CELL BIOLOGY AND METABOLISM:

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Reduction in Transforming Growth Factor β Receptor I Expression and Transcription Factor CBFa1 on Bone Cells by Glucocorticoid*

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Glucocorticoid in excess suppresses bone formation *in vivo* and disrupts bone matrix protein synthesis by osteoblasts *in vitro*. In contrast, transforming growth factor β (TGF-β) potently enhances bone matrix apposition. The rat TGF-β type I receptor gene promoter contains cis-acting elements for transcription factor CBFa1, which increases in parallel with osteoblast differentiation. Here we present molecular data linking these events. We show that previously unexplained effects of glucocorticoid on bone loss may be mediated in part by suppression of CBFa1, with a resultant decrease in the expression and activity of the TGF-β type I receptor on matrix-producing bone cells.

Glucocorticoid-dependent bone loss by disregulated hormone expression or pharmacologic excess causes clinically significant osteoporosis in approximately 50% of affected individuals. Although changes in calcium absorption and effects on nonskeletal tissues contribute to the disease, striking effects occur directly on osteoblasts and at sites of active skeletal matrix deposition and remodeling (1, 2). A chronic reduction in osteoblast activity without corresponding changes in resorption would uncouple normal bone remodeling and decrease skeletal durability. Important genes targeted by glucocorticoid and molecular mediators for these events remain uncertain.

Transforming growth factor β (TGF-β)[1] enhances bone matrix synthesis and repair, and bone contains perhaps the largest store of TGF-β in the body (reviewed in Ref. 3). Bone cells exhibit conventional type II and type III TGF-β receptors (TRII and TRIII) that influence TGF-β binding to type I receptor (TRI) or its activation, both essential for TGF-β-dependent events (4–6). There are few systems where regulation of TRI expression has been examined in detail and where functional changes correlate with these variations. We found TRI levels specifically maintained on differentiated bone cells *in vitro*, despite decreases in TRII and TRIII in response to bone morphogenetic protein 2 (5). In contrast, high levels of glucocorticoid rapidly reduce the proportion of TGF-β binding to TRI on bone cells and correspondingly decrease TGF-β activity (6).

To understand these events further, we cloned the rat TRI promoter and observed higher promoter activity in osteoblast-like cells compared with undifferentiated bone cells or dermal fibroblasts (7). The TRI promoter includes a CpG island, several transcription factor Sp1 binding sites consistent with constitutive expression by many cells, and six cis-acting elements for transcription factors, termed CBFa (7–8).[2] Whereas CBFa2 and CBFa3 are important gene regulators in lymphoid cells (9), CBFa1 expression increases in parallel with osteoblast differentiation *in vitro* (10).[2] Moreover, targeted disruption of the CBFa1 gene eliminates osteogenesis in mice, and insertion, deletion, or missense mutations in CBFa1 occur in humans with the skeletal disorder cleidocranial dysplasia (11). Genes directly affected by CBFa1, especially those important for skeletal development, are difficult to resolve when the factor is absent or dysfunctional and when osteoblasts are absent or hard to detect. Hormone-dependent decreases in CBFa1 could also challenge skeletal integrity and indicate important downstream targets. Because glucocorticoid suppresses TGF-β activity and its binding to TRI on osteoblasts and because binding sequences for CBFa1 occur in the TRI promoter, we postulated molecular links among these events.

EXPERIMENTAL PROCEDURES

Cells—Primary osteoblast-enriched cultures were prepared by timed, sequential collagenase digestion of fetal rat parietal bone using procedures approved by the Yale Animal Care and Use Committee. Cells released during the third through the fifth 20-min collagenase digestion interval exhibit many physical and biochemical characteristics associated with bone-forming cells and are readily distinguished from less differentiated periosteal bone cells. Cells were plated at $3 \times 10^3/cm^2$ and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described (5–8).

Transfections—Cultures at 50% confluence were transfected with luciferase reporter plasmids pEN1.0 (7, 8) containing the fully active TRI promoter, pSXN1C,[2] containing two copies of a CBFa response element inserted in vector pGL3-Promoter, p1233 (12), containing 1.2 kbp of the TRII promoter, or pMMTV-Luc (13), containing a well characterized glucocorticoid response element (GRE) from the mouse mammary tumor virus. No GREs occur in pEN1.0 (7, 8). To assess effects on TRI-dependent TGF-β activity, cultures were transfected with the TGF-β-responsive promoter/reporter construct 3TPLux (4). After 24 h, cultures were treated as indicated in each experiment and rinsed, and extracts were used to measure luciferase expression. There are only minimal variations in co-transfected β-galactosidase expression (± 6%) within experiments, and correcting TRI promoter activity for β-galactosidase expression fails to alter overall results (7, 8). However, TRI reporter constructs are highly expressed by osteoblasts but exhibit significantly reduced activity when co-expressed with secondary reporter constructs, presumably from competition between plasmids for basal expression elements. This study examines inhibitory effectors (glucocorticoids). Therefore, promoter activities were compared in parallel cultures rather than in the same cells in order not to add a

