JOURNAL OF BONE AND MINERAL RESEARCH
Volume 14, Number 7, 1999
Blackwell Science, Inc.

A PCR Analysis of ERα and ERβ mRNA Abundance in Rats and the Effect of Ovariectomy

S.K. LIM,[1] Y.J. WON,[1] H.C. LEE,[1] K.B. HUH,[1] and Y.S. PARK[2]

ABSTRACT

To study the relative abundance and the changes of both estrogen receptor alpha (ERα) and ERβ mRNA before and after ovariectomy in major organs important to the regulation of calcium homeostasis, we compared the degree of mRNA expression of ERα to that of ERβ in rat tissues by performing competitive reverse transcription polymerase chain reaction (RT-PCR) with internal standards. Both ERα and ERβ were highly expressed in the ovary {ERα[(2.2 ± 0.33) × 10^7 copies/μg of total RNA] > ERβ[(1.2 ± 0.33) × 10^5 copies/μg of total RNA]} as we expected. The bone marrow and renal cortex were very important target organs of estrogen because ERα was highly expressed ~2 × 10^5 copies/μg of total RNA, but marrow cells revealed only a very weak expression of ERβ [(0.7 ± 0.21) × 10^2 copies/μg of total RNA]. Both ERα and ERβ were expressed in the trabecular bone [(3.2 ± 0.56) × 10^3 copy/μg of RNA] and [(2.8 ± 0.21) × 10^2 copy/μg of RNA], respectively. However, they were not detected in the cortical bone. In the jejunum, the expression of ERα was not detectable, while ERβ was expressed very weakly [(1.1 ± 0.24) × 10^2 copies/μg of total RNA]. The thyroid gland expressed low copy numbers of ERβ [(6.0 ± 0.23) × 10^2 copies/μg of total RNA], but the parathyroid gland was negative for both ERα and ERβ mRNA. In cultured stromal cells, ERα and ERβ mRNAs were not detected after a 24-h culture; however, the rates of mRNA expression of ERα and ERβ reached ~10^5 copies/μg of total RNA and ~10^2 copies/μg of total RNA, respectively, after 9-, 11-, and 13-day cultures. After ovariectomy, the expression of ERα mRNA decreased abruptly in the bone marrow and renal cortex, and both ERα and ERβ were barely detected in the trabecular bone. In conclusion, ERα might be the main ER in organs important for calcium homeostasis, except in the jejunum. The mRNA expression of ERα in the bone marrow and renal cortex decreased abruptly after ovariectomy, which may partially explain why the effect of estrogen deficiency can be amplified and why trabecular bone loss is more predominant than cortical bone loss shortly after surgical or natural menopause. (J Bone Miner Res 1999;14:1189–1196)

INTRODUCTION

UNTIL RECENTLY, only one type of estrogen receptor (ER) had been identified and the tissue selective effects of estrogen-like molecules have been postulated to involve receptor-associated proteins and/or variations in promoter responsive elements.[1] A novel type of ER (ERβ) was discovered recently and it was found to bind estradiol (E_2) with relatively high affinity and to be capable of activating transcription of a reported gene in the presence of this ligand.[2–4] However, it is still very hard to find major differences between two receptors and the roles of ERβ in the presence of ERα at the same cell.[5] Furthermore, several alternatively spliced ERβ isoforms have also been reported.[6] Therefore, the presence of these two ERs and their isoforms provided more complexity in understanding the pathophysiology of bone loss after menopause.

Estrogen and selective estrogen receptor modulator (SERM) revealed the selective actions of estrogen in different target tissues.[7,8] Recently, Pan et al. reported that ovariectomy induced increased bone resorption and an increased number of osteoclasts per millimeter of trabecular bone surface in ERα knock-out mice.[9] The ERα knock-out mice model might reveal some compensatory role of

[1]Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea.
[2]Seoul Clinical Laboratories, Seoul, Korea.

