Steroids significantly affect skeletal integrity. For example, bone mass decreases with glucocorticoid excess or with estrogen depletion after menopause. Glucocorticoid suppresses gene expression by an essential skeletal tissue transcription factor, Runx2, in rat osteoblasts. We now report that estrogen enhances Runx2 activity in dose- and estrogen receptor-dependent ways independently of changes in Runx2 levels or its DNA binding potential. Estrogen receptor and Runx2 can be collected by co-immunoprecipitation. By two-hybrid gene expression analysis, high affinity complex formation involves portions of Runx2 outside of its own DNA binding domain and the DNA binding domain of the estrogen receptor. Consistent with this interaction, the stimulatory effect of estrogen on Runx2 activity is lost when the DNA binding domain of the estrogen receptor is eliminated. Unlike the stimulatory effect of estrogen and the inhibitory effect of glucocorticoid, androgen fails to increase Runx2 activity, whereas Runx2 potently suppresses gene expression induced by all three steroids. Finally, estrogen increases gene transcription by the transforming growth factor-β type I receptor gene promoter, which contains several Runx binding sequences, and enhances Smad dependent gene expression by transforming growth factor-β in osteoblasts. These results reveal that Runx2 can integrate complex effects on gene transcription in hormone-, growth factor-, and tissue-restricted ways.

Bone is a dynamic tissue that forms and remodels throughout life. Osteoblast-dependent bone formation varies with age, with certain metabolic disease states, or with pharmacological intervention. When osteoclast-dependent bone loss predominates, the structural quality of bone is diminished. This is accompanied by an increase in fracture and pain and a decrease in mobility and function. Loss of bone integrity and its subsequent pathology may therefore follow disparities in growth regulators and molecular events that define the normally balanced bone remodeling cycle (1–3).

Runx2, a member of the Runx family of nuclear transcription factors, first termed PEBP2α, CBFα, AML, or OSF, contains a Runt domain homologous to a transcription factor involved in body segmentation, sex determination, and neurogenesis in Drosophila melanogaster (4–6). Runx2 levels increase greatly during osteoblast differentiation, whereas homozygous gene deletion of Runx2 limits bone formation, and few, if any, differentiated osteoblasts are found in Runx2-deficient mice (7). Importantly, the Runt domain contains sequences essential for DNA binding, and this region seems to be required for heterodimerization with several other nuclear transcription factors. The carboxyl-terminal region of Runx contains a transcription domain as well as a domain that targets its binding to the nuclear matrix. In many instances, Runx transcription factors are thought to increase gene expression through Runx-sensitive response elements (RE) (8), although in some cases, Runx2 may also be inhibitory (6, 8).

Steroid hormones also have complex stimulatory and inhibitory effects on gene expression. This is controlled in part by the way hormone-activated steroid receptors complex with themselves or with other binding partners to form competent or inactive transcription factors. Like other transcription factors, the steroid hormone receptors contain domains important for DNA binding, dimerization, and gene transactivation. Some effects by steroid hormone require direct genomic interactions, and others occur through indirect effects on cellular signaling (9–14).

Several studies suggest that transient exposure to or a low level of the adrenal steroid glucocorticoid permissively alters the expression of some genes associated with osteoblast function (15, 16). In contrast, high levels of glucocorticoid, such as those commonly used therapeutically to suppress inflammatory disease or tissue rejection, cause generalized bone loss leading to fractures as well as gastrointestinal and immunological problems (17, 18). Although in some instances glucocorticoid may enhance Runx2 mRNA expression, in vitro glucocorticoid in excess rapidly depletes the amount of functional Runx2 nuclear protein, with an accompanying inhibitory effect on Runx2-dependent gene expression (19, 20). This seems to reprise the downstream events that occur with glucocorticoid therapy or with Runx2 gene deletion in vivo (7) and would essentially limit osteoblast activity in the adult remodeling skeleton. Bone loss also occurs after menopause or with sex steroid depletion. In ovariectomized animals, bone resorption rates increase, but the balance in bone remodeling can then be restored by hormone replacement therapy. This is believed to occur in part by way of an estrogen-dependent reduction in the expression of lymphokines associated with the development of bone-resorbing osteoclasts through inhibitory effects on tran-
and corrected for protein content. To account for competition among reporter gene activity for another 48 h, treated in serum-free medium, rinsed, and lysed.

