reduced in cells overexpressing Cx45 and also in cells transiently transfected with pcDNA3 or pSFFV-Cx45. 48 h post-transfection, the cells were lysed in 1× passive lysis buffer. Lysates were analyzed for luciferase activity. Values were normalized to β-galactosidase activity. Either removal of the CT element (mtCT) or mutation of the CT element (mutCT) inhibited gap junction-sensitive complex on the CT element.

Formaldehyde Cross-linking and Chromatin Immunoprecipitation—Formaldehyde cross-linking and chromatin immunoprecipitation (ChIP) assay was performed as described by Shang et al. (36). Briefly, ROS17/2.8 and ROS/Cx45 cells were grown to confluence, rinsed twice in PBS, and then allowed to recover in growth media. After 48 h the cells were rinsed in PBS and then lysed in 1× reporter lysis buffer. Luciferase activity was monitored using an Optocomp luminometer. Data were normalized to protein content, as determined by the Bradford method. Transfections were performed in triplicate wells and repeated three times. Western Blotting and Immunoprecipitations—SDS sample buffer was added to luciferase extracts, described above. Samples were sonicated three times for 10 s each. Insoluble material was pelleted, and the supernatants were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat dry milk, probed with goat anti-Sp1 (1:2000) or rabbit anti-Sp3 (1:2000) antibodies, and detected with the appropriate hors eradish peroxidase-conjugated antibodies (1:5000) and ECL detection reagents. Immunoprecipitations were performed using 500 µg of nuclear extract and 3 µg of rabbit anti-Sp1 or rabbit anti-Sp3 antibodies. Immunoprecipitated material was collected using protein A/G plus. Washed immunoprecipitated material was eluted in SDS sample buffer, immunoblotted, and probed with anti-phosphoserine, -phosphothreonine, or -acetyllysine antibodies. The blots were then reprobed with anti-Sp1 or anti-Sp3 antibodies, respectively, to compare the amount of immunoprecipitated material.

Expression of Sp1 (pCMV-Sp1) and Sp3 (pCMV-Sp3, pPacSp3) were kindly provided by Dr. Thomas Suske (Philips-Universitat Marburg, Germany). The pPac0 and pPacSp1 were provided by Dr. Robert Tjian (University of California, Berkeley). The pSFFV-Cx45 plasmid was provided by Dr. Thomas Steinberg. All constructs were sequenced to ensure experiments were performed in a linear range of amplification. PCR primers used are as follows: OC Promoter-F, CCAATTAGTCTCGGCCGAATC; OC Promoter-R, TTGGCTGTGAGGGATCTGGT; OC ORF-F, CCGTGATCGAGAGGGAAGGAG; OC ORF-R, GCGTCACTCGAGACATAC; COLIA1 Promoter-F, TGAGGATCTCTGTCCCTG; COLIA1 Promoter-R, GACACCTGACGCGCTCCCG; COLIA1 ORF-F, GAGAGAGTGCCAACTCCAG; and COLIA1 ORF-R, CACCCCGGAGGAAAACT.

Densitometry of Band Intensity— Autoradiographs were scanned, then allowed to recover in growth media. After 48 h the cells were rinsed in PBS and then lysed in 1× reporter lysis buffer. Luciferase activity was monitored using an Optocomp luminometer. Data were normalized to protein content as determined by the Bradford method. Transfections were performed in triplicate wells and repeated three times. Western Blotting and Immunoprecipitations—SDS sample buffer was added to luciferase extracts, described above. Samples were sonicated three times for 10 s each. Insoluble material was pelleted, and the supernatants were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat dry milk, probed with goat anti-Sp1 (1:2000) or rabbit anti-Sp3 (1:2000) antibodies, and detected with the appropriate horse radish peroxidase-conjugated antibodies (1:5000) and ECL detection reagents. Immunoprecipitations were performed using 500 µg of nuclear extract and 3 µg of rabbit anti-Sp1 or rabbit anti-Sp3 antibodies. Immunoprecipitated material was collected using protein A/G plus. Washed immunoprecipitated material was eluted in SDS sample buffer, immunoblotted, and probed with anti-phosphoserine, -phosphothreonine, or -acetyllysine antibodies. The blots were then reprobed with anti-Sp1 or anti-Sp3 antibodies, respectively, to compare the amount of immunoprecipitated material.
RESULTS

