Estrogen (E2) is involved in mediating many important functions relevant to osteoblast biology through the actions of the estrogen receptors (ER) α and β. To further understand the mechanisms of ER-specific regulation, we used microarray and reverse transcription-PCR analyses of E2-treated U2OS-ERα or -ERβ cells and identified retinoblastoma-binding protein 1 (RBBP1) as a major E2-regulated gene. RBBP1 is a retinoblastoma cofactor involved in the control of osteoblastic proliferation. Although RBBP1 mRNA levels rapidly increased after 2 h of E2 treatment in both U2OS-ER-expressing lines, a sustained induction was only observed in U2OS-ERα cells. Examination of the RBBP1 genomic sequence revealed an ER response element and a Sp1 site located within the first intron. Chromatin immunoprecipitation analyses demonstrated that E2-dependent ERα binding to the intron 1 enhancer region was constitutive, whereas ERβ binding was transient, consistent with the mRNA time course. Interestingly, transient transfection and receptor mutational studies revealed that RBBP1 induction by ERα only requires the Sp1 site, whereas ERβ utilizes both the Sp1 and estrogen response elements binding sites for maximal E2-dependent activation. Stable U2OS transfectants containing a deletion of the ERα activation function 1 (AF1) resulted in a temporal mRNA induction profile similar to that of wild type ERβ. Further, overexpression and chromatin immunoprecipitation analyses also demonstrated that E2-dependent RBBP1 induction is SRC2-dependent for both ER isoforms. These results describe an E2-dependent, ER isoform-specific transcriptional activation of the RBBP1 gene, which in part, is explained by the differential activity of ER AF1 and enhancer element binding.

The transcriptional effects of estrogens (e.g. 17β-estradiol or E2) are largely mediated by two related, but distinct, estrogen receptor (ER) isoforms ERα and ERβ (1–7). The ERs are ligand-inducible transcription factors that bind E2 through a C-terminal ligand-binding domain (or E/F domain). The ER isoforms contain a centrally located and highly conserved DNA-binding domain (or C domain) that associates directly with estrogen response elements (ERE) or indirectly through protein tethering to AP1 or Sp1 (8, 9). Both ERα and ERβ contain two activation functions (AF); a highly divergent N-terminal A/B (AF1) domain and a more conserved C-terminal E/F (AF2) domain that overlaps with the ligand-binding domain (10–13). Activation of transcription is achieved through the recruitment of specific transcriptional coregulators resulting in chromatin remodeling and the recruitment of the basal transcriptional machinery (1, 9, 14–16). Although coregulator recruitment and activation is largely mediated through AF2, the A/B domain (AF1) is known to bind the p160 and p300/CBP (CREB-binding protein) families of coregulators (16–20) as well as p65/p72 (21, 22), steroid receptor RNA activator (23), and MMS19 (25) proteins.

Recent data from our laboratory (26, 27) and others (28, 29) have demonstrated that significant differences exist in gene expression patterns, elicited by ERα and ERβ homodimers and ERα/ERβ heterodimers, in a human osteosarcoma (U2OS) model system. Specifically, only ~20% of all genes regulated through either ER isofrom are regulated by both ER isoforms. The mechanism(s) of ER isoform-specific gene regulation are unknown; however because of the low level of homology between the ERα and ERβ A/B (AF1) domains, differences in transcriptional activation may be largely due to AF1 function (30, 31).

In this study, we show that the RBBP1 gene, the protein product of which is involved in retinoblastoma-mediated suppression of cell proliferation, is differentially regulated by ERα and ERβ in a U2OS model system. Using deletion and mutational analysis, we demonstrate that ERα utilizes a consensus Sp1 sequence, whereas ERβ functions through both the Sp1 and a nearby ERE sequence. Deletion of the ERα AF1 in a stable U2OS transfectant cell line causes the temporal profile to mimic that of wild type ERβ. These data indicate that different

