Journal of Cellular Biochemistry 94:88–97 (2005)

Bone Marrow Stromal Cells Express Two Distinct Splice Variants of ER-α That Are Regulated by Estrogen

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Abstract Estrogen plays a critical role in bone metabolism in both sexes. While the major action of estrogen is to inhibit bone resorption, it is now clear that early osteoblastic (or stromal) cells are a target for estrogen action, mediating the effects of estrogen on bone formation as well as resorption. However, little is known about the expression or regulation of the estrogen receptor (ER)-α in these cells. The expression of ER-α is regulated by a complex set of promoters and ER-α splice variants are present in different tissues. Thus, we sought to define the ER-α splice variants and their regulation by estrogen in the mouse bone marrow stromal cell line, ST-2, which can be induced to differentiate into mature osteoblasts. ST-2 cells expressed the mRNAs and proteins for both the 66 and 46 kDa forms of ER-α; the latter lacks the AF-1 domain and can transduce estrogen signaling in some tissues, while serving as a dominant negative receptor in others. Using primers specific for each of the five 5′-untranslated exons of ER-α, we found that ST-2 cells utilized only the promoters upstream of exons F and C (in contrast to most reproductive tissues, which utilize promoters upstream of virtually all the five exons). Moreover, 17β-estradiol (10^{-8} M) treatment of ST-2 cells markedly diminished levels of the 66 kDa as well as the 46 kDa ER-α proteins, largely through suppression of the transcript arising from the F1 promoter. These data thus indicate that: (1) bone marrow stromal cells express at least two variants of ER-α and (2) estrogen down regulates the ER-α mRNA and protein in these cells. J. Cell. Biochem. 94: 88–97, 2005.

Key words: sex steroids; receptor genes; splicing; transcription; skeleton

Estrogen plays a significant role in bone metabolism in both sexes [Riggs et al., 2002]. While the predominant action of estrogen is to inhibit bone resorption, it is now clear that osteoblastic lineage cells are a major target for estrogen action (for review, see [Spelsberg et al., 1999]). Not only does estrogen regulate the proliferation and differentiation of these cells, it also regulates the production by these cells of key cytokines modulating bone resorption [Spelsberg et al., 1999], including the final effector molecule for osteoclastogenesis, RANKL [Eghbali-Fatourechi et al., 2003], as well as its decoy receptor, OPG [Hofbauer et al., 1999; Saika et al., 2001]. Thus, the effects of estrogen on the pre-osteoblastic (or stromal) cell likely play a significant role in mediating the overall effects of estrogen on bone metabolism.

Despite this, there is currently little information on the expression or regulation of the estrogen receptor (ER) in these cells. Considerable evidence now indicates that of the two ERs (α and β), ER-α is likely the dominant ER regulating bone metabolism at least in mice [Sims et al., 2002]. The ER-α gene, which encodes a 66 kDa protein, is a complex genomic unit composed of several exons upstream of eight coding exons [Flouriot et al., 1998; Kos et al., 2001]. These upstream exons are alternatively spliced to a common acceptor site, located upstream of the translational initiation codon in exon 1, resulting in several mRNA splice

Grant sponsor: NIH; Grant number: AG004875.

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Received 7 June 2004; Accepted 30 July 2004
DOI 10.1002/jcb.20291

variants. These splice variants differ only in the 5′ untranslated regions (5′UTRs), and hence encode the same 66 kDa ER-α protein. To date, six such mRNA variants in the case of human (A–F) [Flouriot et al., 1998] and mouse (A–C, F1, F2, H) [Kos et al., 2000] ER-α and at least four such variants (A1-D) in the case of chicken [Griffin et al., 1998] ER-α have been identified. Each of these splice variants, however, is transcribed from the specific promoter located upstream of the corresponding upstream exon. Thus, the expression of the ER-α gene is regulated by multiple promoters in a tissue and cell specific manner [Flouriot et al., 1998].

In addition to the 66 kDa ER-α, a new ER-α protein isoform has recently been identified and characterized in chickens and humans. The chicken isoform is a 61 kDa protein which lacks 41 NH_2-terminal amino acids present in the 66 kDa protein [Griffin et al., 1999]. The human isoform, on the other hand, is a 46 kDa protein and lacks 173 NH_2-terminal amino acids [Flouriot et al., 2000]. The transcript for the 46 kDa isoform lacks the first coding exon of the ER-α gene. This new transcript originates from either the E or F promoters of the human ER-α gene and is produced by the splicing of the corresponding upstream exon to an acceptor site in exon 2 instead of the acceptor site in exon 1. This alternatively spliced, truncated variant is present not only in MCF-7 breast cancer cells [Flouriot et al., 2000], but also in primary human osteoblasts [Denger et al., 2001]. The 46 kDa variant, unlike the conventional 66 kDa ER-α, lacks AF1 but retains AF2 function. The 46 kDa variant is an effective ligand-inducible transcription factor in cells that support AF2 transactivation function. However, in cells that support only the AF-1 transactivation function, the 46 kDa variant is a powerful inhibitor of the 66 kDa receptor [Flouriot et al., 2000]. The 46 kDa isoform can form homodimers which are capable of forming DNA–protein complexes. It can also form heterodimers with either the full length ER-α or ER-β. When cotransfected with 66 kDa ER-α, it can inhibit the proliferative effect of E_2 acting via the 66 kDa ER-α on SaOs cells, an osteoblastic cell line. Of note, the 46 kDa isoform is present at the same level as the 66 kDa isoform in human primary osteoblasts [Denger et al., 2001].