ERβ in the maintenance of bone mass. Paech et al. showed that antiestrogens, but not 17β-estradiol, can activate a reporter gene downstream of an AP1 site, when ERβ interacts with AP1. Both antiestrogens and 17β-estradiol activated the reporter gene when ERα was present instead of ERβ.[10] Their observation suggested that ERα and ERβ could produce opposite effects in some circumstances. Therefore, the existence of two ERs presents another potential source of tissue-specific estrogen regulation. However, the two receptors have strikingly similar properties in most settings, so the roles of ERβ in the presence of ERα in the same cell remain a puzzle.[5]

Upon interaction with estrogen, ER undergoes a conformational change permitting the displacement of inhibitory proteins.[11] The receptor then spontaneously dimerizes and acquires the ability to interact with specific DNA responses of target organs. ERα and ERβ can be dimerized in several forms such as ERα and ERα, ERα and ERβ, and ERβ and ERβ.[12] The existence of three forms of homo- or heterodimer suggested that different target tissues might respond differently to estrogens or SERMs according to varying ratios of ERα and ERβ. Therefore, it is crucial to know the degree of expression of each ER and the changes of ER expression before and after ovariectomy in organs which are important for the regulation of calcium metabolism, and which might provide some clues to help explain the accelerated bone loss after menopause.

In this study, we compared the degree of mRNA expression of ERα with that of ERβ in each organ and analyzed the changes in their expression 3 weeks after ovariectomy by competitive RT-PCR with 30 bp–deleted internal control mRNA. We found the bone marrow and renal cortex were very important target organs for estrogen and the expression of ERα in both organs was undetectable 3 weeks after ovariectomy. ERβ was also not expressed exclusively in the organs important for regulation of calcium homeostasis, except in the jejunum.

MATERIALS AND METHODS

Materials

Female Sprague-Dawley rats (6–8 weeks old) (Charles River Japan, Co., Tokyo, Japan) were used in this study. Rats were randomly divided into two groups, one ovariectomized (OVX) ($n = 12$ rats) the other non-OVX ($n = 6$ rats). Three weeks later, half of the OVX and all non-OVX rats were sacrificed by cervical dislocation under light ether anesthesia. The remaining OVX rats were then treated with 17β-estradiol (30 μg/kg) for 1 week and sacrificed by the same procedure. The level of serum E_2 was 21.2 ± 3.5 pg/ml and 3.1 ± 0.74 pg/ml in control and OVX rats, respectively. The increase of body weight in OVX rats was greater than that of controls 4 weeks after ovariectomy (from 210 ± 22 g to 298 ± 26 g vs. from 203 ± 20 g to 270 ± 24 g, respectively).

Cell culture

Bone marrow stromal cells were cultured as described previously.[13] The rats were killed by cervical dislocation, and tibiae were aseptically removed and dissected free of adhering tissues. The bone ends were cut off with scissors and the cavity was flushed with 1 ml of alpha modified essential medium (α-MEM) by slow injection from one end of the bone using a sterile 26 G needle. The medium with flushed bone marrow was centrifuged at 1400 rpm for 10 minutes. Cell pellets were resuspended in culture medium and layered on the Ficoll/Hypaque (specific gravity 1.077) (Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 1400 rpm for 30 minutes, an enriched bone marrow stromal cell fraction was obtained from the interface of the culture medium and the Ficoll/Hypaque layer. The cells were seeded into a 35 mm tissue culture dish at a density of 4×10^5 cells/cm^2 and cultured in α-MEM containing 10% FBS, penicillin, and streptomycin (100 U/ml and 100 μg/ml, respectively). The medium was changed twice weekly from the second week. When the cells were grown to 80% confluence, they were subcultured using conventional techniques employing 0.01% trypsin and 0.05% EDTA.

Design of oligonucleotide

The oligonucleotide ERα forward primer-1: 5′AATTTAATACGACTCACTATAGGGA/AATTCTGACAATCGACGCCAG and ERα reverse primer-1: 5′CGCTT/GTGCTTCAAC-ATTCTCCCTCCTC/TGCCCACTTCGTAACACTTGCG were used for the construction of ERα standard template DNA. The oligonucleotide ERα forward primer-2: 5′AATTCTGACAATCGACGCCAG (+472 relative to ATG) and the ERα reverse primer-2: 5′GTGCTTCAACATTCTCCCTCCTC (+794 relative to ATG) were used for amplification of a 344-bp fragment of the rat ERα mRNA as previously reported.[14] The oligonucleotide ERβ forward primer-1: 5′AATTTAATACGACTCACTATAGGGA/TTCCCGGCAGCACCAGTAACC and the ERβ reverse primer: 5′GAGCA/TCCCTCTTTGC-GTTTGGACTA/TCAGTGTCTCTCTGTTTACAGG were used for the construction of ERβ internal standard-template DNA. The oligonucleotide ERβ forward primer-2: 5′TTCCCGGCAGCACCAGTAACC (+38 relative to ATG) and the ERβ reverse primer-2: 5′TCCCTCTTTGCGTTTGGACTA (+279 relative to ATG) were used for amplification of a 262-bp fragment of the ERβ mRNA.