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*This work was supported by National Institutes of Health Grant PO1-AG04875-21 and by grants from the Breast Cancer Research Foundation (New York) and the Mayo Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; RT, reverse transcription; ChIP, chromatin immunoprecipitation; RBBP1, retinoblastoma-binding protein 1; AF, activation function; WT, wild type; FBS, fetal bovine serum; CS-FBS, charcoal-stripped FBS; Dox, doxycycline; ANOVA, analysis of variance; SRC, steroid receptor coactivator.
tial transcriptional activation of RBBP1 by ERα and ERβ and transcription factor binding site utilization are AF1-dependent.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemicals—The U2OS-ERα and -ERβ cell lines, described previously (26), were cultured in phenol red-free Dulbecco’s modified Eagle’s medium/F12 medium containing 10% (v/v) fetal bovine serum (FBS), 1× antibiotic/antimycotic (Invitrogen), 5 mg/liter blasticidin S (Roche Applied Science), and 500 mg/liter zeocin (Invitrogen). The U2OS-ERΔAF1 cell lines, described in Fig. 6, were cultured in the same medium supplemented with 100 mg/liter hygromycin B (Invitrogen). Mouse calvarial osteoblasts were cultured in Dulbecco’s modified Eagle’s medium/F12 plus 10% (v/v) FBS and 1× antibiotic/antimycotic. Steroid treatments were performed either in charcoal-striped fetal bovine serum (CS-FBS) containing Dulbecco’s modified Eagle’s medium/F12 medium (HyClone Laboratories, Logan, UT) or in serum-free medium in the transient transfection assay. Doxycycline (Dox) and 17β-estradiol (E2) were purchased from Sigma.

Reverse Transcription PCR (RT-PCR) Analysis—The U2OS-ERα and -ERβ cell lines were plated in 6-well cell culture dishes at a cell density of 50% and pretreated with 100 ng/ml Dox for 24 h in CS-FBS medium. The cells were then treated with 100 ng/ml Dox plus ethanol control or 10 nM E2 for 1, 2, 4, 6, 8, or 24 h. Mouse calvarial osteoblasts were plated and treated with E2 in the same manner as the U2OS-ER cells. The RNA was harvested using TRIzol reagent (Invitrogen). Four μg of total RNA was heat-denatured at 68 °C for 15 min in a reverse transcription reaction buffer (1× first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂), 50 mM dithiothreitol, 1 μM dNTPs, 500 ng of oligo(dT) primer). Following heat denaturation, 1 unit of mouse Moloney leukemia virus-reverse transcriptase (Invitrogen) was added, and the mixture was incubated at 37 °C for 45 min followed by a 68 °C incubation for an additional 15 min. The cDNA products were diluted to 50 μl with PCR-grade water; 2 μl was used in a PCR reaction using primers specific for human RBBP1 and ERα-actin, and products were separated on a 1.5% agarose gel as described previously (26). Real-time PCR was performed as described previously (32). Primers specific for RBBP1 were as follows: human RBBP1 forward, 5’-GCAAAAAGCTGATCCCGAGCCAGGACTTCCCTGTAC-3’; human RBBP1 reverse, 5’-TCCCGCATGAGAC-GCAAAAGCGTACCCCAAAG-3’. The resulting cell lines are termed U2OS-ERα-ΔAF1 and ERβ-ΔAF1, respectively. The generation of the U2OS-ER-ΔAF1 cell lines was performed using the T-REx system (Invitrogen) as described previously (26). The ER-ΔAF1-pcDNA4/TOR vectors were linearized using a PvuI restriction digest and transfected into the U2OS-Tet8 cells at a density of 50% using FuGENE 6 transfection reagent (Roche Diagnostics). 48 h later, the transfected cells were split and plated in an antibiotic-containing, selective medium. Individual cell clones were isolated and Western blot analysis performed to confirm expression of both ERα-ΔAF1 and ERβ-ΔAF1. The resulting cell lines are termed U2OS-ERα-ΔAF1 and -ERβ-ΔAF1.

Chromatin Immunoprecipitation (ChIP) Assay—U2OS-ERα and -ERβ cells (1 × 10⁷) were seeded into 10-cm plates and treated with Dox for 24 h followed by either ethanol control or 10⁻⁸ E2 for 1, 2, 4, 8, and 24 h in CS-FBS medium. ChIPs were then performed using modifications of the procedure described by Lambert and Nordeen (33) and as described previously (27). Five μg of ERα-specific antibody (HC20; Santa Cruz Biotechnology, Santa Cruz, CA), ERβ-specific antibody (P2571; Invitrogen), SRC1-specific antibody (05–522; Upstate Biotechnology, Charlottesville, VA), or SRC2-specific antibody was used. Standard end point and real-time PCR reactions were performed as described previously using primers specific for the RBBP1 intron 1 enhancer region (27).