The importance of the 46 kDa ER-α variant has recently been highlighted by the description of the ER-α mutant male [Smith et al., 1994] and the initial ER-α knock out mouse [Couse and Korach, 1999]. In both instances, there were homozygous mutations/deletions leading to absent functional 66 kDa ER-α, but these were upstream of the start codon for translation of the 46 kDa ER-α protein. Thus, at least for the initial ER-α knock out mouse, this resulted in continued expression of the transcript and protein for the 46 kDa isoform [Denger et al., 2001], leading to partial responsiveness to estrogen in bone, although higher doses of estrogen were required in these mice as compared to wild type mice in order to elicit skeletal responses [Gentile et al., 2001].

Given the potential importance of estrogen action on stromal cells, we sought to define the ER-α splice variants and the regulation of their expression by estrogen in the mouse bone marrow stromal cell line, ST2, which can be induced to differentiate into mature osteoblasts [Yamaguchi et al., 1996]. Our findings indicate that these cells do express, at the mRNA and protein level, both the 66 and 46 kDa variants of ER-α. We also define the upstream promoters giving rise to the transcripts for the receptor isoforms in these cells, as well as the regulation of one of these promoters by estrogen.

MATERIALS AND METHODS

Cell Culture

ST2 cells were maintained in αMEM (phenol red free) medium containing 10% charcoal stripped fetal bovine serum (FBS). During experiments, 5×10^5 cells were plated in a T75 flask and then grown in αMEM medium containing 1% penicillin/streptomycin (final concentration 100 U/ml penicillin; 100 μg/ml streptomycin) and either 10% regular FBS or 10% charcoal stripped serum (CSS). The cells were treated with or without 17β-estradiol (E_2, 10^{-8} M) for different periods of time at 37°C in 5% CO_2 in air. Media was changed every 3 days.

Whole Cell Extracts

After harvesting, the cells were extracted with a lysis buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Roche Molecular, Indianapolis, IN). The extracts were sonicated for 5 s followed by centrifugation at 14,000 rpm in a microcentrifuge for 5 min. The protein

concentration of the supernatant was measured using the Dc protein assay kit obtained from Bio-Rad (Richmond, CA).

Western Blot Analysis

Proteins from the whole cell extract were denatured at 95°C for 5 min and then resolved on a 10% SDS–polyacrylamide gel alongside of the Rainbow marker (Amersham Pharmacia Biotech, Piscataway, NJ) and electrotransferred onto an immobilon membrane (Millipore, Bedford, MA). The membrane was blocked in Tris–buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dry milk powder. The membrane was then incubated with either a rabbit polyclonal antibody, MC20 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), raised against a peptide mapping to the carboxy terminus of the mouse ER-α, or with anti huER-α antibody, clone ER1D5 (from Immunotech/Beckman, Westbrook, ME) that recognizes the A/B region of the amino-terminal part of huER-α and mER-α, in the blocking reagent. The membrane was then incubated with the appropriate secondary antibody coupled with peroxidase. ER-α proteins were visualized by chemiluminescence using the ECL kit (from Amersham Pharmacia Biotech) following the manufacturer's protocol.

RNA Isolation and cDNA Synthesis

After the desired period of culture, ST2 cells were harvested using trypsin–EDTA. Total RNA was then isolated from these cells using the RNeasy Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. Two micrograms of total RNA was then used for cDNA synthesis with either specific primers for mouse ER-α (mER-α) (5′-GGTGCATTGGTTTGTAGCTG-3′) [White et al., 1987] and mouse GAPDH (mGAPDH) (5′-CATGGACTGTGGTCATGA-3′) [Sabath et al., 1990] or oligodT [$p(dT)_{15}$], using AMV reverse transcriptase (RTase). The RNA, after pre-incubation with the primer at 65°C for 10 min, was mixed with a cocktail containing AMV RTase (25 U from Roche), RNasin (20 U), and dNTPs (2 mM) in a total volume of 20 μl and then incubated at 42°C for 2 h. RTase was inactivated by heating at 95°C for 5 min. The mixture was diluted to 100 μl with water and was used as a source of cDNA for either conventional or real-time polymerase chain reaction (PCR).