Construction of the standard ERα and ERβ template DNA

Total RNA was extracted from the rat ovary and RT-PCR was performed by the standard method for getting ERα and ERβ template DNA. Figure 1 schematically depicts the principle and steps involved in generating internal standards which were used as competitors for RT-PCR of human ERα or ERβ. To prepare internal standard DNAs, RT was done with each forward primer-1 and reverse primer-1 of ERα or ERβ as explained below. The purified RNA (0.3 μg) and RNAse free distilled water (2.5 μl) were boiled for 4 minutes at 94°C in a 500 μl microcentrifuge tube, then chilled on ice for ~1–2 minutes. After a brief spin,

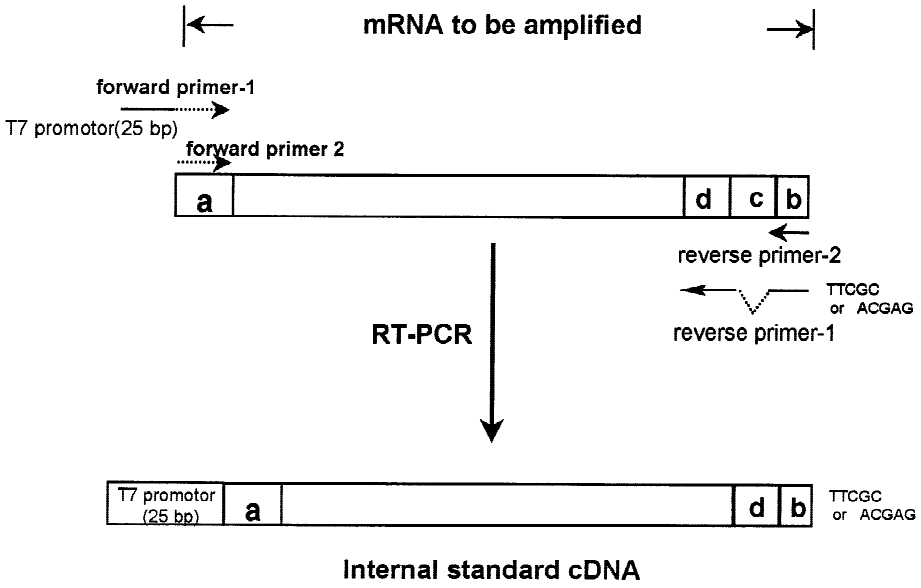

FIG. 1. Synthesis of ER-internal standard cDNA. The sequence of the forward primer-1 was as follows: 5′-T_7 promotor sequence (25 bp) + forward primer-2 (21 bp), and the sequence of the reverse primer-1 was as follows: 5′-CGCTT(5 bp) + reverse primer-2[21 bp (ERα) or 23 bp (ERβ)] + d (22 bp). The c (30 bp) fragment was deleted in the newly synthesized ER-internal standard cDNA.

the following reagents were added to the tube: 1 μl of random hexamers, 1 μl of DTT(100 mM), 4 μl of 5× RT buffer, 8 μl of dNTP(25 mM), 0.5 μl of RNAsin, 1 μl of AMV RT. The mixture was incubated at 37°C for 10 minutes, then at 42°C for 45–60 minutes. The reaction was stopped by boiling for 4 minutes at 94°C, then chilled on ice. Thirty-five cycles of PCR were done as explained below. The following reagents were added to a 500 μl microcentrifuge tube: 10 μl of RTproduct, 2 μl of $MgCl_2$ (25 mM), 4 μl of 10× PCR buffer, 1 μl of forward primer, 1 μl of reverse primer, 0.3 μl of Taq polymerase, and 33.7 μl of DW. The reaction mixture was pulse centrifuged, and PCR amplification was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The prepared samples were denatured at 95°C for 3 minutes. Each cycle consisted of 1 minute denaturization at 95°C, 2 minutes annealing at 52°C, and 3 minutes at 72°C for enzyme extension. The RT-PCR product was run on an agarose gel, and the DNA band was extracted using a DNA purification kit (Qiagen, Inc., Chatsworth, CA, U.S.A.). The synthesized template DNA had the sequence downstream of the T_7 promotor at the 5′ end and contained a 30 bp deletion that ended 28 bp (ERα) or 26 bp (ERβ) from the C-terminal end, respectively.