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[1] The abbreviations used are: TGF-β, transforming growth factor β; TRI, TRII, TRIII, TGF-β receptor types I, II, and III, respectively; GRE, glucocorticoid response element; kbp, kilobase pair(s).

[2] Ji, C., Casinghino, S., Chang, D. J., Chen, Y., Javed, A., Ito, Y., Hiebert, S. W., Lian, J. B., Stein, G. S., McCarthy, T. L., and Centrella, M. (1998) *J. Cell. Biochem.* (in press).

confounder or to compromise TRI promoter/reporter expression further. Studies were repeated a minimum of three times to ensure reproducibility.

RNA Analyses—Total RNA was extracted with acid guanidine monothiocyanate, precipitated with isopropyl alcohol, and dissolved for assay. Transcripts were assessed by RNase protection assay with antisense RNA probes for rat TRI or 18 S rRNA. Probes were combined with 5 μg of RNA, digested with 0.3 unit of RNase A and 14 units of RNase T1, and extracted with proteinase K and 0.5% SDS, and protected RNAs (TRI, 275 nucleotides; 18 S rRNA, 80 nucleotides) were collected in isopropyl alcohol, resolved on a 5% denaturing polyacrylamide gel, and visualized by autoradiography (14). Alternately, total RNA was fractionated on a 1.5% agarose/2.2 M formaldehyde gel, blotted onto charged nylon, and hybridized with a [^{32}P]-labeled cDNA restriction fragment of plasmid λbg7 encoding rat TRIII. Bound material was visualized by autoradiography, and rRNA was assessed by ethidium staining of a parallel sample (5).

TGF-β Binding—TGF-β1 was radioiodinated with chloramine T. Cells were incubated in serum-free medium with 4 mg/ml bovine serum albumin and 150 pM ^{125}I-TGF-β1 for 3 h at 4 °C. Rinsed cultures were chemically cross-linked and extracted in reducing buffer, and samples were fractionated by electrophoresis on 5–15% polyacrylamide gels and examined by autoradiography (5, 14).

Cell and Nuclear Extracts—As described previously (8), cells were collected by scraping and lysed in hypotonic buffer with phosphatase inhibitors, protease inhibitors, and 1% Triton X-100. Nuclei were collected by centrifugation, and supernatants were assessed for TRI protein. Nuclei were resuspended in hypertonic buffer with glycerol, phosphatase, and protease inhibitors and extracted for 30 min on ice. Insoluble material was cleared by centrifugation, and soluble protein was assessed for CBFa1 and Sp1.

Immunoblots—For TRI, cell lysates (100 μg of protein), and for CBFa1, high salt nuclear extracts (40 μg of protein) were fractionated by polyacrylamide gel electrophoresis and electroblotted onto Immobilon P membranes (Millipore). Membranes were washed and blocked in 5% defatted milk, incubated with a 1:2,000 dilution of primary antibody (15), washed, incubated with a 1:3,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase, developed with ECL (Amersham Pharmacia Biotech) reagents, and visualized by chemiluminescence (8).

Electrophoretic Gel Mobility Shift Assay—Double strand oligonucleotide probes were annealed, labeled with [α-^{32}P]dCTP and Klenow fragment of *Escherichia coli* DNA polymerase I, and gel-purified. Nuclear extracts (5 μg of protein) were incubated with ^{32}P probe. To assess transcription factor immunologically, nonimmune (Santa Cruz) or rabbit polyclonal anti-CBFa1 (15) was preincubated with nuclear extract before adding ^{32}P probe. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and analyzed by autoradiography (8).

Collagen and Non-collagen Protein Synthesis—Cultures were pulsed with [^{3}H]proline for the last 2 h of incubation. Cell layers were lysed by freeze-thawing and extracted in 0.5% Triton X-100. Samples were precipitated with trichloroacetic acid and chilled, and insoluble material was collected by centrifugation. [^{3}H]Proline incorporation into collagen and non-collagen protein was measured by differential sensitivity to bacterial collagenase free of nonspecific protease activity (5, 6, 14).