Polymerase Chain Reaction

A 25 μl reaction mix for PCR contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 pmol of each sense and anti-sense primers, 2 μl cDNA as template, 0.5 U of Taq DNA polymerase and dimethyl sulfoxide (DMSO, 5% final concentration). The template was initially denatured at 94°C for 3 min. PCR was performed for 35 cycles with annealing and elongation at 72°C for 3 min and denaturation at 94°C for 1 min. To determine the promoter usage the sense/antisense primers shown below were used [Kos et al., 2000]:

Sense primers:

- 5′CTTCTCTAATCGCAGGCTCTACTCT 3′ (from exon A)
- 5′ CAGCGAATCCAGCAGCGAAGACC 3′ (from exon B)
- 5′ATCACACACCGCGCCACTCGATCAT 3′ (from exon C)
- 5′ CAGGAAGCGCTTCAACAGTTCTTGC 3′ (from exon H)
- 5′ CTCTGGGCGACATTCTTCTCAAGC 3′ (from exon F1).

Antisense primer: 5′ AGGCTGTTGGCACTGAAGGCGGC 3′(from exon 1). To determine the transcript corresponding to the truncated form of ER-α (46 kDa), the sense primer either from exon F1 or from exon C was used and the antisense primer used was 5′ GCCTTGCAGCCTTCGCAGGACCA 3′ (from exon 2).

Real Time-PCR

For quantitation, amplification reactions specific for different splice variants of mER-α mRNAs (and also for mGAPDH mRNA) were carried out by PCR in a Light Cycler (Roche Diagnostics, Indianapolis, IN). A 20 μl PCR mix, for this purpose, contained 1× Light Cycler FastStart reaction mix (a 200 μM mix of dATP, dCTP, dGTP, and dUTP, Fast start Taq DNA polymerase and SYBR green I dye), 3 mM $MgCl_2$, the appropriate primers at 7.5 pmol (Table I) and 5 μl of cDNA. For specific amplification of 66F1F2 and 66C mRNAs the reaction mix also contained 7% DMSO. Reaction products were quantified using a simultaneously amplified series of dilutions of a cDNA sequence (a vector containing RANKL) of known concentration to generate a standard curve for each run.

TABLE I. Sequences of the Primers Used in Quantitative RT-PCR

Murine GAPDH	Sense	5′ CATCACCATCTTCCAGGAGCGAG 3′
	Antisense	5′ GTGCAGGATGCATTGCTGACAATC 3′
46F1	Sense	5′ GGCGACATTCTTCTCAAGCAGGTC 3′
	Antisense	5′ GCCTTGCAGCCTTCGCAGGACCA 3′
66F1F2	Sense	5′ GGCGACATTCTTCTCAAGCAGGGTAC 3′
	Antisense	5′ CTTTGGTGTGAAGGGTCATGGTCAT 3′
66C	Sense	5′ CTCTTGAACCAGCAGGGTGGCCCA 3′
	Antisense	5′ GTAGTTGAACACAGTGGGCTTGCTG 3′

Murine GAPDH primers were chosen considering intron–exon boundaries. The specific primers for 66F1F2, 46F1, and 66C were chosen considering the exon boundaries for each mRNAs.

The amplification profiles were as follows:

- Denaturation at 95°C for 0 s; annealing at 60°C for 7 s, and extension at 72°C for 15 s, for 35 cycles of amplification of a segment of mGAPDH mRNA.
- Denaturation at 95°C for 0 s, annealing at 62°C for 7 s, and extension at 72°C for 15 s, for 35 cycles of amplification of a segment of 46F1 mRNA.
- Denaturation at 95°C for 2 s, annealing and elongation at 72°C for 27 s, for 45 cycles of amplification of segments of 66F1F2 and 66C mRNAs.

RESULTS

Expression of Two Isoforms of ER-α in Mouse Bone Marrow Stromal Cells

ST2 cells were cultured in a medium containing either normal FBS or charcoal stripped serum (CSS) devoid of E_2, and the cells were harvested 3, 6, and 9 days after growth. An aliquot of the whole cell extract from each time point was tested for the presence of ER-α protein by Western blot analysis using an antibody directed against the COOH-terminus of the mouse ER-α protein (Fig. 1). As shown, this antibody detected three main protein bands: the conventional 66 kDa ER-α protein, a protein larger than 45 kDa (we termed this the 46 kDa variant in order to be consistent with the analogous human ER-α isoform), and a third, smaller molecular weight band, the identity of which is not known at present. The levels of both the 66 kDa and the 46 kDa isoforms increased with time when ST2 cells were cultured in a medium containing CSS (which has residual estrogens stripped from it), but were drastically reduced when cultured in FBS (which does contain residual estrogens).

In order to further characterize these isoforms, whole cell extracts of ST2 cells, grown for 6 and 9 days in CSS, were used for Western blot analysis with a monoclonal anti-ER-α antibody directed against the A/B region of the protein (Fig. 2). As is evident, this antibody recognized only the 66 kDa variant but not the 46 kDa variant, indicating that the 46 kDa variant lacks the NH_2 terminus of the ER-α.