Nuclear-free supernatants were analyzed for reporter gene activity (Fig. 2B). There were described previously. Our studies also benefited from the generous gifts of reporter and expression plasmids from other investigators. Dr. Maurer (Oregon Health Sciences University, Portland, OR) provided an expression plasmid for rat androgen receptor (AR). Dr. Ronald M. Evans (Scripps Research Clinic, La Jolla, CA) provided an expression plasmid for human ERα (H9251). Dr. Richard A. Herpesvirus (43–46). Importantly, this osteoblast cell culture model provides a sensitive system to examine specific aspects of ERα on osteoblast activity uncomplicated by the presence of endogenous ERs.

**Estrogen Enhances Runx2 Activity**—Earlier studies revealed that even in the presence of functional ERs, estradiol does not alter basal IGF-I expression. However, it dose dependently decreases the effect of hormones that enhance IGF-I synthesis by a way of C/EBP-ER in exon 1 of the IGF-I gene (23, 37). In direct contrast to its inhibitory effect on C/EBP activity, estradiol increased Runx dependent gene expression in osteoblasts. Treatment increased estradiol dependent gene expression by plasmid SXN1C, where two copies of a Runx RE from the TβRI gene promoter were inserted into pGL3-promoter but had no effect on parental pGL3-promoter activity (Fig. 2A). Stimulation seemed to be largely the result of an increase in endogenous Runx2 activation because estradiol caused an analogous effect when osteoblasts were transfected with limiting transcriptional components, control cultures were transfected with a compensating amount of empty vector. Transfection efficiency was assessed in parallel in cells transfected with positive and negative reporter plasmids as described previously (16, 23, 35–37).

**Protein Extracts**—Cells were rinsed, harvested by scraping and centrifugation, and lysed in hypotonic buffer supplemented with phosphatase and protease inhibitors and 1% Triton X-100 as described previously. Nuclei and cytoplasm were separated by centrifugation. Nuclei were extracted in hypotonic buffer with glycerol and phosphatase and protease inhibitors, and the nuclear proteins released in this way were separated from insoluble material by centrifugation (16, 23, 37).

Electrophoretic Mobility Gel Shift Assay—Double-stranded DNA probes were prepared by annealing complementary oligonucleotides. Overhangs were filled with dNTPs and radiolabeled with [32P]dCTP using the Klenow fragment of DNA polymerase I (New England Biolabs). Nuclear extract was incubated on ice with 32P-labeled probe. In some samples, nuclear extract was pre-incubated with antisera for 1 h before adding 32P-labeled probe. Radioactive complexes were resolved on a 4% non-denaturing polyacrylamide gel and visualized by autoradiography (16, 23, 37).

**Immunoprecipitations and Western Blots**—Cell extracts were combined with pre-immune IgG or specific antibody and immunoprecipitates were collected with Protein A Sepharose (Pierce). Total cell extract or immunoprecipitates were fractionated by electrophoresis on a 12% SDS-polyacrylamide gel, blotted onto Immobilon P membranes (Millipore), probed with primary antibody, and visualized with secondary antibody and chemiluminescence (16, 37).