Connexin-sensitive Protein-DNA Complexes Form on the Proximal Osteocalcin Promoter—Overexpression of Cx45 in ROS17/2.8 cells, which are normally highly coupled by gap junctions formed by Cx43, reduces gap junctional permeability to negatively charged dyes (2, 28). Confirming our previous report (30), overexpression of Cx45 in ROS17/2.8 cells also decreases osteocalcin gene transcription, as assessed by real time PCR (Fig. 1A). To identify the core element and understand the molecular mechanisms of Cx45-mediated transcriptional repression, we first mapped the element responsible for the Cx45 induced down-regulation of osteocalcin promoter basal activity, using promoter deletion constructs cloned upstream of a luciferase reporter. A ∼3-fold repression of osteocalcin promoter activity was observed with all deletion constructs tested, indicating that the gap junction-sensitive transcriptional repression was contained within the −92 to +32 rat osteocalcin promoter (Fig. 1B). Further shortening of this region at the 5′ end resulted in decreased basal activity that precluded detection of an inhibitory effect. As an alternative strategy, we used EMSA to search for binding activities that were altered by Cx45 overexpression. Nuclear extracts were isolated from ROS17/2.8 and from several clonal ROS cell lines stably overexpressing Cx45 (ROS/Cx45 clone C and clone E in Fig. 2; clone D in Fig. 3). A protein-DNA complex whose binding activity was altered by overexpression of Cx45 was identified in a CT-rich stretch of DNA in the −74 to −56 osteocalcin promoter (Fig. 2). The slow migrating complex assembling on the −74 to −56 (CT element) oligonucleotide probe was reproducibly increased −2-fold in ROS/Cx45 extracts compared with ROS17/2.8 in EMSA (Fig. 2B, lanes 1 versus 2). Alteration in binding activity on the CT element was indeed sequence-specific, EMSAs were subsequently performed using radiolabeled CT element, −120 to −100 and −146 to −125 oligonucleotides. As predicted, the putative connexin-sensitive complex assembled on the CT oligonucleotides (Fig. 3A, lanes 2 and 3, arrowhead), and its binding activity was increased in ROS/Cx45 extracts relative to native cells. The −120 to −100 (spanning C/EBP and hox-binding sites) and −146 to −125 (spanning a fibroblast growth factor-response element and a runx2-binding site) oligonucleotide probes assembled equal binding activities using the same nuclear extracts (Fig. 3A, lanes 4–9), demonstrating the specificity of the change observed on the CT element. To validate further the specificity of increased binding in the extracts, radiolabeled AP1 cognate oligonucleotides were used as a negative control (i.e. a complex that is not regulated by perturbation in gap junctional communication). Comparison of AP1 binding between ROS17/2.8 and ROS/Cx45 extracts revealed identical binding activity, whereas binding of the slow migrating complex to the CT element was increased in Cx45-overexpressing cells (Fig. 3B), establishing the CT element as containing a putative connexin-response element (CxRE).