E_2 Down Regulates the Expression of Both 66 and 46 kDa Variants

To determine if the down regulation of the two isoforms of ER-α in regular FBS media was due to the presence of residual estrogens, we cultured ST2 cells in either CSS or CSS supplemented with E_2 (10^{-8} M) for 3, 6, 9, and 12 days. An aliquot of the whole cell extract from each time point was tested for the presence of ER-α isoforms by Western blot using the anti-ER-α antibody (MC20) (Fig. 3). As shown, the levels of both the 66 and 46 kDa variants were markedly reduced in the media containing CSS + E_2 than those in CSS alone (Fig. 3).

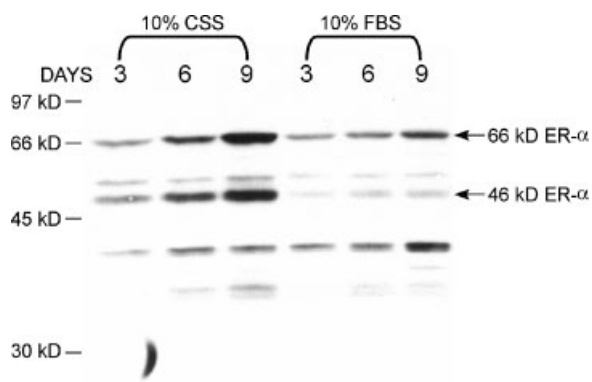

Fig. 1. Detection of the presence of ER-α isoforms in ST2 cells. ST2 cells were grown for different time periods (3, 6, or 9 days) in either CSS or FBS and whole cell extracts (50 μg) were used for Western blot analysis with a rabbit polyclonal antibody (MC20) raised against the COOH-terminus of mouse ER-α. The antibody recognized three main bands: the 66 kDa band, a band larger than 45 kDa (the homologue of the human 46 kDa ER-α isoform), and an unidentified, lower molecular weight band.

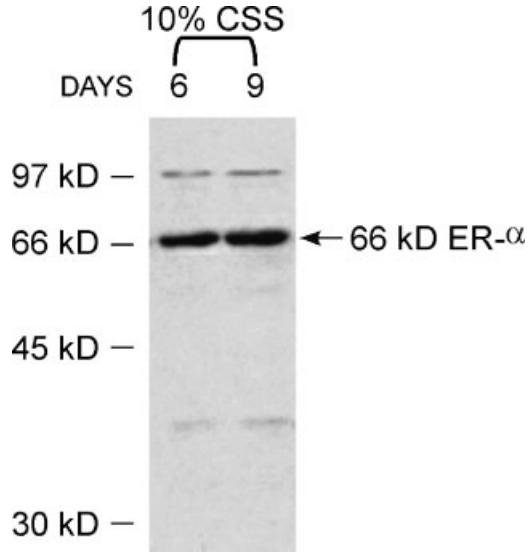

Fig. 2. Detection of ER-α protein in ST2 cells using an NH_2-terminal anti-human ER-α antibody. Proteins of the whole cell extract (100 μg) of ST2 cells, grown in CSS for 6 and 9 days, were used for Western blot analysis using an anti-human ER-α antibody raised against the NH_2-terminus of huER-α. Only the 66 kDa isoform of ER-α was identified, indicating that the 46 kDa isoform in ST2 cells lacks the NH_2-terminus of ER-α.

ST2 Cells Utilize the F1 and C Promoters of ER-α

To determine the promoter(s) utilized by ST2 cells, PCR was performed using an ER-α specific cDNA, prepared from total RNA from ST2 cells cultured in CSS media for 6 days, with a sense primer from different upstream exons (A, B, C, F1, and H) and an antisense primer from exon 1 (see Fig. 4). This revealed the presence of amplified products, representing the transcripts corresponding to the 66 kDa ER-α, only when the reaction mix contained the sense primers specific for either exon F1 or exon C, indicating that ST2 cells utilize only these two promoters of ER-α (we had positive controls for primers from A, B, and H exons for which data are not given) (Fig. 5A). Two DNA bands were amplified by PCR using the sense primer specific for exon F1. Sequencing of these two bands (data not shown) confirmed the presence of two transcripts containing either exon F1 (66F1) or exon F1 followed by exon F2 (66F1F2) attached to the splice acceptor site, which is 72 bp upstream of the initiating ATG, at the beginning of exon 1 (see Fig. 4). These two variants differ by 109 bp, which is the length of the exon F2. The DNA band that was amplified using the sense primer specific for exon C was also sequenced and its identity confirmed (data not shown).

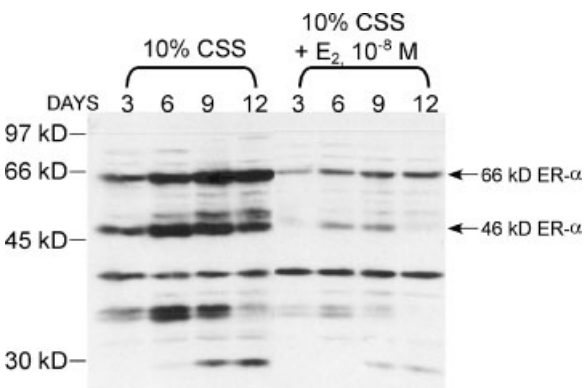

Fig. 3. Effect of E_2 on the expression of ER-α proteins in ST2 cells. Proteins of the whole cell extract (100 μg) of ST2 cells grown for different periods of time (3, 6, 9, or 12 days) in CSS with or without E_2 (10^{-8} M) were used for Western blot analysis utilizing polyclonal antibody (MC20), raised against the COOH-terminus of mouse ER-α. E_2 down-regulated both 66 and 46 kDa isoforms of ER-α in ST2 cells.