Production of the DNA-binding ER Mutants—Construction of the ERα and ERβ P-box mutation expression plasmids was performed using two-step PCR. Briefly, primers were designed to mutate Glu210 and Gly214 to alanines for ERα and Glu167 and Gly169 to alanines for ERβ. The ERα double mutation was as reported previously (34). Because ERα and ERβ share 97% identity within the DNA-binding domain, the same mutation was made in ERβ. Resultant products were mixed and amplified using specific flanking primers containing an N-terminal FLAG epitope tag. The resulting PCR fragment was subcloned into the BamHI/Xhol and HindIII/Xbal sites of pcDNA4/TOR for the ERα and ERβ P-box mutations, respectively.

Development of the ER-ΔAF1 Expression Constructs and the U2OS-ER-ΔAF1 Cell Lines—Construction of the ERα-ΔAF1 and ERβ-ΔAF1 expression plasmids was performed using PCR. Briefly, primers were designed to amplify amino acids 174–595 of human ERα and amino acids 130–530 of human ERβ. An in-frame N-terminal FLAG epitope was included in the forward primers for validation of protein expression (32). The resulting PCR fragment was subcloned into the BamHI/Xhol and HindIII/Xbal sites of pcDNA4/TOR for ERα-ΔAF1 and ERβ-ΔAF1, respectively. The generation of the U2OS-ER-ΔAF1 cell lines was performed using the T-REx system (Invitrogen) as described previously (26). The ER-ΔAF1-pcDNA4/TOR vectors were linearized using a PvuI restriction digest and transfected into the U2OS-Tet8 cells at a density of 50% using FuGENE 6 transfection reagent (Roche Diagnostics). 48 h later, the transfected cells were split and plated in an antibiotic-containing, selective medium. Individual cell clones were isolated and Western blot analysis performed to confirm expression of both ERα-ΔAF1 and ERβ-ΔAF1. The resulting cell lines are termed U2OS-ERα-ΔAF1 and -ERβ-ΔAF1.

Transient Transfection Analyses—U2OS-ERα and U2OS-ERβ cells were plated at a density of 50% in 6-well plates. RBBP1 intron 1 enhancer constructs and ER mutant constructs were transiently transfected in triplicate using FuGENE 6 transfection reagent (Roche Diagnostics) at a concentration of 250 ng/well in CS-FBS-containing medium supplemented with 100 ng/ml Dox for ER induction. The next day, cells were washed twice with 1× phosphate-buffered saline and treated with 100 ng/ml Dox and either ethanol control or 10 nM E2 in serum-free medium for an additional 24 h. Cells were harvested, and 1 μl of protein extract was assayed using luciferase assay reagent (Pro-
**RBBP1 Is Regulated by Estrogen in Osteoblasts**

**RESULTS**

Regulation of RBBP1 by E2 in Osteoblastic Cells—Our previous microarray data demonstrated that RBBP1 was regulated 4.01-fold following 24 h of E2 treatment in the U2OS-ERα cell line, whereas no regulation was detected in the U2OS-ERβ cell line (27). In contrast, at 2 h of E2 treatment, RBBP1 was regulated 3.74- and 2.55-fold in the U2OS-ERα and -ERβ cell lines, respectively (data not shown). To further understand the complex kinetics of RBBP1 induction by both ER isoforms, we performed an E2 time course from 0 to 24 h of treatment.

ChIP analysis was conducted to determine binding of the ER isoforms in the context of chromatin structure and the extent of binding over a period of time to the RBBP1 intron 1 enhancer region. Fig. 3 demonstrates that significant binding, in vivo, of both ERα and ERβ is observed following 1 h of E2 treatment. More specifically, ERα exhibits an E2-dependent binding to the RBBP1 intron 1 enhancer region throughout the 24-h time course, whereas ERβ displays E2-dependent binding only at 1 h of E2 treatment. It is interesting to note that ERβ binding is minimal at 2 h following E2 treatment and that the RBBP1 mRNA (see Fig. 1) does not completely decrease to near control levels until 8 h of E2 treatment, suggesting that mRNA stability is playing a role in these differential chronologies. These data are in general agreement with the RT-PCR data (Fig. 1), which suggest that the sustained decrease in the expression of RBBP1 in U2OS-ERα cells and the temporary increase of RBBP1 expression in U2OS-ERβ cells correlate with and are probably dependent on ER binding to the RBBP1 intron 1 enhancer region.