Densitometry—Relative differences on autoradiograms were assessed with a ScanMan densitometer and SigmaGel® (Jandel, San Rafael, CA).

Statistical Analysis—Data were analyzed after multiple determinations for reproducibility. Biochemical data were expressed as means ± S.E. Statistical differences were assessed by analysis of variance with commercial software (SigmaStat®). After this, analysis was by the Student-Newman-Keuls method and considered significant with P values <0.05.

RESULTS

Glucocorticoid Decreases TRI Promoter Activity—Transfection construct pEN1.0, containing the 3′ 1-kbp region of the rat TRI promoter, directs maximal reporter gene expression in primary and continuous osteoblast-enriched cultures (7, 8). Treatment for 24–48 h with the natural glucocorticoid hydroxycortisone or with the pharmacological glucocorticoid dexamethasone reduced TRI promoter activity by approximately 50% (Fig. 1, *A–C*). Treatment for less than 24 h did not cause significant reductions in reporter gene expression (Fig. 1*A* and other data not shown). This assay depends on competition between genomic and episomal elements for trans-acting factors, on reporter gene half-life, and on a balance between promoter plasmid expression and variations in specific trans-acting factors. Therefore, it may not reflect the timing of TRI mRNA expression with precision. Nevertheless, the effect was not nonspecific since neither steroid reduced reporter gene expression by the TRII promoter construct p1233 (12), and both agents enhanced expression by promoter construct pMMTV-Luc, containing a potent GRE (13) (Fig. 1*D*).

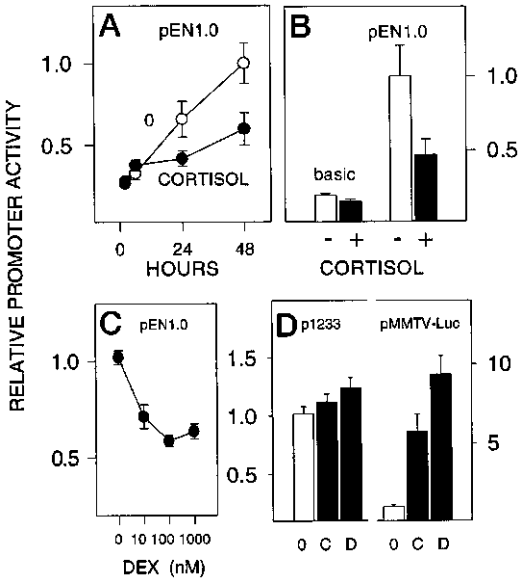

FIG. 1. **Glucocorticoid reduces TRI promoter activity.** Primary osteoblast-enriched cultures were transfected with promoter/reporter plasmid constructs [0.1 μg/cm^2], incubated for 24 h for plasmid expression, and treated with glucocorticoid, and luciferase expression was determined and corrected for protein content. *A*, cultures were transfected with plasmid pEN1.0 containing the 3′ 1.0-kb portion of the rat TRI promoter. Reporter activity was measured at the times indicated in control (○) and 100 nM cortisol-treated cultures (●). *B*, cultures were transfected with basic vector (pGL3-Basic; Promega) or with pEN1.0, and reporter gene expression was evaluated after 48 h in control (−) and 100 nM cortisol-treated (+) cultures. *C*, cultures were transfected with pEN1.0, and reporter gene expression was evaluated after 48 h in cultures treated with control medium (*0*) or the amounts of dexamethasone (*DEX*) indicated. *D*, cultures were transfected with plasmid p1233 containing the 3′ 1.2-kbp portion of the human TRII promoter (12) or with plasmid pMMTV-Luc containing a 7.8-kbp fragment from the mouse mammary tumor virus promoter with a well characterized GRE (13), each with 5-fold activity above pGL3-Basic and treated with control medium (*0*), 100 nM cortisol (*C*), or dexamethasone (*D*). For direct comparisons among constructs, data were corrected by comparison to control cultures after 48 h of treatment, noted in all panels as 1 unit of relative promoter activity. Data represent results from a minimum of three separate studies and eight replicate cultures per condition.