To determine the presence of transcripts for the 46 kDa variants, we then performed PCR with an antisense primer from exon 2 (since the 46 kDa variant lacks the NH_2-terminus) and a sense primer either from exon F1 or from exon C (the only two promoters utilized by ST2 cells) with an ER-α specific cDNA prepared from ST2 cells grown in CSS for 6 days (see Fig. 4). As shown in Figure 5B for the exon F1 primers, this demonstrated the presence of the two larger fragments corresponding to the 66 kDa (66F1 and 66F1F2) as well as the two smaller fragments corresponding to the 46 kDa (46F1 and 46F1F2) transcripts. Sequencing of one of the smaller bands confirmed the presence of a transcript containing upstream exon F1 attached to exon 2 (46F1). Sequencing of the other band showed the presence of the transcript containing upstream exon F1 followed by upstream exon F2 attached to exon 2 (46F1F2) (data not shown). Figure 5C shows the findings using the upstream exon C primers, demonstrating the presence of only the transcript corresponding to the 66 kDa isoform (66C). Thus, the transcripts that code for the truncated variant (46 kDa) arise only from the F1 promoter by alternative splicing of the corresponding upstream exon to exon 2 (see Fig. 4).

E_2 Down-Regulates the Transcripts From the F1 Promoter but not From the C Promoter

We next studied the effects of E_2 on the RNA levels of the 66 kDa variant produced from the

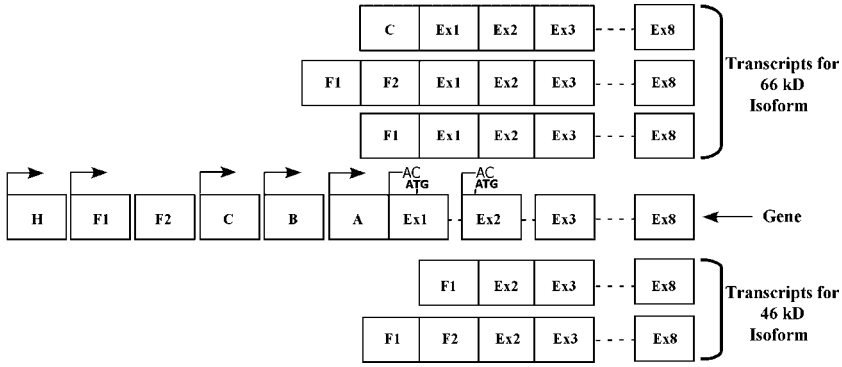

Fig. 4. Schematic representation of the mouse ER-α gene and the transcripts generated by alternate splicing of upstream exons. Upstream untranslated exons are designated with uppercase letters, starting with A. Splicing of the upstream exon to the common acceptor site (AC) in exon 1 (containing the initiating ATG) produces the transcripts corresponding to the 66 kDa isoform, while splicing of the upstream exon to the AC in exon 2 (containing an inframe ATG serving as the initiation codon) produces transcripts corresponding to the 46 kDa isoform. Exon F1 is spliced either directly to the AC or to exon F2 and then to the AC. For consistency with the nomenclature used for the human gene, the mouse exon that contains the translation initiation codon ATG is considered as exon 1 (information was adapted from Kos et al., 2000; Denger et al., 2001).

F1 and C promoters. Since the level of the 66F1F2 transcript was much higher than that of the 66F1 transcript (data not shown), we performed real time-PCR on cDNA from ST2 cells, grown for 9 days in either CSS or in CSS along with E_2, using primers specific for the 66F1F2 and 66C transcripts. As shown in Figure 6, E_2 treatment resulted in a marked reduction in the level of the transcript arising from the F1 exon promoter ($P = 0.0006$) (Fig. 6A), but had no effect on the level of the transcript arising from the C exon promoter (Fig. 6B), indicating that the F promoter (but not the C promoter) is estrogen responsive. Consistent