**RESULTS**

**ER-dependent Gene Expression in Cultured Osteoblasts**—ERα expression parallels late stage osteoblast differentiation and mineralization in culture (39–42). Consistent with this, primary cultures of fetal rat osteoblasts express little or no endogenous ERα at a time in culture when they are susceptible to transfection with plasmids encoding steroid dependent reporters or regulatory gene products (Fig. 1A). Nonetheless, they respond rapidly and potently to estradiol after transfection with an expression plasmid encoding ERα (Fig. 1B). Therefore, these easily obtained and well characterized osteoblast cultures can be made estrogen responsive in vitro, replicating the hormone-sensitive status of osteoblasts that occur in more mature organisms in vivo (43–46).
found heterodimers of ER/H9251 modify gene expression in complex ways (6, 8, 37, 48). The ability of Runx2 to associate with DNA that contains a consensus ERE. Extracts were incubated with either non-immune IgG, or ERα antibodies MC-20 (anti-mouse carboxyl terminus; mouse, human, and rat reactive) or H-184 (anti-human amino terminus; mouse, human, and rat reactive) (Santa Cruz Biotechnology). The identity of the invariant, non-reactive complexes in extracts from osteoblasts that lack ERα expression is unknown. B, osteoblasts were co-transfected with empty vector (V) or ERα and reporter plasmid containing consensus ERE and then treated for 24 h with 10 nm estradiol. Estradiol expression was measured in cytoplasmic extracts and corrected for protein content. Estradiol significantly increased gene expression by ERα in ERα-transfected cells. Data represent results from a minimum of nine replicate samples and three separate studies with different culture preparations.

Therefore, estradiol enhanced Runx2 transcriptional activity, whether Runx2 used its own DBD to drive a native Runx RE or used the added GAL4 DBD to drive the 5XGAL4 RE system. These results are consistent with no obvious stimulatory effects by estradiol on total Runx2 levels in this short time frame or on estradiol nuclear localization domain encoded by the MVN1 transfection vector (47).

Importantly, in addition to COS-7 cells, similar complexes between MVN1-ERα and M1-Runx2 or its fragments occurred when the two-hybrid gene expression assay was performed in primary cultures of osteoblasts (Fig. 4A). To define the regions of ERα that associate with Runx2, MVN1 fusion proteins that contained fragments of ERα, as indicated in Fig. 4B, were then co-transfected with full-length M1-Runx2. Functional complex formation with Runx2 occurred with only discrete fragments of ERα. Notably, the C region or the DBD of ERα alone seemed to be a highly proficient binding partner by comparison with other ERα domains.

**Combined but Independent Effects by Estrogen and Protein Kinase A Activation on Runx2**—Like their stimulatory effects on C/EBPβ, prostaglandin E2 (PGE2) and parathyroid hormone enhance Runx2 activity in a protein kinase A-dependent manner (37, 52). However, in direct contrast to the potent inhibitory effect of estrogen on protein kinase A-activated C/EBPβ, Runx2 activity increased even further in ERα-transfected osteoblasts treated with both estradiol and PGE2 (Fig. 5A). Consistent with the results presented in Fig. 4, co-expression of MVN1-ERα increased M1-Runx2 activity in untreated cells, as defined by the two-hybrid gene expression system. However, treatment with estradiol and PGE2 further enhanced M1-Runx2 activity in complex with MVN1-ERα, by comparison with either hormone alone (Fig. 5B, left).

To substantiate the importance of the ERα DBD for these effects, the C region was eliminated in plasmid MVN1-ERα[-C]. In this context and without hormone treatment, M1-Runx2 activity (Fig. 5B, right) was reduced to approximately the level found in osteoblasts transfected with untagged ERα (Fig. 5A, right). Moreover, whereas the stimulatory effect of PGE2 on M1-Runx2 persisted in cells co-transfected with MVN1-ERα[-C], estradiol failed to enhance M1-Runx2 activity without or with PGE2 (Fig. 5B, right). To address the C domain of ERα within the context of endogenous Runx2 activity, osteoblasts were co-transfected with plasmids containing the Runx-sensitive reporter plasmid SXN1C and either full-length ERα or ERα[-C] subcloned into vector M1. In this way, the VP16 transactivation domain tag would not complicate activation of endogenous Runx2. Consistent with the results in Fig. 5B, SXN1C failed to respond to estradiol in cells transfected with M1-ERα[-C] but remained sensitive to PGE2 (Fig. 5C). To confirm the loss of a functional C domain in ERα[-C] plasmids, osteoblasts were co-transfected with M1-ERα or M1-ERα[-C] and ERα reporter plasmid. M1-ERα[-C] failed to increase estradiol-dependent promoter activity, inasmuch as gene expression in this system requires binding by the C domain of ERα to consensus ERE (Fig. 5D).