Glucocorticoid Suppresses TRI mRNA and Protein Levels—Glucocorticoid also reduced steady state TRI mRNA levels. Dexamethasone at 100 nM decreased TRI mRNA by 20% within 6 h, by 40% at 16 h, and by 65% after 48 h. As shown in Fig. 2*A*, dexamethasone caused a dose-dependent inhibitory effect after 48 h of treatment. This was not from general inhibition of transcription or differential RNA recovery, since glucocorticoid enhanced TRIII mRNA without proportional changes in rRNA transcripts (Fig. 2*B*). Consistent with lower TRI promoter activity (Fig. 1, *A–C*), less TRI mRNA (Fig. 2*A*), and a corresponding decrease in ^{125}I-TGF-β binding to TRI (Fig. 2*C*), glucocorticoid decreased TRI protein levels by 50–60% within 24–48 h and by greater than 90% after 72 h (Fig. 2D).

Glucocorticoid Decreases Nuclear Factor CBFa1 Levels—We previously identified several inactive regions, 6 CBFa binding sequences, and 16 possible Sp1 binding sequences in the TRI promoter (7, 8).[2] Therefore, we examined the effect of glucocorticoid on CBFa1 and Sp1 binding to ^{32}P-labeled consensus

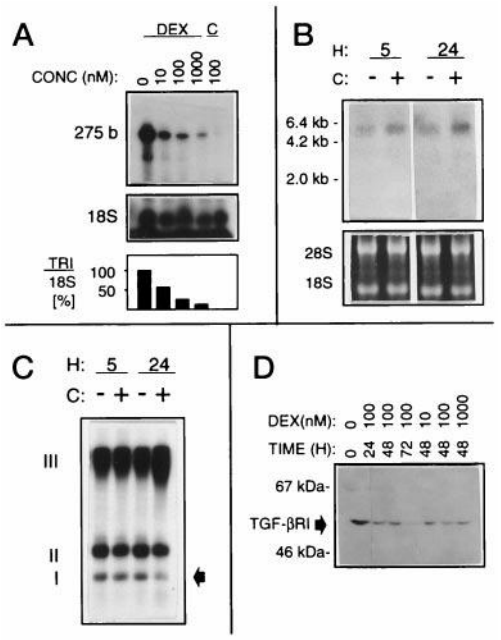

FIG. 2. **Glucocorticoid reduces TRI expression.** Primary osteoblast-enriched cultures were treated with glucocorticoid as indicated, and extracts were prepared to assess the effects on TRI. *A*, cultures were extracted after a 48-h treatment with the amounts of dexamethasone (*DEX*) or cortisol (*C*) indicated, and steady state TRI mRNA and 18 S rRNA levels were evaluated by RNase protection assay (14). *CONC*, concentration; *b*, bases. *B*, cultures were treated for 5 or 24 h (*H*) with control medium (−) or 100 nM cortisol (*C*) and extracted, and 20 μg of RNA were fractionated by agarose gel electrophoresis and probed by Northern analysis with a 3.9-kilobase pair rat-specific TRIII cDNA probe (5). *Numbers* on the left indicate migration of a standard DNA ladder. The *panel* below shows ethidium-stained rRNA bands. *C*, cultures were treated for 5 or 24 h with control medium (−) or 100 nM cortisol (+), incubated with 50 pM ^{125}I-TGF-β1, cross-linked, and extracted, and TGF-β receptors in 100 μg of cell extract were displayed by polyacrylamide gel electrophoresis and autoradiography (5, 14). *Roman numerals* on the left indicate TRI, TRII, and TRIII. *Arrow* indicates loss in TGF-β binding at TRI. *D*, cultures were treated with the amounts of dexamethasone and for the times indicated and extracted, 100 μg of cell extract was fractioned by polyacrylamide gel electrophoresis, and TRI protein levels were assessed by Western analysis with anti-TRI antibody (Santa Cruz Biotechnology, Inc.). *Numbers* on the left indicate migration by molecular mass standards, and the *arrow* indicates the position of the 53-kDa TRI protein. Analogous results occurred in a minimum of two separate studies.