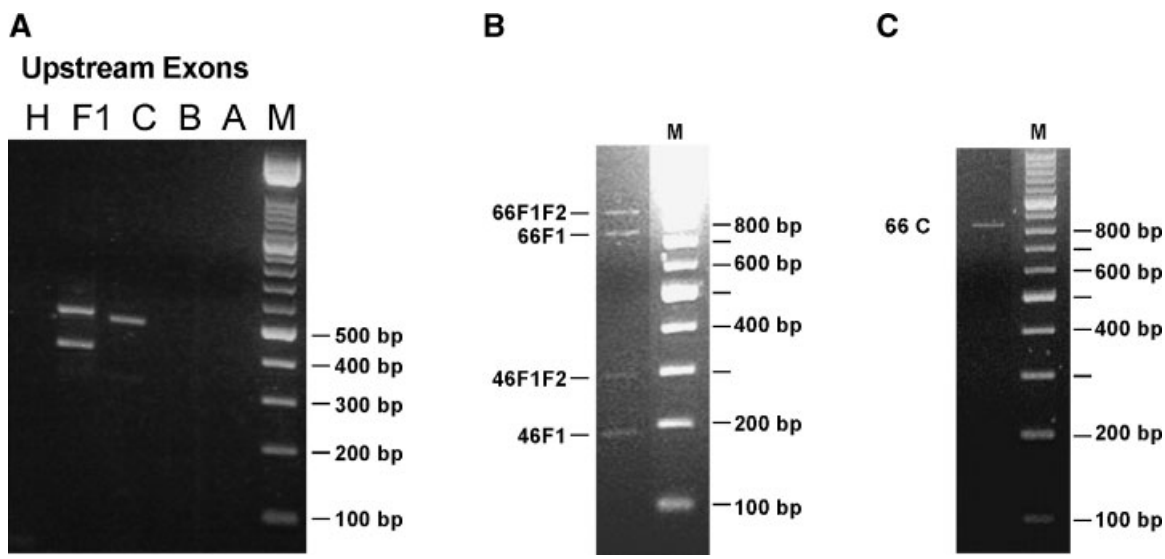

Fig. 5. Determination of promoter usage by ST2 cells for the generation of transcripts for ER-α variants. Agarose gel electrophoresis of the PCR products from reactions using a mouse ER-α specific cDNA prepared from total RNA obtained from ST2 cells cultured in CSS media for 6 days. **A**: Identification of transcripts for 66 kDa variant. PCR was performed with a sense primer from different upstream exons (H, F1, C, B, A) and an antisense primer from exon 1, in the presence of 5% DMSO (to overcome the secondary structures created due to the presence of the high GC content at the NH_2 terminus of the mouse ER). The presence of amplified products (derived from the transcripts of 66 kDa) in the reaction mix that contained primers specific for either exon F1 or exon C indicates that ST2 cells utilize only the promoters upstream of exon F1 and exon C of mouse ER-α. M = 100 bp DNA ladder. **B** and **C**: Identification of transcripts for 46 kDa variant. PCR was performed using antisense primer from exon 2 and a sense primer from exon F1 (B) or exon C (C) in the presence of 2.5% DMSO. Note that the exon F1 primers result in the amplification of transcripts corresponding to both the 66 and 46 kDa isoforms, whereas the exon C primers result in the amplification of only the transcript corresponding to the 66 kDa isoform. M = 100 bp DNA ladder.

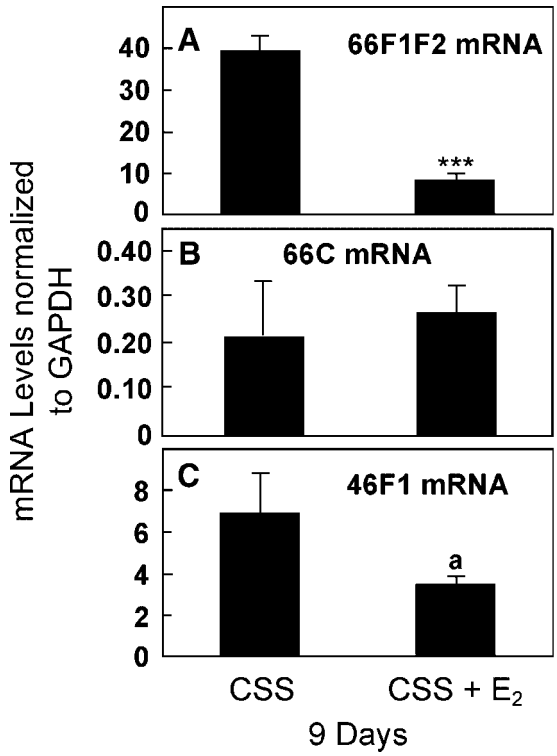

Fig. 6. Effect of E_2 on the levels of the mRNAs coding for either 66 or 46 kDa ER-α in ST2 cells. Levels of 66F1F2 mRNA (**A**), 66C mRNA (**B**) (corresponding to 66 kDa ER-α), and 46F1 mRNA (**C**) (corresponding to 46 kDa ER-α) were quantitated by real time PCR with cDNAs prepared from total RNA of ST2 cells cultured in CSS with or without E_2 (10^{-8} M) for 9 days using specific primers for each transcript. E_2 down regulated 66F1F2 and 46F1 mRNA levels, but had no effect on the 66C mRNA levels ($n = 3$, $^{***}P < 0.001$; $^{a}P = 0.117$).

with this, E_2 also reduced the level of the transcript from the F promoter coding for the 46 kDa variant [although the *P*-value for this (0.117) did not achieve statistical significance] (Fig. 6C).