**Estrogen Specificity**—To assess steroid specificity within the context of Runx2 activation, osteoblasts were then treated with androgen or glucocorticoid. Analogous to our findings with estradiol as shown in Fig. 1, fetal rat osteoblasts exhibit maximal sensitivity to androgen when transfected to express AR and reporter plasmid driven by consensus ARE (Fig. 6A) or...
Estrogen Focuses TGF-β Activity—To determine whether an estrogen-dependent increase in Runx2 activity has downstream effects, osteoblasts were co-transfected with ERα and reporter plasmid driven by full-length TGF-βRI gene promoter that contains four Runx binding elements. Consistent with evidence that Runx2 accounts for 50% decrease that occurs with glucocorticoid treatment (19) (Fig. 5C). Therefore, Runx2 suppresses the activity of estradiol, glucocorticoid, and androgen, whereas these same steroid hormones independently increase, decrease, or have no effect on Runx2 activity. This suggests that control and counter-control between Runx2 and each of these steroids is focused in very different ways.

Runx2 Suppresses Estrogen Activity—Because complex formation with Runx2 involves the DBD of ERα (Figs. 4 and 5), Runx2 could possibly counter-regulate estrogen activity. Indeed, forced expression of Runx2 dose-dependently decreased the effect of estradiol on reporter gene expression driven by ERE (Fig. 7A). Consistent with this, and the normal presence of Runx2 in osteoblasts (5, 28, 35), transfection with Runx2 antisense to limit endogenous Runx2 levels (37, 38) further increased ERE reporter gene expression by 2.5–3-fold (Fig. 7B).

MMTV-Luc.° However, cortisol potently activates GRE-dependent gene expression in these osteoblast cultures through endogenous glucocorticoid receptor (GR) (19, 53) (Fig. 6A) and is not activated further by transfection with GR expression vector. Unlike estradiol, androgen failed to activate Runx2 in osteoblasts transfected to express plasmid SXN1C, and consistent with earlier studies (19), glucocorticoid was inhibitory (Fig. 6B). Androgen also failed to activate M1-Runx2 by way of the heterologous 5XGAL4 reporter. However, expression of M1-Runx2 in the context of the 5XGAL4 reporter rescued osteoblasts from the loss of endogenous Runx2 that occurs with glucocorticoid treatment (19) (Fig. 6C).

Runx2 and Runx2 Suppresses Estrogen Activity—Because complex formation with Runx2 involves the DBD of ERα (Figs. 4 and 5), Runx2 could possibly counter-regulate estrogen activity. Indeed, forced expression of Runx2 dose-dependently decreased the effect of estradiol on reporter gene expression driven by ERE (Fig. 7A). Consistent with this, and the normal presence of Runx2 in osteoblasts (5, 28, 35), transfection with Runx2 antisense to limit endogenous Runx2 levels (37, 38) further increased ERE reporter gene expression by 2.5–3-fold (Fig. 7B).

Analogous inhibitory or stimulatory effects by forced expression of Runx2 or Runx2 antisense occurred on androgen- and glucocorticoid-dependent gene expression (Fig. 7, A and B). Therefore, Runx2 suppresses the activity of estradiol, glucocorticoid, and androgen, whereas these same steroid hormones independently increase, decrease, or have no effect on Runx2 activity. This suggests that control and counter-control between Runx2 and each of these steroids is focused in very different ways.