**Analysis of the RBBP1 Intron 1 Enhancer Region Delineates Different Requirements for ERα and ERβ Binding and Promoter Activation**—To determine the role of the ERE and neighboring trans-acting sites in E2 regulation of RBBP1 by the ER isoforms, various RBBP1 intron 1 enhancer constructs were PCR-amplified, cloned into a luciferase reporter vector, and transiently transfected in both U2OS-ERα and -ERβ cell lines. Both ERs activate the RBBP1 +412 to +1140 construct, which contains the ERE and Sp1 sites (Fig. 4A). Loss of the +950 to +1140 sequences, including most of the Sp1 site, completely abolished ERα-dependent activation, whereas only a slight effect was observed on ERβ-dependent activation. This suggests that the
RBBP1 Is Regulated by Estrogen in Osteoblasts

RBBP1 Is Regulated by Estrogen in Osteoblasts

Figure 2. The RBBP1 intron 1 region contains both ERE and Sp1 binding sites. A, a schematic diagram of the RBBP1 intron 1 enhancer region (+400 to +1140, relative to the transcriptional start site) is depicted. B, the ERE (+899) and Sp1 (+949) sequences from the RBBP1 intron 1 enhancer are depicted with comparisons to the consensus binding sites.

Figure 3. ChIP analysis of the RBBP1 intron 1 enhancer region demonstrates ER binding. A, ChIP assays were performed in U2OS-ERα and ERβ cell lines treated with 10 nM E2 for the indicated times using antibodies specific for either ERα or ERβ as described under “Experimental Procedures.” The resultant products were separated using agarose gel electrophoresis. B, real-time PCR analysis was employed on samples from three independent ChIP experiments. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with control treatment (0 h) within each cell line.

Sp1 site within this sequence is critically important for ERα but not ERβ-dependent transcriptional activation.

To specifically characterize the effects of the ERE and Sp1 sites in E2-dependent activation in the same context, mutations of the ERE and Sp1 sites, alone or in combination, were created in the +851 to +1140 context and transiently transfected in the U2OS-ER cell lines. Fig. 4B demonstrates that mutation of the Sp1 site completely abolishes E2-dependent activation in the U2OS-ERβ cell line, consistent with the deletion of the Sp1 site in Fig. 4A. Interestingly, no significant effect was observed with the ERE mutation in the U2OS-ERα cell line. Mutation of either the Sp1 or ERE decreased ERβ-specific activation by ~50% but still exhibited a statistically significant E2 induction, whereas mutation of both sites completely abolished E2-dependent activation in the U2OS-ERβ cell line. These data suggest differential mechanisms for ERα-versus ERβ-dependent activation, wherein ERα exclusively utilizes the Sp1 site and ERβ utilizes both the Sp1 and ERE sites for maximal activation.

ERα-dependent Activation of the RBBP1 Intron 1 Enhancer Region Is Independent on ERE Binding—Because the RBBP1 intron 1 enhancer region contains both an ERE and Sp1 element, we determined the relative contribution of these elements in the RBBP1 wild type +851 to +1140 context. Jakacka et al. (34) created a mutation in the mouse ERα DNA-binding domain, which inhibited ER binding to an ERE but did not affect signaling through non-ERE sites. We therefore created the same mutation in the human ERα and ERβ DNA-binding domains (Fig. 5A) to generate constructs in which signaling through the RBBP1 ERE was eliminated yet still maintained the ability to signal through the RBBP1 Sp1 site. Fig. 5B demonstrates that both the ERα and ERβ P-box mutations significantly inhibit activation through a consensus ERE element when compared with the wild type receptor. Interestingly, the ERα P-box mutation had no effect on transcriptional activation of the RBBP1 +851 to +1140 construct, whereas the ERβ P-box mutation decreased activation by ~60% (Fig. 5C). This is in agreement with Fig. 4B demonstrating that activation of the RBBP1 intron 1 enhancer region through ERα is ERβ-independent, whereas activation through ERβ is at least partially ERα-dependent.