DISCUSSION

We demonstrate in the present study that mouse bone marrow stromal (ST2) cells express both the mRNA and protein for the 66 and 46 kDa ER-α isoforms. Moreover, E_2 down-regulates the production of both isoforms, principally by reducing the level of the transcripts arising from one of the two ER-α promoters (the F1 promoter) utilized by these cells. Although, the expression of both 66 and 46 kDa variants in human primary osteoblastic cells [Denger et al., 2001] and the utilization of F1 promoter by the primary osteoblasts [Denger et al., 2001] and MG-63 osteoblastic cell line [Lambertini et al., 2003] have been reported earlier, to our knowledge, this is the first investigation of the expression of ER-α isoforms and their regulation by estrogen in the context of bone marrow stromal cells.

Previous studies using tissues and animal species have indicated that the expression of ER-α gene is generally under the control of E2 in a tissue specific manner. E2, for example, up-regulates ER-α in endometrium, myometrium, and pituitary, whereas downregulates ER-α in mammalian liver, hypothalamus, and mammary gland [Simerly and Young, 1991; Boyd et al., 1996; Friend et al., 1997; Ing and Tomesi, 1997; Zou and Ing, 1998]. E2 regulates ER-α also in breast cancer cell lines. It down regulates ER-α mRNA level in MCF-7 cells, while upregulates it in EMF-19, ZR-75, and T47-D cells [Saceda et al., 1988; Westley and May, 1988; Read et al., 1989; Clayton et al., 1997]. Our observation of the downregulation of both ER-α isoforms by E2 in ST2 cells at the mRNA and also at the protein level, however, is not only interesting but also novel, since no such information has been reported earlier. The regulation of ER-α expression, again, has been shown to occur at the transcriptional and also at the post-transcriptional level. In endometrium, E2 upregulates ER-α expression by stabilizing ER mRNA via a discrete RNA sequence present at the 3′-untranslated region [Mitchell and Ing, 2003]. In MCF-7 cells, on the other hand, E2 down regulates ER-α by both transcriptional repression and mRNA destabilization [Kaneko et al., 1993; Saceda et al., 1998]. Interestingly, analysis of the Celera mouse genomic data base showed the presence of an ERE half site and an AP1 site in the promoter region upstream of the F1 exon (data not shown. Such sites are also present upstream of the F1 exon of human ER-α gene [Flouriot et al., 1998; Lambertini et al., 2003]). Thus, E_2 could be decreasing transcription from this promoter through one or both of these DNA elements since, in addition to its ability to bind to EREs, E_2 can also act via protein/protein interactions with c-fos and c-jun through AP1 sites. Also consistent with our finding of a lack of regulation by estrogen of the transcript arising from the exon C promoter, there was no ERE or AP-1 site upstream of exon C in the Celera data base. However, future experiments with reporter gene assays are needed to ratify this hypothesis.

As noted earlier, it is important to understand the expression and regulation of ER-α in bone marrow stromal cells, since these cells are at a key control point for estrogen action on bone. They produce many of the major cytokines known to be regulated by estrogen that are important for the initiation and maintenance of osteoclastogenesis, such as IL-1, IL-6, M-CSF, and IL-7 (for review, see [Riggs et al., 2002]). In addition, these cells are critical for the support of osteoclastogenesis through their production of RANKL, which has recently been shown to be regulated by estrogen in vivo [Eghbali-Fatourechi et al., 2003]. Osteoblastic lineage cells also produce the major known inhibitor of osteoclast development, OPG, and this is known to be regulated by estrogen [Hofbauer et al., 1999; Saika et al., 2001]. Finally, estrogen can induce the production by stromal cells of BMP-6 [Rickard et al., 1998] and BMP-2 [Zhou et al., 2003], two of the major growth factors that enhance osteoblast differentiation. Thus, the stromal cell orchestrates bone resorption by osteoclasts through its production of pro-resorptive cytokines and via the RANKL/RANK/OPG system, is the precursor cell to the mature osteoblast that is responsible for bone formation, and all of these processes are regulated by estrogen. However, our observation about the expression of both isoforms and their regulation by E2 is based on experiments using one stromal cell line, ST2. Further experiments with either other stromal cell lines or stromal primary cultures isolated from bone marrow are, therefore, needed, to draw unequivocal conclusions about how the stromal cells and hence the osteoblasts and bone derived from them, regulate their sensitivity to estrogen.