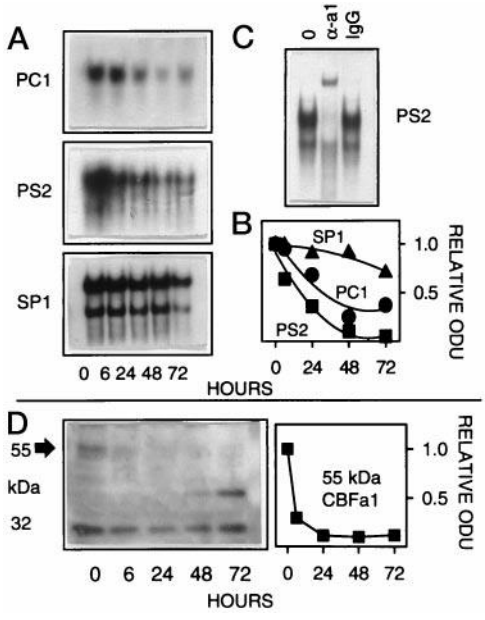

FIG. 3. **Glucocorticoid suppresses transcription factor CBFa1.** Primary osteoblast-enriched cultures were treated with glucocorticoid as indicated, and nuclear extracts were prepared to assess nuclear factors. *A*, cultures were treated with 100 nM dexamethasone for the times indicated, 5 μg of nuclear protein was combined with ^{32}P-labeled oligonucleotides PC1 (5′-CGTATTAACCACAATACTCG-3′), PS2 (5′-AAGGAAGTTGAACCGCGGGATTGGACCG-3′, CBFa binding sites *underlined*), or SP1 (5′-GTACATTCGATCGGGCGGGGCGAGCGATC-3′, Sp1 binding site *underlined*), and DNA/protein complexes were displayed by polyacrylamide gel electrophoresis and autoradiography. *B*, complex formation was evaluated by densitometry. For direct comparison among probes, data were corrected relative to untreated cultures, noted as 1 relative optical density unit (*ODU*). *C*, nuclear protein (5 μg) was combined with ^{32}P-PS2 with no addition (*0*), 1 μg of anti-CBFa1-specific antibody (α-a1), or nonimmune rabbit IgG (IgG) and analyzed as in *A*. *D*, nuclear extracts from cultures treated with 100 nM dexamethasone for the times indicated were fractionated on 10% polyacrylamide gels, blotted onto Immobilon P polyvinylidene difluoride membranes, probed with anti-CBFa1 antibody, visualized by chemiluminescence, and evaluated by densitometry. Analogous results occurred in a minimum of two separate studies.

oligonucleotide probes (Fig. 3*A*). Binding by osteoblast-derived nuclear factor to CBFa binding sequences in probes PC1 (5′-AACCACA-3′) defined with lymphocyte and osteoblast extracts (9–10)[2] and PS2 (5′-AACCGCG-3′) from the rat TRI promoter[2] decreased by 40–70% within 24 h of exposure to glucocorticoid and by 70–90% after 48 h. In contrast, nuclear factor binding to probe SP1 with Sp1 binding sequence (5′-GGGCGGGG-3′) (8) decreased by 10% or less after 48 h of glucocorticoid treatment and by only 30% after 72 h (Fig. 3*B*). Studies with isoform-specific antibodies (15) reveal that CBFa1 forms the principal high molecular mass complex between nuclear protein from osteoblast-enriched cultures and TRI oligonucleotide PS2 (Fig. 3*C*) or other consensus DNA binding sequences for CBFas (9–10).[2] By Western blot analysis (Fig. 3*D*), glucocorticoid rapidly and potently reduced full-length 55-kDa CBFa1, increased the appearance of a 46-kDa immunoreactive protein, and produced less variability in a 32-kDa immunoreactive band. The latter two proteins may be processed CBFa1 fragments or cross-reactive gene products. A small increase in complex formation appears to recur between 48 and 72 h of glucocorticoid treatment, but it is not yet known if it derives from the 46-kDa immunoreactive protein that accumulates during this treatment interval.

To demonstrate that loss of CBFa1 critically reduces CBFa-dependent promoter activity in osteoblasts, cultures were transfected with promoter/reporter construct pSXN1C, which contains two copies of a CBFa response element derived from the TRI promoter inserted into vector pGL3-Promoter.[2] pGL3-Promoter has a viral promoter but lacks enhancer sequence and thus exhibits significant but limited endogenous basal activity. Therefore, reporter expression above that produced by the viral promoter relies on the presence of the two CBFa binding sequences in pSXN1C. pGL3-Promoter increased reporter gene expression by approximately 10-fold above pGL3-Basic (empty vector with no promoter or enhancer sequences). Consistent with the presence of CBFa1 in osteoblast-enriched cultures, pSXN1C increased reporter gene expression by 20–25-fold. When full-length CBFa1 levels were reduced by glucocorticoid treatment, reporter expression in cultures transfected with pSXN1C was not significantly different from that in cultures transfected with pGL3-Promoter (Fig. 4). These results are consistent with the presence of multiple CBFa binding sequences in the TRI promoter (8), with increased TRI promoter activity in cells expressing higher levels of CBFa1 (7, 11),[2] and with reduced TRI promoter activity in cells expressing a dominant negative CBFa2 mutant protein.[3]

[3] M. Centrella, unpublished observation.