The ERα AF1 Domain Is Important for Sustained Activation of RBBP1 Intron 1 Enhancer Activity—To test the hypothesis that the ER A/B domain (AF1) was involved in mediating ER isoform-specific activation of the RBBP1 gene, N-terminal deletions of both ER isoforms were created (Fig. 6A). The ERαΔAF1 construct, starting at Met174 of ERα, matches the previously described 46-kDa form of ERα, which is expressed in normal osteoblasts (36, 37). Therefore, wild type and ERΔAF1 constructs were transiently cotransfected with the RBBP1 +851 to +1140 reporter plasmids (Fig. 4B) in U2OS cells. The data presented in Fig. 6B closely resembles the transfection data from the U2OS-ER stable transfectants (Fig. 4B), wherein the activity of ERα is purely Sp1-dependent (ERE mutation has no effect), and maximal activity of ERβ is dependent on both the ERE and Sp1 sequences. Coexpression of ERαΔAF1 with the wild type RBBP1 +851 to +1140 construct (Fig. 6C) leads to an increased -fold activation by E2 over wild type ERα (4.96-versus

Figure 4. ChIP analysis of the RBBP1 intron 1 region demonstrates ER binding. A, ChIP assays were performed in U2OS-ERα and ERβ cell lines treated with 10 nM E2 for the indicated times using antibodies specific for either ERα or ERβ as described under “Experimental Procedures.” The resultant products were separated using agarose gel electrophoresis. B, real-time PCR analysis was employed on samples from three independent ChIP experiments. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with control treatment (0 h) within each cell line.

Figure 5. ChIP analysis of the RBBP1 intron 1 region demonstrates ER binding. A, ChIP assays were performed in U2OS-ERα and ERβ cell lines treated with 10 nM E2 for the indicated times using antibodies specific for either ERα or ERβ as described under “Experimental Procedures.” The resultant products were separated using agarose gel electrophoresis. B, real-time PCR analysis was employed on samples from three independent ChIP experiments. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with control treatment (0 h) within each cell line.

Figure 6. ChIP analysis of the RBBP1 intron 1 region demonstrates ER binding. A, ChIP assays were performed in U2OS-ERα and ERβ cell lines treated with 10 nM E2 for the indicated times using antibodies specific for either ERα or ERβ as described under “Experimental Procedures.” The resultant products were separated using agarose gel electrophoresis. B, real-time PCR analysis was employed on samples from three independent ChIP experiments. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with control treatment (0 h) within each cell line.
RBBP1 Is Regulated by Estrogen in Osteoblasts

**FIGURE 4.** Mutational analysis of the RBBP1 intron 1 enhancer region results in ER isoform-specific effects. A, U2OS-ERα and -ERβ cells were transiently transfected in triplicate with the indicated RBBP1 intron 1 enhancer region constructs as described under "Experimental Procedures." -Fold regulation relative to ethanol control was determined for each of the constructs, which were assayed in at least three independent experiments. Individual errors were less than 10% of the mean for each experiment. The black bars represent the mean ± S.D. of n number of trials. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with the +851 to +1140 construct. B, cells were treated, transiently transfected, assayed, and analyzed with either ERE or Sp1 mutations in the +851 to +1140 context. Asterisks denote significance at the p < 0.01 level (ANOVA) compared with +851 to +1140 wild type construct. C, the wild type sequences of the ERE and Sp1 sites of the RBBP1 intron 1 enhancer region is depicted with the mutations listed in bold.

1.99-fold), indicating that the ERα AF1 may play a suppressor role in E2-dependent activation of RBBP1. Interestingly, the ERα-ΔAF1 construct is now sensitive to the RBBP1 ERα mutation as is normally observed with wild type ERβ (Fig. 6, compare ERβ WT (B) with ERα-ΔAF1 (C)). No significant change was observed with the ERβ-ΔAF1 compared with wild type ERβ with any of the RBBP1 constructs. These data suggest that the ERα AF1 is involved in mediating specific interactions with the RBBP1 Sp1 site and inhibition of the use of the RBBP1 ERE sequence because deletion of the ERα AF1 now allows signaling through the ERE.