Estrogen Focuses TGF-β Activity—To determine whether an estrogen-dependent increase in Runx2 activity has downstream effects, osteoblasts were co-transfected with ERα and reporter plasmid driven by full-length TβRI gene promoter that contains four Runx binding elements. Consistent with evidence that Runx2 accounts for ~50% of TβRI gene expression in osteoblasts (28), estradiol activated the TβRI gene promoter by 40–50% relative to the 40–50% decrease that occurs with glucocorticoid treatment (19) (Fig. 8A) and caused a corresponding

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2 T. L. McCarthy and M. Centrella, unpublished observations.
3 M. Centrella, unpublished observations.
increase in TβRI protein (Fig. 8B). Furthermore, the stimulatory effect of estradiol on Runx2 activity and on TβRI expression allowed an analogous increase in Smad-dependent gene expression in response to TGF-β in cells co-transfected with plasmid SBE4 (Fig. 8C). However, although estradiol enhanced TβRI gene promoter activity and the TGF-β-stimulated Smad pathway, it suppressed reporter gene expression by 3TP-Lux in TGF-β-treated cells (Fig. 8D),
perhaps by its previously noted inhibitory effect on AP-1 activity in some situations (54–56).

DISCUSSION

Although many studies focus on estrogen as an inhibitor of bone resorption, others suggest additional effects on bone formation. Recent evidence predicts that estrogen may act in part on osteoblasts to inhibit apoptosis (24–27) or to regulate AP-1-related transcription factors by specific kinase-dependent pathways (25, 27, 57). In this report, we show that estradiol increases gene transactivation by Runx2, an essential transcription factor for skeletal tissue development (5, 6). The effect of estradiol requires a functional ER and occurs without changes in Runx2 expression or its affinity for DNA. Moreover, the stimulatory effect of estradiol on Runx2 activity is further enhanced by PGE2, a potent kinase activator in osteoblasts (34, 58), although their primary mechanisms of action are independent.

It is important to note that many aspects of our current study involved exogenous gene transfection. This required the use of cells at a stage in culture at which they do not yet express high levels of ERs (39–42). To correct this, we transiently transfected or co-transfected osteoblasts to express ER/H9251. Other investigators addressed this deficiency by creating osteoblast-like cell lines that were stably transfected to express ER/H9251 under selective pressure from antibiotics or transforming viral gene elements. We chose to avoid possible complications from antibiotic toxicity, unknown effects from stable gene integration, and phenotypic drift by cells in continuous culture with our approach. Even so, it is difficult to know how the level of ER/H9251 expression in any transfected cell model compares with that in adult bone because, even in vivo, levels of ERs vary considerably with age and with anatomical bone location (43–46). However, expression of ERs by transient gene transfection seemed appropriate and predictable, because the ability of estrogen to drive reporter gene expression by ERE was steroid-
specific and varied by less than 10% in our studies.

Other reports suggest links between estrogen and Runx2 through an increase in the number of Runx2-expressing cells (59). Furthermore, selective ER modulators with modes of action that are often distinct from estrogen itself seem to enhance Runx2 gene promoter activity (60, 61). We found no increase in Runx2 protein or its DNA binding potential after estrogen treatment. Important differences between estrogen and its mimetics may result in part from variations in ER conformation that occur after engagement with different ligands, or from their abilities to activate or suppress different genomic RE (62–64). Moreover, effects on Runx2 gene promoter activity or mRNA expression may not readily correspond to the level of functional Runx2 protein or to its gene activation potential (19, 37, 52, 65, 66). Our studies therefore identify a new pathway that may account in part for the anabolic effect of estrogen on skeletal tissue cells and a decrease in bone formation after estrogen withdrawal.

We found that ERα and Runx2 interact through the DBD of ERα. Interactions have been reported between ERα and several other transcription factors, including retinoid receptors (67), forkhead transcription factors (68), Smads (69, 70), and Stat5 (71). The interaction between ERα and Stat5 also maps to the ERα DBD (71). Other transcriptional regulators seem to control Runx transcription factors by an interaction that requires, in whole or in part, the Runt domain (4, 6, 8, 37, 72), whereas ERα does not seem to associate in an efficient way with the Runt domain of Runx2 by itself (current study). As modeled in Fig. 9, the focused interaction that we find with the DBD of ERα retains and expands Runx2 activity, although it limits ERα-dependent gene expression through consensus ERE. This distinction between direct and indirect control of gene expression by ERα is reminiscent of mutations in GRα that prevent its ability to form homodimers. In this instance, a lack of gene expression through GRE is compatible with life, whereas overall gene deletion of GRα is lethal (73, 74). Similarly, inactivating mutations in the ERα gene in mice cause steriodogenesis and selective defects in reproductive and mammary tissue (71, 75). Thus, effects on genes driven by elements other than the consensus steroid RE also seem to be critical, if not essential, targets of steroid hormone action in bone-forming cells.