Glucocorticoid Reduces TGF-β Effects—Up to 48 h of treatment with glucocorticoid did not reduce basal protein synthesis (Fig. 5*A*) or stimulatory effects induced by platelet-derived growth factor (6), indicating that decreases in TRI and CBFa1 were not from nonspecific inhibitory effects. Nonetheless, glucocorticoid caused a small decrease in basal collagen synthesis. Type I collagen, a major product of bone-forming osteoblasts, comprises about 90% of the organic bone matrix. It is the basic element of a network where other skeletal components assemble and must be restored to areas resorbed during bone remodeling (1, 2) and is greatly enhanced by TGF-β (3, 5, 6, 14). Consistent with a reduction in TRI expression, stimulation by TGF-β was significantly suppressed by glucocorticoid (Fig. 5, *B* and *C*). Because bone matrix type I collagen expression may be regulated directly by changes in CBFa1 (16–19) and, therefore, independently of variations in TRI, we also examined the effect of glucocorticoid on reporter gene expression induced by the TGF-β-responsive promoter/reporter transfection construct 3TPLux (4). Glucocorticoid had no effect on basal 3TPLux expression but significantly reduced the marked stimulatory effect of TGF-β treatment (Fig. 5*D*).

DISCUSSION

CBFa1 levels progressively increase with expression of the osteoblast phenotype (10)[2] in parallel with the increases that we reported earlier on TRI promoter activity, TRI mRNA, and cell surface TRI protein (5, 7). We now show that glucocorticoid rapidly suppresses functional CBFa1 in nuclear extracts from osteoblast cultures. Importantly, loss of CBFa1 by glucocorticoid treatment corresponds with decreases in TRI promoter activity, TRI mRNA and protein levels, and TGF-β-dependent biological effects. Western blots with anti-CBFa1 specific antibody show a decrease in full-length CBFa1 and an increase in protein of reduced molecular mass in glucocorticoid-treated bone cells. In contrast, Northern blots do not show coinciding reductions in CBFa1 mRNA,[4] suggesting that some of this effect may be post-transcriptional and perhaps produced by changes in CBFa1 protein stability.

The decrease in cell surface TRI in glucocorticoid-treated cells is consistent with our previous evidence for a short half-life of TRI mRNA of approximately 6–7 h (14). A lower level of TRI expression, driven principally by constitutive regulatory elements in the TRI promoter (8), should not by itself fully eliminate osteogenic cell activity. Clearly, genes other than TRI are important for osteoblast function. Like osteocalcin, a protein expressed predominantly or exclusively by osteoblasts, some may be directly regulated by CBFa1 (10, 11, 16–20). For example, basal type I collagen expression is increased in cells transfected to express high levels of CBFa1 (16) and is decreased in osteoblasts after 48 h of glucocorticoid treatment (6, 21) when CBFa1 levels are near maximally suppressed. Even so, the large increase in collagen synthesis induced by TGF-β treatment is even more strikingly reduced. Accordingly, the stimulatory effect of TGF-β on collagen synthesis is significantly lower in bone-derived cells that endogenously express fewer osteoblast-associated features (5, 14), have lower osteogenic potential *in vitro* (22), exhibit less TRI by relation to TRII and TRIII (5, 14), and express less CBFa1.[2] Loss of TRI levels also limits the stimulatory effects of TGF-β on general protein synthesis and on expression of 3TPLux (a reporter gene sensitive to fluctuations in TGF-β receptors; Ref. 4), neither of which are directly reduced by glucocorticoid treatment. Therefore, variations in osteogenic events normally regulated by TGF-β, which may be rapidly limited by the short functional half-life of TRI on osteoblasts (14), may be sufficient to disrupt at least in part the balanced bone remodeling cycle.

Whereas TGF-β enhances replication and the synthesis of type I collagen and other matrix components by bone cells that are not yet fully differentiated, it inhibits biochemical activities associated with later aspects of osteoblast differentiation and mineralization *in vitro* (3, 5). Therefore, loss of TRI in response to glucocorticoid would effectively suppress stimulatory effects by local TGF-β on osteoblast number and production of organic bone matrix components required for later mineral deposition. However, on well differentiated bone cells, lower levels of TRI would also reduce the sensitivity of these cells to TGF-β and enable mineralization in regions of bone where the organic matrix is already sufficiently formed. In this regard, targeted