Because the data in Fig. 6 suggest that the deletion of the ERα AF1 results in an "ERβ-like" pattern of E2-inducible expression, we determined the effect of ERO-ΔAF1 on endogenous RBBP1 expression. Therefore, we constructed stable U2OS cells lines expressing either the ERα- or ERβ-ΔAF1 construct. As seen in Fig. 7A, the U2OS-ERα-ΔAF1 and -ERβ-ΔAF1 cell lines express similar levels of ER to those observed in U2OS-ER wild type cell lines and correspond to the expected size (~46 kDa). We next examined the temporal E2 induction profile of endogenous RBBP1 in the U2OS-ERα-ΔAF1 and -ERβ-ΔAF1 cell lines. The U2OS-ERα-ΔAF1 cell line exhibited a transient increase in RBBP1 expression peaking at 2 h of E2 treatment, whereas the U2OS-ERα cell line exhibited a sustained increase in RBBP1 expression (Fig. 7B) as observed previously in Fig. 1. Interestingly, the U2OS-ERα-ΔAF1 expression pattern is similar to that of U2OS-ERβ. The U2OS-ERβ-ΔAF1 cell line exhibits an overall decreased level of RBBP1 expression at 2 h of E2 treatment with minor induction observed at later time points. Collectively, these data suggest that the ERα AF1 confers ERO-specific patterns of regulation in both transient transfection analysis and endogenous RBBP1 expression.

**SRC2 Preferentially Enhances E2-induced RBBP1 Intron 1 Enhancer Activity**—To investigate the possible role of a differential SRC coactivator recruitment to explain the ER isoform-specific regulation of RBBP1, we overexpressed SRC1 and SRC2 with the +851 to +1140 construct in both U2OS-ER cell lines. SRC3 was not considered because a previous study demonstrated little or no SRC3 expression in osteoblasts (32). Fig. 8A demonstrates that SRC1 overexpression has no effect on E2-dependent transcriptional coactivation, whereas SRC2 has a significant effect in both U2OS-ER cell lines. In fact, SRC1 overexpression appears to inhibit the E2-dependent response to both ER isoforms. We repeated the experiment using the vitellogenin A2 ERE element to verify that our SRC overexpression was effective (38). Fig. 8B demonstrates that both SRC1 and SRC2 equally and effectively increase E2-dependent transcription with both ER isoforms with the vitellogenin A2 ERE. ChIP analysis was performed to determine SRC recruitment to the RBBP1 intron 1 enhancer region and demonstrated that no detectable SRC1 was recruited, whereas significant SRC2 protein was recruited in an E2-dependent manner (Fig. 8, C and D), which supports the transient transfection data (Fig. 8A). These results confirm that SRC2 is the preferred coactivator recruited to the RBBP1 intron 1 enhancer region. Interestingly, mutation of the RBBP1 ERE to the vitellogenin A2 consensus, in the RBBP1 +851 to +1140 context, does not
rescue the ability of SRC1 to function in RBBP1 activation (data not shown). This demonstrates that not only the ERE sequence but also the surrounding DNA context is important for the induction of RBBP1 by E2.

**DISCUSSION**

This paper presents evidence that ER isoform-specific transcriptional activation of the RBBP1 gene is because of the AF1 activity. We chose the RBBP1 gene because previous microarray and RT-PCR analyses demonstrated that RBBP1 was a highly E2-regulated gene, and ERα and ERβ exhibited a differential induction pattern. Furthermore, RBBP1 is a known Rb cofactor and may have an involvement in the control of osteoblastic proliferation and differentiation. We demonstrate that the RBBP1 gene displays a unique regulatory response by which a sustained induction is observed with ERα, whereas only a transient induction is seen with ERβ. This pattern was paralleled by ChIP assays demonstrating that differential ER occupancy of the RBBP1 intron 1 enhancer region was most likely responsible for the observed expression time course. Mutational analysis demonstrated different cis-acting requirements for activation of RBBP1 by the ER isoforms. ERα required the presence of the Sp1 site, whereas ERβ was capable of E2 induction through either the ERE or Sp1 sites. Interestingly, deletion of the ERα AF1 domain elicited a temporal transcription profile similar to that of ERβ, suggesting that differences in the ER AF1 domains play a critical role in E2-dependent activation of the RBBP1 gene.