Identification of the CT Element as a Connexin-sensitive Unit—To establish that the CT element is in fact a gap junction-sensitive transcriptional unit, the −74 to −56 region of the osteocalcin promoter, spanning the CT element was cloned into an RSV minimal promoter upstream of a luciferase reporter gene (CT-RSVLUC). Cotransfection of CT-RSVLUC and a Cx45 expression vector (pSFFV-Cx45) or a control vector (pcDNA3) into ROS17/2.8 cells revealed a 2.4-fold decrease in transcription in cells overexpressing Cx45 (Fig. 4A). To verify that this effect was gap junction-mediated, ROS17/2.8 cells were treated with 100 μM oleamide, a chemical inhibitor of gap junctional communication, 24 h after transfection with CT-RSVLUC reporter. Treatment with the gap junction inhibitor mimicked the effect of Cx45 overexpression, repressing 2.2-fold the CT element transcription activity (Fig. 4A). By contrast, the RSV minimal promoter reporter was unaffected by overexpression of Cx45 or by oleamide treatment. Furthermore, deletion of the CT element in the −92 to +32 osteocalcin homologous promoter context (−92ΔCT-OCLUC) or mutation of the CT element (−92mtCT-OCLUC) abrogated the sensitivity of the reporter to the overexpression of Cx45 (Fig. 4B). The activity of the empty reporter (pGL2) was unaffected by Cx45 overexpression. We also verified that transient transfection of Cx45 in this experimental setting resulted in efficient expression of Cx45,
as detected by immunoblotting in the pSSFV-Cx45-transfected cells only (Fig. 4A, inset). These results corroborate the notion that the CT element present in the proximal osteocalcin promoter is a gap junction-sensitive transcriptional unit.

Identification of Sp1- and Sp3-containing Complexes on the Osteocalcin CT Element—EMSA analysis using wild type and mutated CT cognate oligonucleotides allowed identification of a core element within the CT sequence to which the gap junction-sensitive complex binds. Nuclear extract was incubated with radiolabeled CT or a series of radiolabeled mutated CT oligonucleotides (Fig. 5). The gap junction-sensitive complex was unable to bind mut 9, mut 12, and mut 15 oligonucleotides and could only weakly bind mut 6 oligonucleotide. Binding was unaffected on the mut 3 oligonucleotide. These results define the minimal binding cognate as a CCTGCCCCTCCTGC motif located in the −70 to −57 region of the osteocalcin proximal promoter. This sequence shares considerable homology with reported CT-rich Sp-binding sites (28, 37–41).

To identify components of the gap junction-sensitive complex assembling on the CT element, supershift analysis and competition studies were performed with various antibodies and cognate binding sites for several transcription factors. The binding activity could not be supershifted by incubation with antibodies against c-Jun, ATF-1, ATF-2, or c-Fos (data not shown), all known to bind to the proximal osteocalcin promoter. However, incubation with 50- or 100-fold molar excess of unlabeled CT or Sp cognate oligonucleotides competed for binding of the gap junction-sensitive complex, whereas a 100-fold molar excess of...
a mutated Sp cognate did not (Fig. 6A, lanes 1–6). Furthermore, binding activity was not competed for by unlabeled Mzf1 cognate, or by the −146 to −125 osteocalcin promoter oligonucleotides, a region upstream of the CT element (Fig. 6A, lanes 7–10), suggesting that members of the Sp family of transcription factors may bind to the gap junction-sensitive CT element.

In support of this hypothesis, a decrease in binding and supershift of complex was observed when Sp1 or Sp3 antibodies were added to the binding reactions (Fig. 6B, lanes 2 and 6 for Sp1; lanes 3 and 7 for Sp3). By contrast, a supershift was not produced by an irrelevant antibody (Fig. 6B, lanes 4 and 8). Therefore, Sp1 and Sp3 are components of the gap junction-sensitive transcriptional complex assembled by the CT element in the osteocalcin promoter. A more robust supershift of Sp3 was produced in ROS/Cx45 nuclear extracts (Fig. 6B, lane 7) compared with ROS17/2.8 cells (Fig. 6B, lane 3). The amount of Sp1 supershifted from the complex was similar among the two cell types (Fig. 6B, lanes 2 and 6). These data suggest that the increase in binding activity may be a result of increased amounts of Sp3 present in the gap junction-sensitive complex in ROS/Cx45 cells.