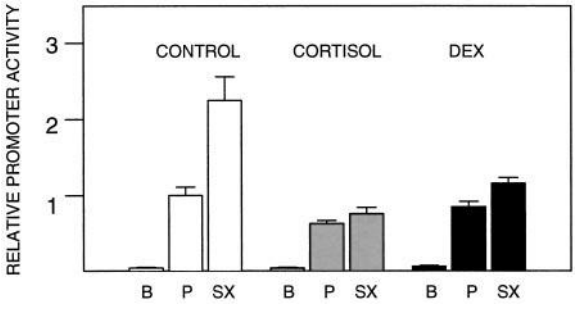

FIG. 4. **Glucocorticoid suppresses CBFa-dependent promoter activity.** Primary osteoblast-enriched cultures were transfected with promoter/reporter plasmid constructs pGL3-Basic (*B*), pGL3-Control (*P*), or pSXN1C (*SX*), treated for 48 h with control medium, 100 nM cortisol, or dexamethasone (*DEX*), as indicated, and luciferase expression was assessed as in Fig. 1. Data were corrected relative to pGL3-Control, noted as 1 unit of relative promoter activity. Results are from 2 separate studies and 7–10 replicate cultures per condition.

[4] T. L. McCarthy, D. J. Chang, and M. Centrella, unpublished observation.

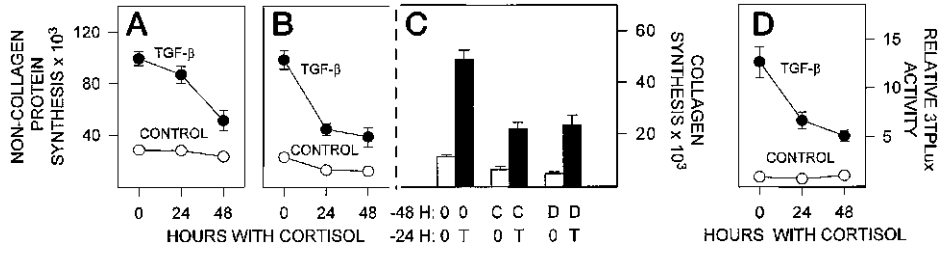

FIG. 5. **Glucocorticoid suppresses TGF-β-dependent activity.** Primary osteoblast-enriched cultures were treated with 100 nM cortisol for 0, 24, or 48 h in *A*, *B*, and *D* or for 24 h with control medium (0), 100 nM cortisol (*C*), or dexamethasone (*D*) in *C* and then treated with control medium (○) or 120 pM TGF-β1 (●) for a subsequent 24 h. In *A*, *B*, and *C*, cultures were labeled with 5 μCi/ml [^{3}H]proline during the last 2 h of culture, and extracts were prepared to assess total non-collagen (*A*) and collagen (*B* and *C*) protein synthesis by differential sensitivity to purified bacterial collagenase (5, 14). Data are from a minimum of 4 separate studies and 16 replicate cultures per condition and show total isotope incorporation (dpm/culture). In *D*, cultures were transfected with the TGF-β-dependent promoter/reporter construct 3TPLux before glucocorticoid and TGF-β treatments, extracted, and analyzed for luciferase reporter gene expression. Data are results from a minimum of three separate studies and nine replicate cultures per condition. *T*, TGF-β.

overexpression of TGF-β2 by late stage osteoblasts under control of the osteocalcin promoter correlates with abnormal bone formation (23), and paradoxically, mineralized nodule formation is enhanced *in vitro* by low dose or transient exposure to glucocorticoid in some culture models (24). In the primary cell cultures used in our studies, dexamethasone dose-dependently suppresses nodule formation *in vitro*.[3] This result is consistent with studies in rat bone cell cultures where nodule formation was reduced by the presence of antisense oligonucleotide to a domain common to all CBFa subunits (10). Therefore, despite appropriate stimulatory or permissive effects that occur with normally controlled expression or release of TGF-β and glucocorticoid, inappropriate expression of either agent or their receptors could disrupt the normal osteogenic process.

CBFa1 knockout animals exhibit complete loss in mineralized skeletal components and few if any differentiated osteoblasts (17, 18). Consequently, it is not possible to assess directly the importance of CBFa1 on specific genes expressed by bone-forming cells or their precursors with those animals. Forced overexpression of CBFa1 has been correlated with a number of osteoblast-related genes (16), but it is not yet clear if all of these effects are direct. More subtle events are more likely to regulate the gain or loss of CBFa1 expression during normal bone growth or metabolic bone disease. Our results provide the first evidence for glucocorticoid-dependent variations in CBFa1 expression in bone and suggest that its loss rapidly downregulates TRI expression by bone cells. To date, little is still known about the mechanisms that control TRI expression in bone or in any tissue. Thus, our studies also provide new evidence for molecular mechanisms that can account for changes in TRI expression in combination with a functional consequence in a physiologically relevant target tissue. Persistent decreases in matrix accumulation, endogenously or in response to locally released TGF-β, may contribute substantially to bone fragility in glucocorticoid-dependent osteoporosis. Identifying and understanding these control mechanisms may assist the development of new ways to circumvent bone loss associated with this and other aberrations in CBFa1 expression or its activity.

