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Gap Junctional Communication Modulates Gene Expression in Osteoblastic Cells

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Bone-forming cells are organized in a multicellular network interconnected by gap junctions. In these cells, gap junctions are formed by connexin43 (Cx43) and connexin45 (Cx45). Cx43 gap junctions form pores that are more permeable to negatively charged dyes such as Lucifer yellow and calcein than are Cx45 pores. We studied whether altering gap junctional communication by manipulating the relative expression of Cx43 and Cx45 affects the osteoblast phenotype. Transfection of Cx45 in cells that express primarily Cx43 (ROS 17/2.8 and MC3T3-E1) decreased both dye transfer and expression of osteocalcin (OC) and bone sialoprotein (BSP), genes pivotal to bone matrix formation and calcification. Conversely, transfection of Cx43 into cells that express predominantly Cx45 (UMR 106–01) increased both cell coupling and expression of OC and BSP. Transient cotransfection of promoter–luciferase constructs and connexin expression vectors demonstrated that OC and BSP gene transcription was down-regulated by Cx45 cotransfection in ROS 17/2.8 and MC3T3-E1 cells, in association with a decrease in dye coupling. Conversely, cotransfection of Cx43 in UMR 106–01 cells up-regulated OC and BSP gene transcription. Activity of other less specific osteoblast promoters, such as osteopontin and osteonectin, was less sensitive to changes in gap junctional communication. Thus, altering gap junctional permeability by manipulating the expression of Cx43 and Cx45 in osteoblastic cells alters transcriptional activity of osteoblast-specific promoters, presumably via modulation of signals that can diffuse from cell to cell. A communicating intercellular network is required for the full elaboration of a differentiated osteoblastic phenotype.

INTRODUCTION

Bone remodeling is a lifelong process, necessary to replace aging bone tissue and to repair injuries. Adequate remodeling of the skeletal tissue requires the coordinated activity of bone-resorbing and -forming cells. Cells can communicate via soluble factors, and the bone microenvironment is abundant in cytokines and growth factors with paracrine and autocrine functions. Increasing evidence indicates that direct cell-to-cell interactions are also critically involved in a variety of fundamental processes in bone physiology, such as support of osteoclastogenesis by stromal cells (Udagawa *et al.*, 1989), fusion of osteoclast precursors (Mbalaviele *et al.*, 1995), induction of osteoblast cytokines by T cells (Tanaka *et al.*, 1995), and osteogenic cell proliferation (Van der Plas and Nijweide, 1988) and hormonal response (Van der Plas and Nijweide, 1988; Van der Molen *et al.*, 1996). Direct intercellular contact and communication are of obvious relevance to bone-forming cells, i.e., osteoblasts, which form an epithelium-like network over the bone surface and are interconnected by abundant junctional structures, including adherens and gap junctions (Doty, 1981; Palumbo *et al.*, 1990).

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Gap junctions are aqueous intercellular channels that allow the diffusion of small molecules and ions from cell to cell. Each gap junction pore is formed by juxtaposition of two hemichannels in adjacent cells, and each hemichannel is composed of a hexameric array of transmembrane proteins called connexins (Beyer *et al.*, 1990; Sáez *et al.*, 1993). Different connexins have different molecular permeabilities and differing abilities to interact with each other. We have previously found that the rat osteosarcoma cell line ROS 17/2.8 expresses the gap junction protein connexin43 (Cx43) and is well dye coupled, whereas the rat osteogenic sarcoma cells UMR 106–01 express predominantly connexin45 (Cx45) and is poorly dye coupled. Furthermore, expression of Cx43 in UMR 106–01 cells increases dye coupling, while expression of Cx45 in ROS 17/2.8 cells reduces dye coupling by 50% (Steinberg *et al.*, 1994; Koval *et al.*, 1995). These two osteoblastic cell lines also differ in their ability to produce bone matrix proteins: ROS 17/1.8 cells produce most osteoblast-specific markers, including osteocalcin (OC), bone sialoprotein (BSP), and alkaline phosphatase (AP), in basal and stimulated conditions (Rodan *et al.*, 1989), but UMR 106–01 expresses little, if any, OC and BSP in resting conditions. Likewise, an immortalized mouse cell line MC3T3-E1, which is able to differentiate in culture (Sudo *et al.*, 1983), also express Cx43 and is well coupled (Yamaguchi *et al.*, 1994). In the present studies, we have found that manipulation of gap junctional communication by overexpression of Cx45 in ROS 17/2.8 and MC3T3-E1 cells and Cx43 in UMR 106–01 and MC3T3-E1 cells alters basal expression of osteoblast genes in a reciprocal manner. Our data directly demonstrate that gap junctional communication modulates transcriptional activity of osteoblast-specific promoters, thus pointing to a fundamental physiological role of intercellular communication for the function of specialized tissues, such as bone.

MATERIALS AND METHODS

Cell Models

The osteogenic sarcoma cell line ROS 17/2.8 was provided by Dr. Gideon Rodan (Merck Research Laboratories, West Point, PA). ROS 17/2.8 cells have been shown to express several osteoblastic features, including production of osteocalcin and other matrix proteins (Majeska *et al.*, 1980, 1985). These cells were cultured in MEM-F12 containing 10% heat-inactivated FBS (Summit Biotechnology, Fort Collins, CO). The UMR 106–01 cells were a gift of Dr. Nicola C. Partridge, St. Louis University (St. Louis, MO). These cells are derived from the rat osteogenic sarcoma cell line UMR 106, which has been characterized as having an osteoblastic phenotype (Partridge *et al.*, 1983; Forrest *et al.*, 1985). They were also maintained in MEM supplemented with 10% heat-inactivated FBS and antibiotics. Subcultures until passage 30 were used in these studies. The mouse calvaria osteoblasts MC3T3-E1 were obtained from the American Type Culture Collection (Rockville, MD), and grown in MEM-F12 medium, as described for the ROS 17/1.8 cells. This cell line represents phenotypically immature osteoblasts, derived from spontaneous immortalization of calvaria cells selected by the 3T3 passaging protocol (Sudo *et al.*, 1983).