Sp1 and Sp3 Have Opposite Effects on the Osteocalcin Promoter Activity—To corroborate the role of Sp factors in the gap junction-sensing transcriptional complex, we next analyzed the functional effect of Sp1 and Sp3 on the CT element. As shown in Fig. 7A, cotransfection of the CT-RSVLUC reporter with pCMV-Sp1 expression construct in ROS17/2.8 cells resulted in 2-fold induction of transcription relative to control cells cotransfected with empty pCMV vector, whereas cotransfection of CT-RSVLUC with pCMV-Sp3 resulted in repression of transcription. Importantly, Sp3 expression antagonized Sp1-induced transcriptional activation of the CT element when pCMV-Sp1 and pCMV-Sp3 were cotransfected into ROS17/2.8 cells. Thus, Sp1 and Sp3 have antagonistic effects on the CT element. Deletion of the CT element from the homologous promoter context (−92ΔCT-OCLUC) blocked the effect of Sp1 and Sp3 on reporter transcription, and mutation of the CT core sequence (−92mtCT-OCLUC) blunted the effects of Sp1 and Sp3 (Fig. 7B). The failure of the mutation in the −92mtCT construct to abrogate completely transactivation/repression is probably secondary to the small 2-bp mutation introduced in the CT core sequence, which is most likely insufficient to completely prevent binding of Sp1/Sp3 to the CT element. These data demonstrate that Sp1 can activate transcription specifically from the CT element and that Sp3 can antagonize Sp1-induced transactivation. In order to more clearly establish the roles of Sp1 and Sp3 function on the CT element, cotransfection studies were also performed in the Drosophila SL2 cell line, which lacks endogenous Sp1 and Sp3. SL2 cells were cotransfected with either empty RSVLUC minimal promoter-reporter construct or CT-RSVLUC heterologous promoter reporter construct, and with pPac0 (empty vector), pPacSp1, pPacSp3 expression constructs, or both pPacSp1 and pPacSp3 together. In the SL2 cell background, Sp1 activated transcription from the CT element to an even larger degree (>50-fold) than that observed in the ROS17/2.8 cells, whereas Sp3 repressed transcription 1.8-fold, and when coexpressed with Sp1, Sp3 antagonized Sp1 transcriptional activation (Fig. 7C). The empty RSVLUC vector was unaffected by Sp1 or Sp3 (data not shown).

Recruitment of Sp1 and Sp3 on the Osteocalcin Promoter Is Gap Junction-sensitive—By having shown that Sp1 and Sp3 interact physically and functionally with the osteocalcin CT element, we next assessed the recruitment of Sp1 and Sp3 to the CT element in different gap junctional communication environments using formaldehyde cross-linking and ChIP. EMSA data suggested an increase in the abundance of Sp3 in ROS/Cx45 compared with ROS17/2.8 extracts in supershift experiments, and the ChIP assay would provide a more precise quantitative estimation of the relative abundance of Sp1/Sp3 assembled on the CT element. ROS17/2.8 and ROS/Cx45 cells were analyzed for promoter occupancy using anti-Sp1 and anti-Sp3 antibodies, which specifically and nearly equally immuno-
Data are from representative experiments and are presented as reduced in cells overexpressing Cx45 and in cells treated with oleamide.

As a positive element in the communication-deficient cells. As a positive.

ROS/Cx45 cells, Sp3 was the predominant occupant of the CT element in ROS17/2.8 cells, perhaps slightly favoring Sp1 occupancy (Fig. 8). Although both Sp1 and Sp3 antibodies also immunoprecipitated the CT element in ROS17/2.8 cells, Sp3 was the predominant occupant of the CT element in the communication-deficient cells. As a positive control, a region of the collagen I α1 (col1A1) promoter containing two canonical Sp binding cognates was used in the ChIP assay (42, 43). Interestingly, recruitment of Sp1/Sp3 mimicked the pattern observed with the osteocalcin promoter; Sp1 and Sp3 antibodies could immunoprecipitate the col1A1 promoter in ROS17/2.8 cells, whereas only Sp3 antibodies could immunoprecipitate the col1A1 promoter in ROS/Cx45 cells. These data suggest that regulation of Sp1/Sp3 recruitment to transcriptionally active complexes may be a general mechanism for gap junction-sensitive modulation of gene promoters. Indeed, we have shown previously that, like osteocalcin, collagen I mRNA and protein are down-regulated in primary osteoblasts derived from mice genetically deficient of Cx43 (25).