Our studies also provide insights into the ER-α promoter usage by these cells. Like human, mouse ER-α gene also has six upstream exons (A, B, C, F1, F2, H) [Kos et al., 2000] which are alternatively spliced to a common acceptor site in exon 1 generating different splice variants, each of which codes for the 66 kDa isoform. Since each of the upstream exons is preceded by a promoter sequence, the presence of a particular splice variant is, therefore, indicative of the utilization of the corresponding promoter. Since exon F2 is always attached to exon F1 in the case of mouse ER-α [Kos et al., 2000], we tested for the presence of five splice variants (A, B, C, F1, and H) to determine the promoters used for the transcription of ER-α in ST2 cells. Our results indicate that the ST2 cells utilize only the C and F1 promoters to generate the 66 kDa ER-α transcripts. Although the variants produced from the C and F1 promoters are the major variants present in mouse tissues [Kos et al., 2000], various human and mouse reproductive and nonreproductive tissues do utilize multiple promoters to control the expression of the ER-α gene [Flouriot et al., 1998; Kos et al., 2000]. In contrast, SaOs and HOS TE 85, two human osteoblastic cell lines, utilize the E and F promoters [Flouriot et al., 1998], and human primary osteoblastic cells [Denger et al., 2001] and MG-63 cell line [Lambertini et al., 2003] utilize only the F promoter. These data, along with our findings in ST2 cells, point to a restricted usage of ER-α promoters by stromal/osteoblastic lineage cells compared to reproductive tissues. The physiological significance of this in terms of the differential sensitivity of various tissues to estrogen remains to be determined.

Since ST2 cells utilize only the C and F1 promoters, we also tested whether the transcripts corresponding to the truncated variant (46 kDa) were also derived from these two promoters in this cell line. Our results indicate that the ST2 cells contained the transcripts for the truncated variant originating exclusively from the F1 promoter. The transcripts from this promoter contained either exon F1 attached to exon 2 (46F1) or exons F1F2 together attached to exon 2 (46F1F2). Sequencing of the 46F1 transcript revealed that the upstream exon F1 binds to a common acceptor site 464 bp down stream of the initiating ATG (with A of the ATG as +1) [White et al., 1987], almost at the beginning of exon 2. These transcripts contain two ATGs at positions +532 and +538 (with A of the ATG for 66 kDa assigned the +1 position) which are in frame with the rest of the reading frame of the mouse ER-α. Both ATGs are in a favorable Kozak context for initiation of translation [Kozak, 1989]. This predicts an approximately 47.9 kDa product using either of these ATGs, which lacks 177 NH_2-terminal amino acids present in the 66 kDa isoform. Since we have not tested the primers from other upstream exons, besides F1 and C, we can not exclude the possibility that the 46 kDa isoform is arising also from other promoters like H, B, or A. Of note, the transcript for the truncated version of ER-α is also generated from the F promoter in MCF-7 [Flouriot et al., 2000] and

human osteoblastic cells [Denger et al., 2001], as is the truncated ER-α detected in the bones of the initial ERKO mouse [Denger et al., 2001]. This truncated ER-α isoform is a minor component in MCF-7 cells [Flouriot et al., 2000], but accounts for about 50% of the total ER-α transcripts in human osteoblastic cells [Denger et al., 2001]. Our data show that in ST2 cells, the 66 and the 46 kDa isoforms are present in comparable amounts when grown in CSS media, with lower amounts of the 46 kDa relative to the 66 kDa isoform when the cells are grown in FBS or in CSS media plus E_2, due to the fact that the 46 kDa transcripts probably arising exclusively from the estrogen sensitive F1 promoter.

Recently, Ciana et al. [2003] have reported elegant studies in which transgenic mice expressing a ERE luciferase reporter gene under the control of activated ERs were imaged to demonstrate ER activity in vivo. In terms of bone, two novel findings were that (1) skeletal tissues appeared to have significant ERE luciferase activity (up to 50–60% of maximal activation) even in the absence of ligand (i.e., following ovariectomy or in sexually immature mice) and (2) that in contrast to reproductive tissues, where ERE luciferase activity essentially paralleled changes in circulating E_2 levels during the estrus cycle, ERE activity in bone was, in fact, lower during proestrus (when circulating E_2 levels are highest) than during diestrus (when circulating E_2 levels are considerably lower). In the context of our findings in ST2 cells, although these cells were grown in non-differentiation media and we were not looking at the issue of ER-α variants in other more mature osteoblast lineage cells, a plausible hypothesis is that skeletal sensitivity to estrogen may be regulated in vivo, in part, through variations in ER-α levels during the estrus (or in humans, the menstrual) cycle. Thus, since bone appears to be one of the tissues where there is significant ligand-independent activation of the ER, perhaps through growth factors and their receptors [Ma et al., 1994; Kato et al., 1995], marked increases in ER activity driven by high circulating E_2 levels (as are present in certain phases of the estrus or menstrual cycle) may, in fact, be detrimental to the skeleton. This may, for example, result in excessive suppression of normal bone remodeling. As such, restricted promoter usage and regulation of at least one of these promoters by estrogen may be an adaptive mechanism whereby the skeleton protects itself against the surges of circulating estrogen levels that are necessary for reproductive function, but which may be potentially deleterious to bone. Clearly, further studies are needed to address this possibility.

In summary, our studies demonstrate that mouse bone marrow stromal cells express two major variants of ER-α, utilize a more restricted set of ER-α promoters than classical reproductive tissues, and that one of the two promoters used in these cells is sensitive to estrogen. Both the relative amounts of the 66 and 46 kDa isoforms as well as the regulation by estrogen of the F1 promoter may be mechanisms by which these cells regulate sensitivity to estrogen, although further studies are needed to examine this issue.

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