Clones of ROS 17/2.8 or UMR 106–01 cells stably expressing either chick Cx45 (ROS/Cx45) or rat Cx43 (UMR/Cx43), respectively, were generated by transfection with the pSFFV-Neo vector containing the reading frames of either connexin, as detailed in previous reports (Steinberg *et al.*, 1994; Koval *et al.*, 1995). For transient transfections, both these Cx43 and Cx45 constructs in pSFFV-Neo, as well as a construct generated by inserting chick Cx45 in pZeocin (Promega, Madison, WI), were used.

Chemicals

Reagents for molecular biology were purchased from Promega. Synthetic oligonucleotides were obtained from Life Technologies (Grand Island, NY). [α^{32}P]-dCTP was purchased from Amersham (Arlington Heights, IL). Antibodies against Cx43 were produced by immunizing rabbits with synthetic peptides corresponding to amino acids 252–271 of rat Cx43 (Beyer and Steinberg 1991; Kanter *et al.*, 1992). Antibodies against Cx45 were produced by immunizing rabbits with a Cx45 carboxyl terminus–6His fusion protein, which was generated using the pET15b vector and purified using previously described methods (Koval *et al.*, 1995). 1,25$(OH)_2D_3$ was a kind gift from Dr. Milan Uskokovic (Hoffman LaRoche, Nutley, NJ). Calcein acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR), dissolved in DMSO to a concentration of 1 mg/ml (1 mM), aliquoted, and stored at −20 C in the dark. The membrane permanent dye PKH-26 was purchased from Zynaxis Cell Science (Malvern, PA), and it was dissolved in aqueous solutions according to the manufacturer's instructions, as described below. All other chemicals and the tissue culture media were from Sigma Chemical (St. Louis, MO), unless otherwise indicated.

Alkaline Phosphatase Assay

A previously described method was used (Cheng *et al.*, 1994b). Briefly, cell layers in confluent dishes were scraped into 0.5 ml of 50 mM Tris, pH 7.4, and alkaline phosphatase activity was measured in the cell lysate as *p*-nitrophenol produced from *p*-nitrophenyl phosphate. Enzyme activity was expressed in nanomoles/min/mg protein. Protein was determined using the method of Bradford (1976). Experiments were performed in quadruplicate dishes.

Cell Proliferation

Following a published procedure (Reid *et al.*, 1988), cells were seeded at a density of 2×10^5 cells/well and incubated in MEM-F12 or MEM containing 10% FCS. One day later, the medium was replaced with fresh, serum-free growth medium and incubated for an additional 24 h. Fresh medium containing 2 mM [methyl-^{3}H]-thymidine (1 mCi/ml) and 10% FCS was then added for the last 24-h incubation. The amount of isotope incorporated in proliferating cells was measured in trichloroacetic acid-precipitated material and corrected for protein content. Experiments were performed in triplicate wells.

RNA Blots

Poly-A RNA was purified from cell extracts using the Mini RiboSep kit (Collaborative Biochemical Products, Bedford, MA) as described previously (Cheng *et al.*, 1994a,b). Samples (10 μg/lane) were separated on 1% formaldehyde agarose gels by electrophoresis, blotted onto nylon membranes, and UV cross-linked. The membranes were hybridized using [^{32}P]-labeled probes made by a random primed oligonucleotide method (Boehringer Mannheim, Indianapolis, IN) in 40% formamide, 10 mM Tris, 5×SSC, 125 mg/ml salmon sperm DNA, 1.25× Denhardt's solution, at 42°C, and washed twice in 2×SSC, 0.1% SDS at 42°C, followed by one high-stringency wash in 0.2×SSC, 0.1% SDS at 52°C for 25 min. The following cDNA probes were employed: mouse OC, mouse OP, rat AP, mouse BSP, rat

GAPDH, chicken Cx45, and rat Cx43. The sources and preparation of all these probes have been previously reported (Civitelli *et al.*, 1993; Cheng *et al.*, 1994b). The relative amounts of mRNA were quantitated by densitometric analysis of the autoradiographic bands, after normalization for the intensity of GAPDH.

Dye Coupling

Two methods were used to assess gap junctional permeability to negatively charged dyes. Intercellular transfer of microinjected Lucifer yellow was used for homogeneous cell population of stable connexin transfectants and parent clones, as described previously (Steinberg *et al.*, 1994; Koval *et al.*, 1995) on cells grown on a glass coverslip. Fluorescence was monitored using a charge-coupled device camera with an image intensifier (Dage MTI, Michigan City, IN) and an image-processing system (Georgia Instruments, Roswell, GA). The number of adjacent cells containing dye 3–5 min after the injection was recorded as a measure of dye coupling.

To assess the degree of coupling in transiently transfected cells, in which the exogenous connexin is expressed only in ~15% of the cells, the newly developed "parachute assay" was employed (Ziambaras *et al.*, 1998). This method is based on transfer of calcein from preloaded, donor cells to recipient, acceptor cells. Briefly, ROS 17/2.8 or UMR 106–01 cells were preloaded with calcein using the acetoxymethyl-ester form of the dye and added on top of a monolayer of the same cell type at the end of a 72-h incubation with the appropriate expression vectors. The cells in the monolayer were previously labeled with the permanent membrane dye, PKH-26. After the parachuted cells were allowed to settle and dye transfer had occurred (~2 h), cells were released from the culture dishes, and the number of calcein-loaded, donor cells and PKH-26–labeled, acceptor cells were counted by FACS. The proportion of double-labeled cells, representing the acceptor cells that have taken calcein via gap junctions, relative to the total number of potential acceptor cells, was expressed as "transfer ratio," which represents a quantitative estimation of the degree of coupling in the entire population, and takes into account the donor:acceptor cell ratio, a critical variable in each experiment (Ziambaras *et al.*, 1998).