Accordingly, real time PCR performed using col1A1 gene-specific primers revealed a 3.9-fold reduction in mRNA abundance in ROS/Cx45 compared with ROS17/2.8 cells (Fig. 9A). Furthermore, a heterologous minimal promoter driven by a portion of the col1A1 promoter spanning two Sp-binding cognates was repressed when gap junctional communication was perturbed by overexpression of Cx45 or by treatment with the gap junction inhibitor oleamide (Fig. 9B).

Finally, we examined whether interference with gap junction function via the overexpression of Cx45 alters the abundance and/or post-translational processing of Sp1 or Sp3, thus accounting for their altered recruitment to the CxRE. No significant change in protein levels for either transcription factor was detected in nuclear extracts from ROS17/2.8 and ROS/Cx45 cells immunoblotted using anti-Sp1 and anti-Sp3 antibodies (Fig. 10A).

Immunoprecipitation of nuclear extracts from ROS17/2.8 cells transfected with pcDNA3 or Cx45 was then performed, followed by Western blotting with phospho-threonine, phosphoserine (Fig. 10B), or acetyl-lysine antibodies (Fig. 10C). There was a 3.6-fold decrease in threonine phosphorylation of Sp1 (normalized to the amount of immunoprecipitated Sp1) when gap junctional communication was reduced (Fig. 10B). Likewise, serine phosphorylation of Sp1 was reduced 9.4-fold in ROS/Cx45 cells (Fig. 10B). On the other hand, the acetylation of Sp3 was increased 1.9-fold (normalized to the amount of immunoprecipitated Sp3) in cells whose gap junctional communication was disrupted (Fig. 10C). These data demonstrate an alteration in post-translational modification of Sp1/Sp3 when gap junctional communication is perturbed.

**DISCUSSION**

We and others (25–27, 29, 30) have demonstrated previously that disruption of gap junctional communication among osteoblasts results in the misexpression of numerous genes, including down-regulation of osteocalcin. However, the molecular mechanism by which gap junctional communication modulates gene expression is unknown. In this work, we have identified a minimal element in the −70 to −57 region of the proximal rat osteocalcin promoter that is sensitive to changes in gap junctional communication. DNA binding activity and transcription from this element are both affected by changes of gap junctional communication induced by expression of an exogenous connexin, Cx45, in an endogenous Cx43 background. The chemical inhibitor of gap junctions, oleamide, recapitulates the transcriptional repression of osteocalcin, indicating that the down-regulation is indeed gap junctional communication-dependent. This CT-rich region of the promoter, which we name the CT element, or CxRE, is relatively well conserved among both the mouse Oγ1 and Oγ2, and the rat osteocalcin promoters (44, 45). A high degree of conservancy also extends to the human osteocalcin gene promoter (44), suggesting functional conservation as well. Thus, the CT element/CxRE represents the first identified transcriptional unit that senses changes in gap junctional communication.

Interestingly, deletion or mutation of the CT element generates only a 2-fold de-repression of the −92 to +32 osteocalcin promoter, whereas overexpression of Cx45 causes a 3–4-fold repression from the −92 to +32 osteocalcin promoter (compare Fig. 1B to Fig. 4B). The cause of this apparent discrepancy may be multiple. First, in addition to the Sp1/Sp3-containing complex, another faster migrating complex binds to this CT-rich element, and deletion or mutation of the CT element will alter the activity of this second complex, which in turn may account for the lack of full de-repression. Second, slight variation in Cx45 expression levels among experiments after transient transfection may result in variable degrees of de-repression when CT is deleted or mutated. Indeed, overexpression of Cx45 results in 2-fold repression of osteocalcin transcription in the context of the endogenous gene promoter, commensurate to the 2-fold repression of CT-driven transcription and the 2-fold de-repression when the CT element is deleted or mutated. Finally, we cannot completely rule out that disruption of gap junctional communication may have minor effects on other areas of the osteocalcin promoter, although these data clearly demonstrate that the CT element/CxRE is the major gap junctional sensitive element present in the osteocalcin promoter.