In vitro transcription for synthesis of internal standard ERα or ERβ mRNA

The extracted DNA was transcribed using T7 RNA polymerase applying the Riboprobe in vitro Transcription System (Promega, Co., Madison, WI, U.S.A.). The RNA concentration was determined by spectrophotometry.

Purification of total RNA

Female Sprague-Dawley rats (6–8 weeks old) were sacrificed by cervical dislocation and tissue was collected. Cancellous bone was surgically collected from the distal metaphysis, and cortical bone was collected from the distal diaphysis. Tissue samples were immediately processed for total RNA isolation. Total RNA of each tissue or cultured marrow stromal cells was extracted by using RNeasy Mini kit (Qiagen). Only RNA samples exhibiting an A260/A280 ratio >1.6 and showing integrity of the RNA by electrophoresis were used in further experiments. After quantitation, RNA was diluted to 100 ng/μl in diethylpyrocarbonate (DEPC) H_2O for RT-PCR. Before RT-PCR analysis, total RNA was electrophoresed on a 1% formaldehyde agarose gel to check for intact 28S and 18S ribosomal bands. All experiments were approved by this Institute's Animal Care Committee.

Competitive RT-PCR

Competitive RT-PCR was done as explained below. The following reagents were added to a 500 μl microcentrifuge: 14.8 μl of DW, 3 μl of 10× buffer, 3 μl of 2 mM dNTP, 3 μl of 10 pmol each primer (forward primer 2 and reverse primer 2), 1.5 μl of 100 mM DTT, 0.3 μl RNAsin, 0.3 μl of AMV RT, 0.1 μl of Taq polymerase, 3 μl (300 ng) of extracted tissue RNA, and 1 μl of internal standard mRNA. The mixture was incubated at 42°C for 60 minutes and was stopped by boiling for 5 minutes at 95°C. Thirty-three cycles of PCR were done continuously. Each cycle consisted of 1 minute denaturization at 94°C, 1.5 minutes annealing at 62°C, and 2 minutes extension at 72°C for en-

zyme extension. The cycling was followed by a final extension at 72°C for 5 minutes. All samples were added to 1 μl 10× loading dye and size was fractionated by electrophoresis in a 3.5% meta-agarose gel. Visualization after ethidium bromide staining (30 minutes in 0.5 μg) was performed by Polaroid MP-4 camera (Clifton, NJ, U.S.A.) and the amount of DNA band was analyzed. The RNA was serially diluted from 0 to 10^7 copy/μl (0, 10^3, 10^4, 10^5, 10^6, and 10^7 copy/μl, respectively). The RT reaction for β-actin was performed at 65°C for 25 minutes followed by a two-step PCR reaction (95°C 10 s, 65°C 20 s) in 2.2 mM $MgCl_2$ for 33 cycles.[15] Products were visualized by running 10 μl of 100 μl total reaction volume on a 3.5% metaphor-agarose/1× TBE gel for 1.5 h at 100 V. The copy number of internal standard mRNA was calculated as:

$$\text{copies}/\mu\text{l} = \text{OD}_{260} \times 40(\text{ng}/\mu\text{l}) \times 10^{-9} \times 6 \times 10^{23}/(\text{standard bp} \times 660)$$

After titration of the copy number of internal standard mRNA, 1 μl of mRNA (from 0 to 10^7 copies/μl) was added to each tube for the RT-PCR reaction. Products were visualized by running 10 μl of 100 μl total reaction volume on a 3.5% metaphor-agarose/1× TBE gel for 1.5 h at 100 V. To confirm whether nonvisualized products in 3.5% metaphor-agarose gel are not expressed at all, we repeated RT-PCR without any internal standard mRNA several times. The results were expressed as the mean ± SE from six observations.