Promoter–Luciferase Reporter Constructs

The rat OC and BSP promoter–luciferase reporter constructs, OCLUC and BSPLUC, containing the −637 to +32, or the −795 to +38 5′-flanking sequence relative to the transcriptional start sites of the OC and BSP genes, respectively, were prepared as described previously (Towler *et al.*, 1994). This OC promoter fragment includes all the known regulatory sequences necessary for supporting OC expression in osteoblasts, and the BSP promoter fragment contains all known basal elements for BSP expression. OPLUC was prepared by PCR amplification of the −867 to +33 sequence of the human OP promoter, using genomic DNA as a template. Primers were designed to include *Kpn*I and *Mlu*I restriction sites at the 5′- and 3′-ends of the product, respectively. Each promoter fragment was purified by agarose gel electrophoresis and then subcloned into *Kpn*I/*Mlu*I-digested promoterless pGL2-Basic (GeneLight Plasmid, Promega) upstream of the luciferase reporter gene. The ONLUC construct was obtained by excising the −504 to +63 fragment of the bovine ON gene from the ONREX construct (courtesy of Dr. Marian Young, National Institute of Dental Research, National Institutes of Health) by *Hind*III digestion (Dominguez *et al.*, 1991). The 0.6 kilobase (kb) restriction fragment was purified and subcloned into the pGL2-Basic vector, digested with *Hind*III. Orientation of the insert was assessed by restriction fragment analysis.

Transient Transfection and Luciferase Assay

Transient transfections were performed in triplicate, and transfection efficiency was monitored using the pGL2-Promoter (SV40LUC, Promega) or the cytomegalovirus-β-galactosidase (Promega) vec-

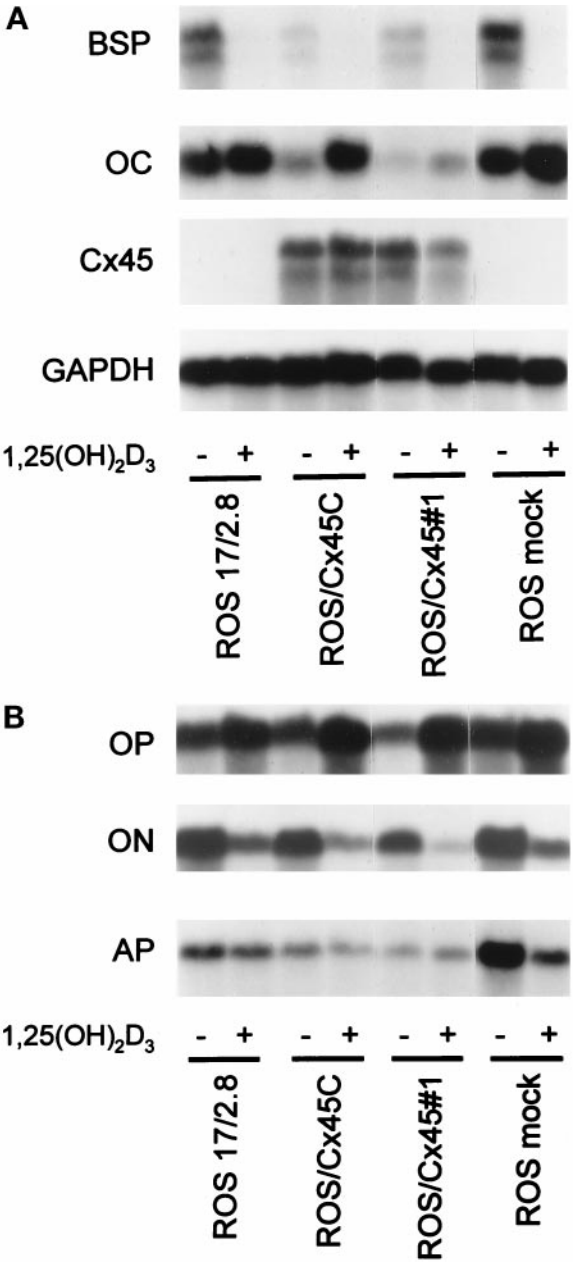

Figure 1. Steady-state mRNA levels of osteoblast products are altered by overexpression of chick Cx45 in ROS 17/2.8 osteoblastic cells. Cells stably expressing chick Cx45 (ROS/Cx45) or mock-transfected cells (ROS mock), as well as their parent cells (ROS 17/2.8), were grown to confluence in the same conditions, in the presence (+) or in the absence (−) of 10^{-8} M $1,25(OH)_2D_3$, and poly-A RNA was separated on agarose gel and blotted onto nylon membranes. Membranes were sequentially hybridized with [^{32}P]-labeled cDNA probes for (A) OC, BSP, Cx45, GAPDH, and (B) alkaline phosphatase (AP), ON, and OP. Between each hybridization step, the membrane was extensively washed and re-exposed to x-rays to ensure complete removal of the previous hybrizidation band.

tors in parallel cultures. For these experiments, osteoblastic cells were plated at high density (3×10^5 cells/well) onto 12-well plates. Twelve to 18 h later, cells were rinsed, and 0.15 ml of serum-free medium containing 0.2 mg/ml DEAE-dextran (Promega) and the appropriate plasmids were added to each well. After an initial 20-min incubation at 37°C, 0.3 ml of serum-free medium was added and the incubation was continued for an additional 30 min. Thereafter, cells were shocked by exposure for 2 min to 10% DMSO in PBS at 20°C, rinsed twice, and incubated in complete medium for 72 h. In some experiments, transfections were performed using the calcium–phosphate coprecipitation method using 1–8 μg/ml circular DNA, followed by glycerol (15%) shock, as previously described (Towler *et al.*, 1994). Cell lysates (0.25 ml/well) were prepared using the Promega Luciferase Assay System, according to the manufacturer's recommendations, and luciferase activity was measured in an Optocomp luminometer (MGM Instruments, Hamden, CT).