The present work also establishes that the complex binding
to the CT-rich CxRE contains the Sp1 and Sp3 transcription factors. Sp1 is a ubiquitously expressed zinc finger-containing transactivator that regulates numerous constitutive and inducible genes (46, 47). Sp3 is also widely expressed but can activate or repress gene transcription depending on cell and promoter context (48). Sp1 and Sp3 regulate transcription via binding to identical cognate DNA elements with similar affinities (49). In the osteocalcin promoter, Sp1 and Sp3 have antagonistic actions. Although Sp1 is a strong activator of transcription from the CT element, Sp3 is a repressor and inhibits Sp1-induced transactivation, a mechanism also reported in other cell systems (50, 51). The CT element/CxRE we have identified as cognate for Sp1/Sp3 binding in the proximal osteocalcin promoter differs from the canonical GC and GT boxes where Sp family members typically bind (52). However, other CT-rich elements have been identified in other cell types as binding Sp1 (37, 38, 53–55). Therefore, the pyrimidine-rich core motif, CCTGCCCCTCCTGC, represents an alternative site for Sp1/Sp3-mediated transcriptional regulation.

More recently, Yeung and co-workers (56) reported Sp1 binding to the CT element in the human osteocalcin promoter in prostate cancer cells. They were also able to link Sp1 activity to misexpression of the osteocalcin gene, suggesting that Sp1 may be involved in the development of a differentiated osteoblastic phenotype. This notion is fully in line with the present data, which define Sp1 as a strong activator of osteoblast gene expression. Unfortunately, ablation of the Sp1 gene in mice is embryonically lethal (47), thus precluding the analysis of lack of Sp1 on bone. However, Sp3 null mice die shortly after birth and do exhibit defects in late tooth development and impaired

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**FIG. 11. Model of gap junctional regulation of gene transcription from CxREs.** A, ROS17/2.8 cells, which are coupled exclusively by Cx43, transmit signal molecules among cells through gap junctions. These signals are propagated to the nucleus where they modulate transcription at CxREs. In this highly coupled environment, Sp1 is serine- and threonine-phosphorylated in pcDNA3-transfected cells, but the degree of phosphorylation was markedly reduced in cells transfected with Cx45. Immunoprecipitates were re-probed with Sp1 antibodies to normalize for the amount of precipitated material. The ratio of phosphorylated Sp1 divided by immunoprecipitated (IP) Sp1 band intensities is noted below each lane. B, immunoprecipitation of Sp3 from nuclear extracts from ROS17/2.8 cells transiently transfected with pcDNA3 or pSFFV-Cx45. Nuclear proteins were immunoprecipitated with Sp3 antibodies, immunoblotted, and probed with anti-phosphothreonine and anti-phosphoserine antibodies. Sp1 is serine- and threonine-phosphorylated in pcDNA3-transfected cells, but the degree of phosphorylation was markedly reduced in cells transfected with Cx45. Immunoprecipitates were re-probed with Sp1 antibodies to normalize for the amount of precipitated material. The ratio of phosphorylated Sp1 divided by immunoprecipitated (IP) Sp1 band intensities is noted below each lane.
Ossification (57, 58). Surprisingly, osteocalcin production by osteoblasts is down-regulated when Sp3 is ablated (57). However, this seemingly paradoxical result may be an indirect consequence of Sp3 deletion, because many genes are controlled by Sp1/Sp3 transcription factors (58). The emerging strong role of Sp1/Sp3 regulation of bone-forming cells apparently contrasts with the rather ubiquitous distribution of these transcription factors. Although it is unlikely that Sp1/Sp3 plays a dominant role in controlling bone formation, they may integrate different and perhaps more general regulatory modes, for example intercellular signaling through gap junctions. In the osteoblast cell context, the physical proximity of the CT element/CxRE with OSE1, which confers osteoblast-specific transcriptional regulation to the osteocalcin promoter (44, 45, 59), may allow recruitment of tissue-specific factors into a higher order complex also containing Sp1 and Sp3, and thus provide both tissue specificity and Sp-mediated regulation of transcription. Although this model remains speculative, a polymorphism in one Sp1-binding site in the col1A1 promoter has been correlated to decreased bone mass and osteoporotic fractures in humans (60).