RESULTS

Construction of standard ERα and ERβ template DNA

ERα and ERβ template DNA were constructed through RT-PCR by using the forward primer-1 designed to have the T7 promotor sequence at the 5′ end and the reverse primer-1 designed to generate 30 bp (c portion in Fig. 1) deletion that ended 28 bp (ERα) or 26 bp (ERβ) from the C-terminal end, respectively. After quantitation, RNA was diluted to 300 ng/μl in DEPC H_2O for RT-PCR.

RT-PCR of ovarian ERα and ERβ

A RT mixture was amplified for 15, 20, 25, 30, 33, or 35 cycles with the specific primers, respectively. After 33 cycles, the rate of amplification decreased gradually and it approached a plateau at 35 cycles (data not shown). Thirty-three cycles were found to be the most appropriate because amplification under those conditions was within the linear range and was also submaximal. All experiments described hereafter employed amplification for 33 cycles. Internal standard cDNA amplifications competed with target cDNA amplification. A 345 bp ERα and a 315 bp ERα-internal standard cDNA were obtained by using both ERα-forward primer-2 and ERα-reverse primer-2 (Fig. 2, lane 1). A 262 bp ERβ and a 232 bp ERβ-internal standard cDNA were obtained by using both ERβ-forward primer-2 and ERβ-reverse primer-2 (Fig. 2, lane 3). In addition, RT-PCR using β-actin primers was performed on all RNA samples as a control for tube-to-tube variability and differences in the amount of RNA (Fig. 2, lanes 2 and 4).

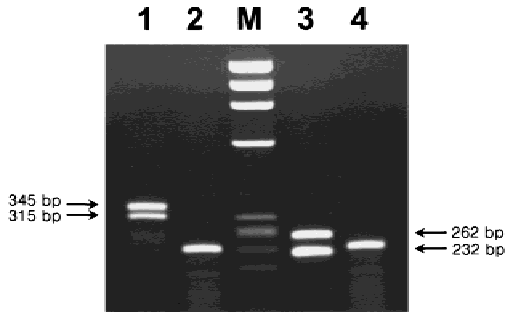

FIG. 2. RT-PCR from total RNA of the ovary using each forward primer-2 and each reverse primer-2 with ERα or ERβ internal standard m-RNA. Lane 1, ERα (345 bp) and ERα internal standards (315 bp); lanes 2 and 4, β-actin internal standard; M, size marker (Φ × 174/*Hae*III); lane 3, ERβ (262 bp) and ERβ internal standard (232 bp).

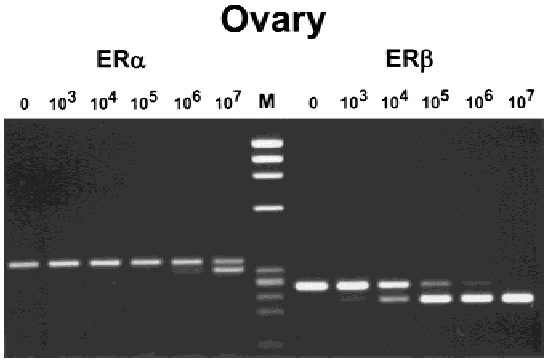

FIG. 3. Competitive RT-PCR from the ovary using each forward primer-2 and each reverse primer-2 with a serial copy number of ERα or ERβ internal standard m-RNA. M, size marker (Φ × 174/*Hae*III).

Competitive RT-PCR

Compared with ERβ, a significantly high expression of the ERα gene was detected in the ovary (Fig. 3 and Table 1). ERα was expressed in the ovary [$(2.2 \pm 0.33) \times 10^7$ copies/μg (RNA), renal cortex [$(3.1 \pm 0.32) \times 10^5$ copy/copies/μg of RNA], bone marrow [$(2.3 \pm 0.24) \times 10^5$ copies/μg of RNA] and uterus [$(3.3 \pm 0.43) \times 10^3$ copies/μg of RNA]. ERβ was expressed in the ovary [$(1.2 \pm 0.33) \times 10^5$ copies/μg of RNA], thyroid [$(6.0 \pm 0.23) \times 10^2$ copies/μg of RNA], and jejunum [$(1.1 \pm 0.24) \times 10^2$ copies/μg of RNA]. After ovariectomy, the expression of ERα was undetectable in the bone marrow and it dropped markedly to nearly 1/1000th of the pre-OVX value in the renal cortex (Fig. 4). However, we could not see any remarkable change of ERα expression in the uterus. In contrast to the change of ERα