Immunoblots

Cells were cultured on 100-mm tissue culture plates to 75–85% confluence and incubated with the experimental compounds as appropriate (Civitelli *et al.*, 1993; Steinberg *et al.*, 1994). Cells were solubilized in 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.5% BSA, 50 mM Tris, pH 8, containing a cocktail of protease and phosphatase inhibitors. Protein concentration in each sample was determined before electrophoresis using the method of Bradford (1976). Proteins were separated by electrophoresis on 10% polyacrylamide gels, and transferred to polyvinylidene membranes (Immobilon P, Millipore, Bedford, MA) using a tank transfer apparatus (Trans-blot Cell, Bio-Rad, Richmond, CA). After blocking with 5% nonfat milk, the membranes were incubated with the anti-Cx45 antibody overnight at room temperature, and then washed in PBS and incubated with an anti-rabbit antibody conjugated to HRP (Tago, Burlingame, CA). The immune reaction was detected by exposing the membranes to autoradiography film (Hyperfilm, Amersham, Arlington Heights, IL) in the presence of luminol using the ECL detection kit (Amersham), according to the manufacturer's recommendations.

Immunofluorescence

A previously described method was used (Cheng *et al.*, 1994a; Steinberg *et al.*, 1994). Briefly, cells were fixed in 3% paraformaldehyde, permeabilized in 0.5% Triton-X100 buffer, and incubated in 2% heat-inactivated goat serum to reduce nonspecific binding. The coverslips were then incubated with the appropriate dilutions of a rabbit anti-connexin antibody, followed by a biotinylated anti-rabbit secondary antibody, and rhodamine-conjugated streptavidin. After mounting on glass slides, the coverslips were sealed for fluorescence microscopy.

RESULTS

To test whether changes in gap junctional communication affect osteoblast gene expression, we first studied the phenotypic features of stable ROS/Cx45 cell transfectants. As reported previously, overexpression of chick Cx45 reduces intercellular transfer of Lucifer yellow or calcein by more than 50% compared with parent ROS 17/2.8 cells, without affecting the expression of endogenous Cx43 (Koval *et al.*, 1995). Steady-state mRNA levels for OC and BSP were markedly lower in two ROS/Cx45 clones compared with the parent cells and mock-transfected cells (Figure 1A). Slightly lower AP and osteonectin (ON) mRNA levels were also observed in the ROS/Cx45 cells, whereas osteopontin (OP) mRNA was, if anything, slightly higher than in parent cells (Figure 1B). Despite the different basal levels of bone matrix protein mRNA expression, ROS/Cx45 transfectants retained their responsiveness to $1,25(OH)_2D_3$. The abundance of OC transcripts was considerably increased by 24 h incubation with $1,25(OH)_2D_3$ (10^{-8} M) in each cell line, whereas BSP mRNA was undetectable in $1,25(OH)_2D_3$-treated cells (Figure 1A). As expected, decreases in AP and ON and an increase of OP mRNA expression were also observed after $1,25(OH)_2D_3$ treatment in all cell lines (Figure 1B). Importantly, the vitamin D metabolite did not affect either Cx45 mRNA expression (Figure 1A), or dye coupling in any of these cells (our unpublished results).

The above results are summarized in a more quantitative manner in Table 1. The ability to transfer Lucifer yellow was decreased >50% in the clones expressing Cx45 compared with parent cells, and the decreased dye coupling was associated with signifi-

Table 1. Osteoblast phenotypic markers in ROS 17/2.8 cells and ROS/Cx45 transfectants

Cell line	Cx45 abundance[a]	Dye coupling (coupled cells/cell)[b]	Matrix protein mRNA[c]					AP activity (mmol/min/mg)	Cell proliferation (dpm $\times 10^3$/ μg protein)
			OC	BSP	OP	ON	AP		
ROS 17/2.8	0	11.2 ± 6.6 (59)	100	100	100	100	100	1942 ± 89	106.5 ± 6.3
ROS/Cx45C	100 ± 13	3.6 ± 3.7[d] (74)	44 ± 18[d]	81 ± 45[d]	127 ± 17[d]	64 ± 2[d]	48 ± 3[d]	474 ± 99[d]	118.8 ± 13.0
ROS/Cx45#1	87 ± 31	4.8 ± 2.7[d] (40)	29 ± 20[d]	65 ± 25[d]	132 ± 6[d]	62 ± 18[d]	32	519 ± 160[d]	96.0 ± 8.6
ROS/mock	0	n.d.	120	132	138	125	107	2182 ± 112	127.9 ± 31.0

Data are the average ± SD (n = 2, if SD not shown); n.d., not done. ROS/Cx45C and ROS/Cx45#1 represent two different, stably transfected ROS/Cx45 clones.

[a]Cx45 protein abundance relative to ROS/Cx45C, assessed by immunoblot in three experiments.

[b]Assessed by cell-to-cell diffusion of microinjected Lucifer yellow. In parenthesis is the number of cells microinjected.

[c]Intensity of specific mRNA band relative to ROS 17/2.8, after normalization to GAPDH (n = 3).

[d]$p < 0.05$ vs. ROS 17/2.8 (*t*-test for unpaired samples).

cantly lower (55–75%) basal OC and BSP mRNA abundance relative to well-coupled parent cells. Whereas ON mRNA was decreased ~35% in ROS/Cx45 transfectants, OP mRNA was slightly increased (~20%), in comparison with parent cells. Consistent with the mRNA data, AP activity was also greatly reduced in ROS/Cx45 clones. Importantly, overexpression of chick Cx45 did not alter the proliferation potential of these cells. Because these results were obtained in cells cultured under identical conditions and at the same confluence status, we conclude that changes in gap junctional communication alter basal expression of osteoblast phenotypic genes.

Since these data were obtained on the stable ROS/Cx45 transfectants, clone selection represents a potential confounder that may influence steady-state mRNA levels in the transfectants. In addition, expression of the osteoblastic phenotype is not normally regulated in these transformed cell lines. To address these issues and to determine whether the changed steady-state mRNA levels in ROS/Cx45 transfectants were a consequence of gene transcription modulation, we transiently cotransfected a chick Cx45 expression construct and constructs containing the cis-regulatory regions of the OC, BSP, ON, and OP genes, upstream of the luciferase reporter gene into either ROS 17/2.8 or MC3T3-E1 cells. In ROS 17/2.8 cells cotransfected with Cx45, both OC and BSP promoter activities were reduced to 44 ± 12% and 42 ± 11% (n = 4), respectively, relative to cells cotransfected with the vector only (Figure 2A). In contrast, Cx45 had minimal effect on an SV40–luciferase construct. Transcriptional activity of an osteonectin promoter–luciferase construct was also decreased, whereas OP promoter activity was not significantly affected in ROS 17/1.8 cells cotransfected with chick Cx45 (our unpublished results).