Our results therefore predict that Runx2 expression in growing or remodeling bone in part limits those aspects of estrogen activity that require binding by ERα directly to DNA but expands its gene targets in a tissue-restricted way. Runx2 also suppresses gene expression through ARE and GRE, even though androgen has no effect on Runx2 activity in osteoblasts (current study) and glucocorticoid is inhibitory (19). Consequently, changes in the balance of Runx2 and steroid hormone receptors in osteoblasts could integrate gene expression in complex but focused ways. For example, a high level of glucocorticoid could significantly limit development of the osteoblast phenotype in bone cells where Runx2 expression is evolving but

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4 M. Centrella and T. L. McCarthy, unpublished observations.
have negligible effects on Runx2-sensitive genes in more differentiated osteoblasts, where Runx2 levels are high. Consistent with this, the inhibitory effect of glucocorticoid was not evident in osteoblasts, in which Runx2 was sustained by gene transfection. Protein hybridization and domain mapping studies showed that Runx2 also interacts with the DBD of GR,4 consistent with its ability to suppress gene expression at GRE. Again, the interaction between androgen and Runx2 in osteoblasts seems to be limited to inhibitory effects on AR activity through ARE. Our results are similar to those of earlier studies showing complex formation between full-length Runx2 fusion protein and either AR or GR. However, they also differ in part because those authors found that overexpression of Runx2 in monkey kidney cells failed to suppress the effect of androgen or glucocorticoid on gene expression driven by steroid hormone RE fused to a fragment of the thymidine kinase gene promoter (51). Nevertheless, in osteoblasts, Runx2 suppressed gene expression driven by consensus ERE, ARE, or MMTV-Luc, a well characterized androgen and glucocorticoid sensitive reporter plasmid. We have not yet mapped interacting domains between Runx2 and AR to learn whether this is similar to our current findings with ER and GR.

One of several target gene products for Runx2 in osteoblasts is TgRI (28, 35). Indeed, loss of Runx2 by glucocorticoid excess (19) or by transfection with Runx2 antisense expression plasmid (38) limits TgRI expression and suppresses the stimulatory effect of TGF-β on collagen synthesis. This would therefore reduce the amount of the major organic component of the framework for mineral deposition in bone and subsequently limit bone formation or repair. Thus, abanic effects by estrogen may occur in part through changes in the balance of TgRI expression that maintains or enhances the effectiveness of local TGF-β in the bone environment. Unlike the inhibitory effects of estradiol on TGF-β-sensitive and bone morphogenetic protein-sensitive Smads that occur in mesangial and breast cancer cells (69, 70), estradiol enhances Smad-dependent gene expression in response to TGF-β in osteoblasts. Interactions between ERs and AP-1 transcription factors are also complex, because estrogen can either increase or decrease the expression of AP-1-sensitive genes (56, 57, 71, 76). We found that estrogen reduced TGF-β activation of the AP-1-sensitive promoter 3TP-Lux. Therefore, estrogen also seems to distinguish specific aspects of TGF-β activity that are downstream of Runx2 activation and TgRI expression.

In summary, our studies present new evidence for abanic effects by estrogen on bone-forming cells through an increase in gene activation by transcription factor Runx2. In contrast, Runx2 limits the so-called “classic” pathway of steroid hormone action by estrogen, androgen, and glucocorticoid. Therefore, changes in the relative synthesis of Runx2 and steroid hormone receptors may regulate the expression of “classic” and “nonclassic” steroid-sensitive gene products during specific stages of osteoblast development in different ways. We predict that similar important interactions may also occur with steroid hormones in those cells in which other Runx gene family members have critical effects on tissue-restricted gene expression.

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