There are numerous reports describing the functional interactions of ERα and Sp1 in the E2-dependent regulation of gene...
RBBP1 Is Regulated by Estrogen in Osteoblasts

expression (39–50). Furthermore, the N-terminal region of ERα is known to physically interact with the C-terminal zinc-finger region of Sp1 (47, 50, 51). This functional and physical evidence suggests that DNA-bound Sp1 recruits the ER, which is necessary for transcriptional activation of numerous genes. Indeed, our data suggest that the Sp1 site is necessary and sufficient for ERα-specific transcription of the RBBP1 intron 1 enhancer region. However, comparatively little is known concerning ERβ and Sp1 interactions and their effects on transcriptional activity. Saville et al. (47) demonstrated that although both ER isoforms bind Sp1 in a ligand-independent manner, only the ERα-Sp1 interaction is transcriptionally competent on a synthetic Sp1 reporter construct, whereas the ERβ-Sp1 interaction fails to activate transcription. In contrast, Salvatori et al. (52) demonstrated that ERβ-Sp1 interactions result in transcriptional activation of an epidermal growth factor receptor promoter construct. Our data using the RBBP1 intron 1 ERE enhancer domain demonstrate that ERβ can support E2-inducible transcription through both the ERE and Sp1 sites. The apparent discrepancy in the literature of the effects ERβ-Sp1 interactions on transcription may be because of cell type and promoter/enhancer context specificity.

Our laboratory has previously reported that in our U2OS-ERα cell models, ERα and ERβ homo- and heterodimers appear to regulate distinct cadres of genes with only 20% of all E2-regulated genes being regulated by both ER isoforms (26, 27). These data have been confirmed by other laboratories (28, 29). Our laboratory reported a similar ER isoform specificity using the selective estrogen receptor modulator 4-hydroxytamoxifen and further showed that E2 and 4-hydroxytamoxifen regulated unique patterns of gene expression. The data presented in this paper begin to delineate the mechanism of these gene-specific inductions by the ER isoforms. Although ER isoform-specific interactions of coregulators cannot be ruled out, at least with respect to the RBBP1 gene, the
differential interactions of the ERs with the ER enhancer domains/elements, the differential time course of ER occupancy on these elements, and the ER AF1 domain appear to play a large role in the ER isoform-specific regulation. Merot et al. (31) previously demonstrated that in well differentiated E2-sensitive tissues/cell lines, ERα activity is largely influenced by AF1. Our data support this model because deletion of ERα AF1 caused an ERβ-like temporal pattern in the E2-dependent activation of the RBBP1 gene.

Previous studies have reported the existence of a natural splice variant of ERα that lacks the first 173 amino acids of ERα (termed hERα46) and is consequently identical to the ERα-ΔAF1 receptor described in this study (36). hERα46 is found to be an effective E2-inducible transcription factor and exhibits a strong inhibitory effect on wild type ERα (hERα66) transcription, possibly because of interference with coregulator binding to the hERα46/hERα66 heterodimer (36, 53). Furthermore, hERα46 form is demonstrated to be naturally expressed in human primary trabecular osteoblasts (37). Data from this study suggest that ERα-ΔAF1 (e.g. hERα46) is capable of RBBP1 activation in U2OS cells, however, in a manner reminiscent of wild type ERβ and not wild type ERα. Because the ER-ΔAF1 cell system in this paper expresses only ER-ΔAF1, future studies are needed to determine the interactions between ER-ΔAF1 mutants and wild type ERs in the same cell on RBBP1 regulation.

We previously reported decreased E2-dependent cell proliferation in the U2OS-ERα cell line, whereas no effect was observed in the U2OS-ERβ cell line (26, 27). The specific gene(s) involved in the suppression of osteoblastic proliferation are currently unknown. RBBP1, in concert with Rb, has been previously shown to decrease E2F1 promoter activity, to decrease cellular proliferation, and to be involved in transcriptional repression (54–56). Interactions of Rb with Runx2, a major regulator of osteogenesis, result in decreased cellular proliferation and increased differentiation (57). Whether RBBP1 has a role, if any, in E2-dependent regulation of cellular proliferation or differentiation requires further investigation.

In conclusion, the current study describes RBBP1 as a novel E2-regulated gene in osteosarcoma cells that exhibit a differential response to the ER isoforms. The ER isoform-specific regulation of RBBP1 expression appears to be caused by the differential occupancy of the ER isoforms on the RBBP1 intron 1 ERE enhancer domain, which in turn appears to involve ER isoform-specific interactions with specific enhancer elements (e.g. ERE, Sp1). Furthermore, it appears that the ERα AF1 is involved in imparting ERα-specific patterns to E2-dependent RBBP1 activation. Further experimentation is required to expand this hypothesis to other ERα-regulated genes.

Acknowledgments—We thank Barbara Getz, Amanda Hemmingsson, and Grant Bender for their excellent technical assistance.

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