This effect of Cx45 overexpression was not limited to the ROS 17/2.8 cells, since closely similar results were obtained in the nontransformed, phenotypically immature mouse MC3T3-E1 cells. Like ROS 17/2.8, the MC3T3-E1 cells are highly dye coupled and express abundant Cx43 (Yamaguchi *et al.*, 1994), as well as OC and BSP (Boudreaux and Towler 1996; Towler, unpublished observation), and support the activity of the OC promoter (Towler *et al.*, 1994; Boudreaux and Towler 1996). As shown in Figure 2B, transient overexpression of chick Cx45 in MC3T3-E1 cells decreased OC and BSP promoter activities to 54 ± 8% and 51 ± 12% (n = 4) relative to vector-transfected cells, respectively. On the contrary, and at variance with the ROS 17/2.8 cells, ON–luciferase activity was not affected (95 ± 14%, n = 4), whereas the OP promoter activity was up-regulated by Cx45 transfection in MC3T3-E1 cells (148 ± 11%, n = 4). Identical results were obtained using either pSFFV-Neo or pZeocin vectors for Cx45 transfection, thus ruling out a spurious effect of the vector itself.

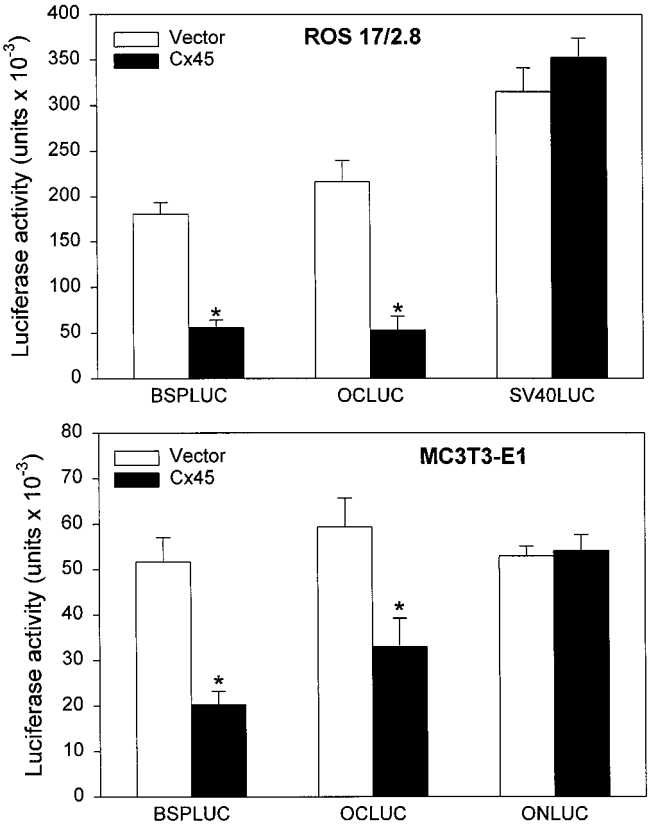

Figure 2. Transient transfection of chick Cx45 decreases transcriptional activity of OC and BSP promoters in ROS 17/2.8 (A) and MC3T3-E1 (B) osteoblastic cells. Cells were cotransfected with either a chick Cx45 expression construct or its vector, and one of the promoter-luciferase constructs, OCLUC, BSPLUC, SV40LUC, or ONLUC, as indicated. Luciferase activity was measured 3 d after transfection. Data are representative of four different experiments and are expressed as the average ± SD of triplicate wells for each condition. *, $p < 0.01$ vs. vector- transfected cells (*t* test for unpaired samples).

To further prove that the relative expression of Cx43 and Cx45 regulates osteoblast gene expression, we repeated similar experiments in another rat osteoblastic cell line UMR 106–01, which is poorly dye coupled and expresses predominantly Cx45 and little Cx43 on the cell surface (Steinberg *et al.*, 1994). First, a clone stably transfected with rat Cx43 (UMR/Cx43) was analyzed by RNA blotting and compared with the parent cells. Basal levels of OC mRNA were very low in this cell line, as would be anticipated based on previous work (Fraser *et al.*, 1988), but they were increased ~30% in the UMR/Cx43 transfectants (Figure 3). In contrast to the ROS 17/2.8 cells, OC mRNA expression was not sensitive to $1,25(OH)_2D_3$ in the UMR 106–01, independently of the level of Cx43 expressed. The OC mRNA levels correlated with higher degrees of coupling and Cx43 abundance in UMR/Cx43, whereas steady-state levels of BSP, OP, and ON

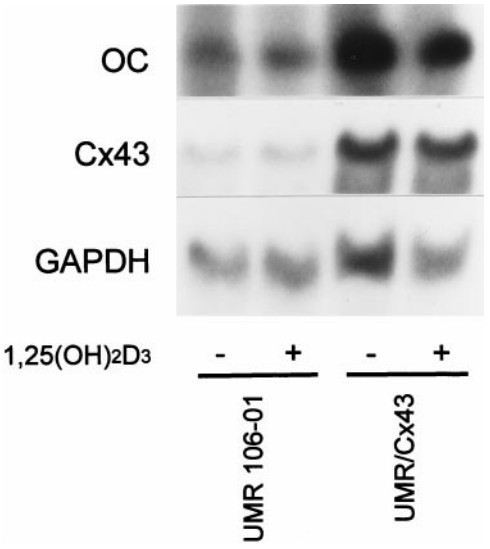

Figure 3. Steady-state OC mRNA levels are increased by overexpression of rat Cx43 in UMR 106–01 osteoblastic cells. UMR 106–01 cells stably expressing rat Cx43 (UMR/Cx43) and their parent cells were grown to confluence in the same culture conditions, and poly-A RNA was separated on agarose gel and blotted onto nylon membranes. Membranes were sequentially hybridized with [^{32}P]-labeled cDNA probes for OC, Cx43, and GAPDH, with extensive washes between each hybridization step.

were not significantly different in the transfectants, as compared with the parent clones (Table 2).