A major novel finding of this work is the demonstration that the ratio of Sp1/Sp3 recruited to the osteocalcin promoter is altered when gap junctional communication is disrupted. In well coupled ROS17/2.8 cells, which express Cx43 only, the ratio of Sp1 and Sp3 assembled on the osteocalcin CT element/ CxRE favors Sp1, resulting in increased transcription. In communication-impaired ROS/Cx45 cells, more Sp3 than Sp1 is recruited onto the CT-rich CxRE, resulting in repression of the osteocalcin promoter (44, 45, 59), may allow recruitment of tissue-specific factors into a higher order complex also containing Sp1 and Sp3, and thus provide both tissue specificity and Sp-mediated regulation of transcription. Although this model remains speculative, a polymorphism in one Sp1-binding site in the col1A1 promoter has been correlated to decreased bone mass and osteoporotic fractures in humans (60).

Modulation of Sp1/Sp3 recruitment to Sp-binding sites by changes in gap junctional communication is very likely due to post-translational effects on these Sp factors, because their relative protein abundance appears unchanged. Sp1 can be modified by both glycosylation and phosphorylation. Alterations of Sp1 glycosylation affects its interaction with basal transcription factors and interferes with Sp1 protein turnover (62, 63). Importantly, Sp1 can be phosphorylated by numerous kinases, including DNA-dependent kinase (64), Sp1 kinase (65), protein kinase A (66, 67), casein kinase II (68), protein kinase C (66, 69), cyclin A/cyclin-dependent protein kinase 2 complex (70), and mitogen-activated protein kinase (65, 67, 71). On the other hand, Sp3 repressive activity is enhanced by acetylation (72). We have shown a decrease in serine/threonine phosphorylation of Sp1 and an increase in Sp3 acetylation upon disruption of gap junctional coupling, suggesting that signals passing through gap junctions regulate signaling cascades that in turn post-translationally modulate Sp1/3 binding affinity and/or transcriptional activity. Based on the pore size selectivity of Cx43, which is permeable by ~1-kDa molecules with a negative charge preference, and Cx45 gap junctions, which are permeable to ~0.4-kDa molecules with a positive charge preference, one could predict that intercellular diffusion of signaling molecules such as cyclic nucleotides or inositol phosphates may be impaired when Cx43 permeability is decreased by interaction with Cx45 (2, 3, 73). We hypothesize that these signaling molecules that can pass through gap junctions when the intercellular channels are open and communication is allowed, activate and maintain signaling cascades that in turn modulate Sp1/Sp3 binding affinity and/or activity, and thus the expression of target genes (Fig. 11). Indeed, such osteoblastic genes as type I collagen, alkaline phosphatase, and bone sialoprotein, in addition to osteocalcin, contain Sp1-binding sites in their promoters, and are down-regulated in response to disruption of gap junctional communication (74–76). Considering the ubiquitous presence of Sp-binding sites in many gene promoters, it is highly likely that this mode of transcriptional regulation is a general mechanism of cell biology.

Understanding the signaling events initiated by signal molecules passing through gap junctions will provide insight into the molecular mechanisms of disease associated with perturbation of gap junctional communication. In this study, we have identified the ubiquitous transcription factors, Sp1 and Sp3, as downstream targets of signaling events altered when gap junctional communication is disrupted. It is likely that modulation of Sp1/Sp3 recruitment to promoters is not cell type-specific and may provide answers to the role of gap junctions in controlling gene expression in tissues other than bone.

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