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REFERENCES

1. Adler, R. A., and Rosen, C. J. (1994) *Endocrinol. Metab. Clin. North Am.* **23,** 641–654
2. Lukert, B. P. (1996) in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism* (Fauvus, M. J., ed) pp. 278–282, Raven Press, Ltd., New York
3. Centrella, M., Rosen, V., Horowitz, M. C., Wozney, J. M., and McCarthy, T. L. (1995) in *Hormonal Regulation of Bone Mineral Metabolism* (Bikle, D. D., and Negro-Vilar, A., eds) pp. 211–226, Endocrine Society, Bethesda, MD
4. Wieser, R., Wrana, J. L., and Massague, J. (1995) *EMBO J.* **14,** 2199–2208
5. Centrella, M., Casinghino, S., Kim, J., Pham, T., Rosen, V., Wozney, J., and McCarthy, T. L. (1995) *Mol. Cell. Biol.* **15,** 3273–3281
6. Centrella, M., McCarthy, T. L., and Canalis, E. (1991) *Mol. Cell. Biol.* **11,** 4490–4496
7. Ji, C., Casinghino, S., McCarthy, T. L., and Centrella, M. (1996) *J. Cell. Biochem.* **63,** 478–490
8. Ji, C., Casinghino, S., McCarthy, T. L., and Centrella, M. (1997) *J. Biol. Chem.* **272,** 21260–21267 *
9. Ito, Y., and Bae, S.-C. (1997) in *Oncogenes as Transcriptional Regulators Vol. 2: Cell Cycle Regulators and Chromosome Translocation* (Yaniv, M., and Ghysdael, J., eds) pp. 107–132, Vol. 2, Birkhaeuser Verlag, Basel, Switzerland
10. Banerjee, C., McCabe, L. R., Choi, J.-Y., Hiebert, S. W., Stein, J. L., Stein, G. S., and Lian, J. B. (1997) *J. Cell. Biochem.* **66,** 1–8
11. Rodan, G. A., and Harada, S.-I. (1997) *Cell* **89,** 677–680
12. Bae, H. W., Geiser, A. G., Kim, D. H., Chung, M. T., Burmester, J. K., Sporn, M. B., Roberts, A. B., and Kim, S.-J. (1995) *J. Biol. Chem.* **270,** 29460–29468
13. Majors, J., and Varmus, H. E. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5066–5070
14. Centrella, M., Ji, C., Casinghino, S., and McCarthy, T. L. (1996) *J. Biol. Chem.* **271,** 18616–18622
15. Meyers, S., Lenny, N., Sun, W., and Hiebert, S. W. (1996) *Oncogene* **13,** 303–312
16. Ducy, P., Zhang, R., Geoffrey, V., Ridall, A. L., and Karsenty, G. (1997) *Cell* **89,** 747–754
17. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y.-H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) *Cell* **89,** 755–764
18. Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Beddington, R. S. P., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. (1997) *Cell* **89,** 765–771
19. Mundlos, S., Otto, F., Mundlos, C., Mulliken, J. B., Aylsworth, A. S., Albright, S., Lindhout, D., Cole, W. G., Henn, W., Knoll, J. H. M., Owen, M. J., Mertelsmann, R., Zabel, B. U., and Olsen, B. R. (1997) *Cell* **89,** 773–779
20. Banerjee, C., Hiebert, S. W., Stein, J. L., Lian, J. B., and Stein, G. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4968–4973
21. Centrella, M., Rosen, V., Wozney, J. M., Casinghino, S. R., and McCarthy, T. L. (1997) *J. Cell. Biochem.* **67,** 528–540
22. Centrella, M., Casinghino, S., Gundberg, C., McCarthy, T. L., Wozney, J., and Rosen, V. (1996) *Ann. N. Y. Acad. Sci.* **785,** 224–226
23. Erlebacher, A., and Derynck, R. (1996) *J. Cell Biol.* **132,** 195–210
24. Bellows, C. G., Aubin, J. E., and Heersche, J. N. M. (1987) *Endocrinology* **121,** 1985–1992