Next, we determined whether overexpression of Cx43 in UMR 106–01 cells up-regulated the OC promoter, as would be predicted if OC gene transcription were sensitive to the relative expression of Cx43 and Cx45. Similar experiments were also performed in MC3T3-E1 cells, which express endogenous Cx43 but at a lower degree than do ROS 17/2.8, and in the latter cell line. As exemplified in Figure 4, basal OC and BSP promoter activities were lower in UMR 106–01 than in ROS 17/2.8 or MC3T3-E1 cells. Cotransfection of OCLUC or BSPLUC with Cx43 increased transcriptional activity compared with vector-transfected cells in both UMR 106–01 (142 ± 16%, and 137 ± 12%, respectively, n = 4) and MC3T3-E1 cells (135 ± 11%, and 117 ± 11%, respectively, n = 6), whereas the SV40LUC construct was unaffected. On the other hand, no significant changes in transcriptional activity were observed by transfecting Cx43 into ROS 17/2.8 cells (our unpublished results). Thus, the relative expression of Cx43 and Cx45 modulates in a reciprocal manner the transcriptional activity of stage-specific promoters in osteoblasts.

Finally, we examined whether, under the conditions used for the gene transcription studies, transient overexpression of either connexin was associated with the predicted changes in dye coupling. First, we demonstrated by immunofluorescence that transfected Cx45 and Cx43 were present preferentially on the cell surface of either ROS 17/2.8 or UMR 106–01, respectively (Figure 5). The proportion of cells exhibiting positive staining was commensurate with a transfection efficiency of ~15%, and the higher abundance of Cx43 stain in UMR 106–01 cells, compared with Cx45 in ROS 17/2.8 cells, reflects expression of both exogenous and endogenous Cx43 in this cell line (Figure 5). Such cellular localization, compatible with functional gap junctions, is identical to that observed in stably transfected clones of the same cell lines (Steinberg *et al.*, 1994; Koval *et al.*, 1995). Because only a minority of cells express the exogenous connexin, classic dye microinjection methods are unsuitable for measuring chemical coupling in these transiently transfected clones. Therefore, to assess the ability to diffuse dyes in these conditions, we employed the newly developed parachute assay followed by FACS analysis (Ziambaras *et al.*, 1998). This method measures the degree of coupling in an entire cell population, expressed as "transfer ratio" (Ziambaras *et al.*, 1998). Thus, if connexin transfection changes gap junctional communication only in a fraction of cells, the contribution of this

Table 2. Osteolbast phenotypic markers in UMR 106 cells and UMR/Cx43 transfectants

Cell line	Cx43 abundance[a]	Dye coupling (coupled cells/cell)[b]	Matrix protein mRNA[c]			
			OC	BSP	OP	ON
UMR 106	1.00	1.6 ± 1.9 (74)	100	100	100	100
UMR/Cx43	11.75	5.4 ± 4.1 (55)[d]	125 ± 13[e]	107 ± 17	101	118

Data are the average ± SD.

[a]Cx43 protein abundance relative to UMR 106-01, assessed by immunoblot in two experiments.

[b]Assessed by cell-to-cell diffusion of microinjected Lucifer yellow. In parenthesis is the number of cells microinjected.

[c]Intensity of specific mRNA bands relative to UMR 106-01, after normalization to GAPDH (n = 3 or 2).

[d]From Steinberg *et al.*, (1994).

[e]$p < 0.05$ vs UMR 106-01 (*t*-test for unpaired samples).

fraction to average coupling in that population will result in a change of transfer ratio relative to control cells. As predicted, the transfer ratio of calcein, a negatively charged dye, from donor to acceptor cells was decreased in ROS 17/2.8 cells after transfection with Cx45 (28.8 vs. 33.7 in vector-control cells) in the same conditions as those used in the luciferase assay (see above). Accordingly, transfer ratio was increased in UMR 106–01 cells transfected with Cx43 (3.4 vs. 2.6 in vector-control cells). These differences are commensurate with a transfection efficiency of ~15%. Similar results were reproduced in three different experiments and are consistent with the hypothesis that reciprocal expression of either Cx45 or Cx43 regulates transcriptional activity of osteoblast-specific promoters via changes in gap junctional communication.

DISCUSSION

We have demonstrated that two different connexins, Cx43 and Cx45, which form gap junction channels with different molecular permeabilities, modulate the expression of specific osteoblastic gene products by regulating the transcriptional activity of their promoters in a reciprocal manner.

In previous studies, we found that Cx45 functions as a partial dominant-negative connexin for Cx43, in that overexpression of chick Cx45 in cells that endogenously express Cx43, such as ROS 17/1.8 cells, greatly decreases gap junctional permeability (Koval *et al.*, 1995). Conversely, overexpression of Cx43 in poorly coupled cells, such as UMR 106–01, increases gap junctional permeability (Steinberg *et al.*, 1994). The present work indicates that these changes in gap junctional communication translate into reciprocal modulatory actions on specific osteoblast promoters. Several lines of evidence suggest that the regulatory effect on osteoblast gene transcription observed after overexpression of Cx45 or Cx43 in cells with different endogenous connexins is most likely the consequence of a changed gap junctional communication. First of all, manipulation of gap junctional communication in three different cell lines by overexpression of either Cx43 or Cx45 consistently demonstrates a direct correlation between dye coupling and OC and BSP gene expression. Concordant direct (or reverse) relationships were also evident between transcriptional activity of OC and BSP promoters and Cx43 (or Cx45) expression. Finally, direct assessment of dye coupling in transiently transfected cells revealed changes in gap junctional communication predicted by Cx43/Cx45 interactions. Therefore, although other, noncommunication-dependent actions of connexins have been described in other cell systems (Duflot-Dancer *et al.*, 1997), this does not appear to be the case for gene-regulatory effects in osteoblasts.

Of the various proteins that define the osteoblast phenotype, only OC and BSP are highly specific for bone-forming cells. Expression of OC is restricted to mature osteoblasts, odontoblasts, and hypertrophic chondrocytes undergoing calcification, whereas BSP is present almost exclusively in bone and placenta (Weinreb *et al.*, 1990; Chen *et al.*, 1992). Although their precise function in bone has not been completely clarified, OC and BSP represent the most specific markers of a fully differentiated osteoblast, and their expression is selectively detected at the onset of mineralization in bone. Basal mRNA expression and transcriptional activity of OC and BSP promoters were largely modulated in opposite directions by overexpression of Cx45 and Cx43, whereas expression of matrix proteins that are less specific for bone or that are less regulated during osteoblast differentiation and mineralization were either less sensitive to changes in gap junctional

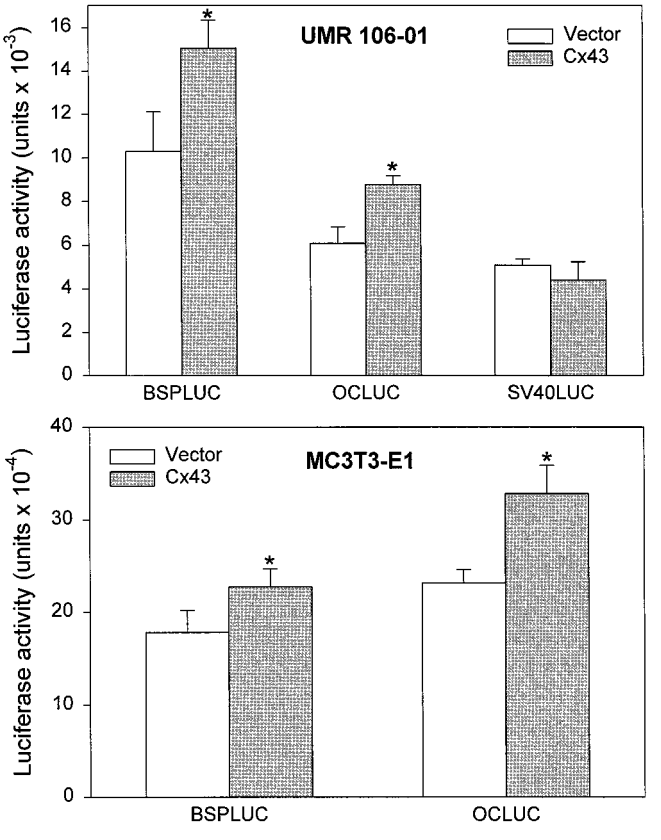

Figure 4. Transient transfection of rat Cx43 increases transcriptional activity of OC and BSP promoters in UMR 106/01 and MC3T3-E1 cells. Cells were cotransfected with either a rat Cx43 expression construct or its vector, and one of the promoter–luciferase constructs, OCLUC, BSPLUC or SV40LUC, as indicated. Luciferase activity was measured 3 d after transfection. Data are representative of four (UMR 106–01) and six (MC3T3-E1) different experiments and are expressed as the average ± SD of triplicate wells for each condition. *, $p < 0.01$ vs. vector-transfected cells (*t* test for unpaired samples).

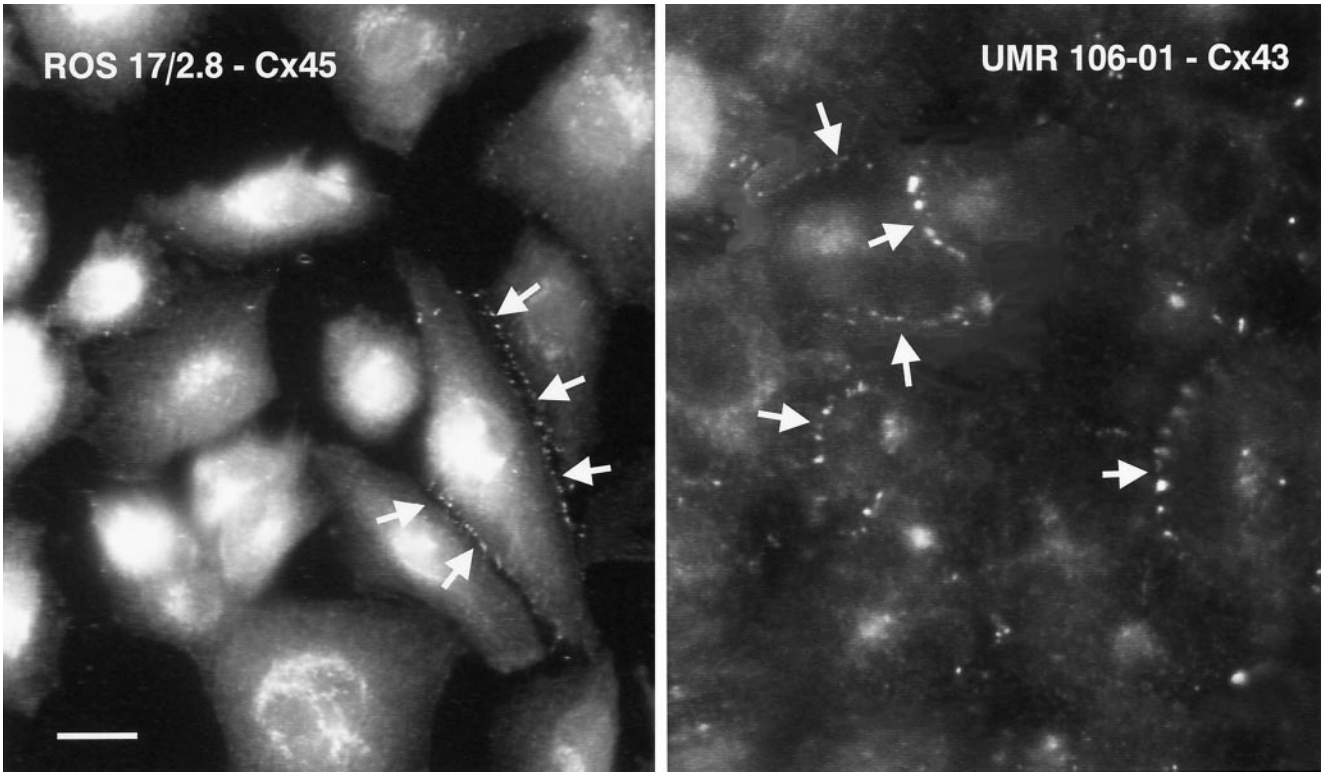

Figure 5. Transient transfection of Cx45 in ROS 17/2.8 and Cx43 in UMR 106–01 cells leads to cell surface expression of either connexin protein in a proportion of cells. Cells were seeded at low density on glass coverslips and incubated with either Cx45 or Cx43 expression constructs for 72 h. After fixation, cells were immunostained with anti-Cx45 (ROS 17/2.8, left) or anti-Cx43 (UMR 106–01, right) antibody, as indicated. Bar, 20 μm.

communication, i.e., ON, or were regulated in opposite directions from OC and BSP, as in the case of OP. These findings may reflect different functions of osteoblast-secretory products during differentiation and matrix maturation (Stein *et al.*, 1990; Stein and Lian, 1993). The almost identical results obtained in two osteoblastic cell lines with similar endogenous coupling and connexin expression (ROS 17/2.8 and MC3T3-E1) strongly indicate that the sensitivity of osteoblastic promoters to gap junctional intercellular communication represents a physiological mechanism, rather than a cell line-specific phenomenon. Furthermore, the correlation between permeability to negatively charged dyes, which in osteoblasts is mediated by Cx43 gap junctions (Civitelli *et al.*, 1993; Steinberg *et al.*, 1994), and OC and BSP gene transcription in the various cell lines and conditions observed in these studies strongly supports the notion that the type of gap junctional communication provided by Cx43 is permissive for a full elaboration of the mature osteoblastic phenotype.

The molecular interaction between Cx45 and Cx43 in forming gap junctions may have important physiological ramifications for bone formation. Connexin45 is present in normal human (Civitelli *et al.*, 1993) and rodent osteoblasts (our unpublished observations), although its abundance is lower than Cx43. In addition, both connexins are expressed in embryonic bone, but with distinct temporal and spatial distribution patterns (Minkoff *et al.*, 1994). Conceivably, the relative expression of Cx45 and Cx43 may allow cells to modulate their gap junctional permeability to levels that cannot be achieved by a single connexin and thus provide a regulatory mechanism required to control gene expression during different phases of bone development and osteoblast maturation. One can hypothesize that inadequate control of intercellular communication among osteoblasts or a reduced number of communicating cells may occur in pathological conditions and contribute to impaired synthesis of new bone and reduced trabecular wall thickness, typical of osteoporotic bone (Parfitt *et al.*, 1983). In partial support of this hypothesis, a correlation between density of bone-lining cells and bone-formation rate has been reported (Brown *et al.*, 1993). More recently, decreased responsiveness to parathyroid hormone has been observed in ROS 17/1.8 cells rendered communication deficient by transfection with an antisense Cx43 cDNA construct (Van der Molen *et al.*, 1996).

The nature of the mechanism that links gap junctional communication to gene expression remains elusive, but it certainly depends on the type of signals that permeate the junctional channel. Based on the pore size selectivity of Cx43 and Cx45 gap junctions (Veenstra *et al.*, 1992), 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. Notably, cAMP plays an important role in supporting OC expression in both MC3T3-E1 (Boudreaux and Towler, 1996) and ROS 17/2.8 osteoblastic cells (Shigeno *et al.*, 1988; Theophan and Price, 1989). The stimulatory activity of cAMP on dye coupling and Cx43 expression in osteoblastic cells (Schiller *et al.*, 1992; Civitelli *et al.*, 1998) may contribute to its regulatory effect on osteoblast phenotype. Alternatively, the affinity of transcriptional factors for specific DNA-binding sites may be sensitive to spontaneous oscillations in intracellular ionic concentration, i.e., calcium (Dolmetsh *et al.*, 1997), or in membrane polarity. Finally, propagation of certain types of calcium waves requires gap junctional communication (Jørgensen *et al.*, 1997).

These and other recent findings suggest that gap junctional communication is critically important for the normal function of many highly differentiated tissues other than bone. For example, the steroidogenic response of adrenal cells to ACTH is dependent on cell density and gap junctional communication (Munari-Silem *et al.*, 1995), and stimulation of smooth muscle contraction by α_1-adrenergic agonists is blunted by pharmacological inhibitors of cell coupling (Christ *et al.*, 1996). More to the point, restoration of cell coupling by transfection of either Cx43 or Cx32 in communication-deficient insulinoma or thyroid cells, respectively, increases insulin production or thyroglobulin expression (Vozzi *et al.*, 1995; Statuto *et al.*, 1997). In the study of Vozzi *et al.* (1995), optimal hormone synthesis was obtained when cell coupling was up-regulated to a level similar to normal pancreatic β-cells; a very high degree of coupling actually decreased hormone production (Vozzi *et al.*, 1995). Instead, we observed a direct relationship between coupling and OC and BSP gene transcription in our cell models. This discrepancy may underline the differences in gap junctional communication achieved in a mixed connexin environment as compared with a single connexin background in insulinoma cells (Vozzi *et al.*, 1995). Nevertheless, these observations demonstrate conclusively that gap junctions provide a fundamental regulatory mechanism controlling gene expression in tissues whose specialized function requires the synchronization of multicellular activity. Coordination of gene expression during osteoblast differentiation and bone remodeling represents an excellent model with which to test this novel physiological role of gap junctional intercellular communication.

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