TABLE 1. EXPRESSION OF ERα AND ERβ IN RAT TISSUES BEFORE AND AFTER OVARIECTOMY

	Alpha		*Beta*	
	Before OVX	*After OVX*	*Before OVX*	*After OVX*
Duodenum	—	—	—	—
Jejunum	—	—	$(1.1 \pm 0.24) \times 10^2$	$(1.2 \pm 0.35) \times 10^2$
Ileum	—	—	—	—
Renal cortex	$(3.1 \pm 0.32) \times 10^5$	$(3.2 \pm 0.23) \times 10^2$	—	—
Marrow stroma	$(2.3 \pm 0.24) \times 10^5$	—	$(0.7 \pm 0.21) \times 10^2$	—
Ovary	$(2.2 \pm 0.33) \times 10^7$		$(1.2 \pm 0.33) \times 10^5$	
Uterus	$(3.3 \pm 0.43) \times 10^3$	$(3.3 \pm 0.34) \times 10^3$	$(0.7 \pm 0.31) \times 10^2$	—
Thyroid	—	—	$(6.0 \pm 0.23) \times 10^2$	$(2.4 \pm 0.37) \times 10^3$
Parathyroid	—	—	—	—
Trabecular bone	$(3.2 \pm 0.56) \times 10^3$	—	$(2.8 \pm 0.21) \times 10^3$	—
Cortical bone	—	—	—	—

Copies/μg of total RNA.

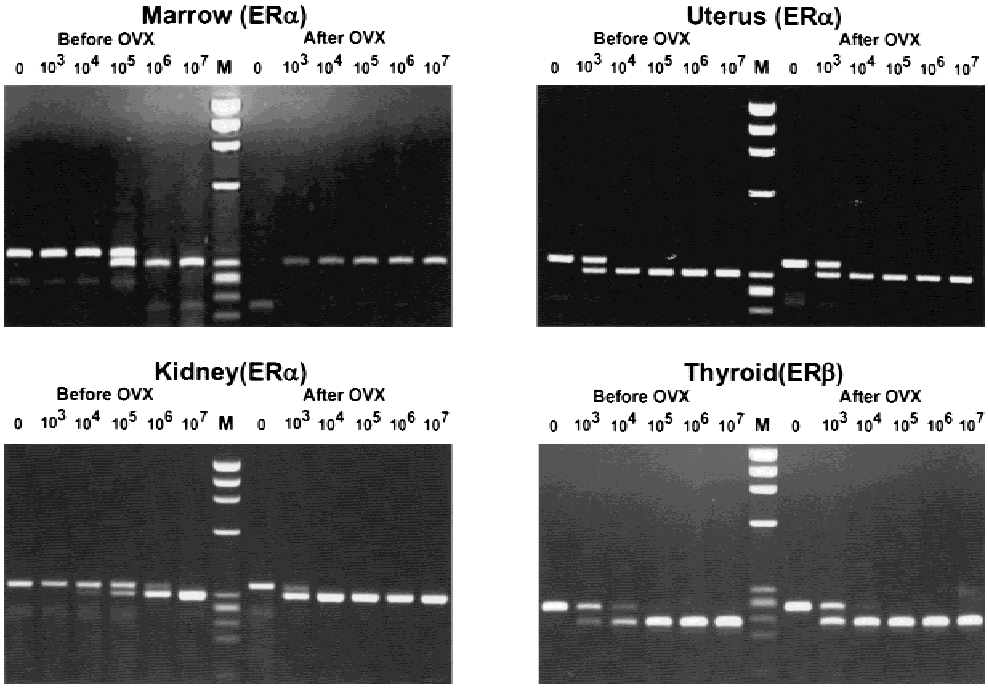

FIG. 4. Competitive RT-PCR from the different tissues using each forward primer-2 and each reverse primer-2 with a serial copy number of ERα or ERβ internal standard m-RNA. M, size marker (Φ × 174/*Hae*III).

expression, there was no change in the expression of ERβ in the jejunum or thyroid after ovariectomy. One week after 17β-estradiol (30 μg/kg) treatment, the rates of ERα expression in the bone marrow and renal cortex increased by 20–30 times of the pretreatment value. However, we could not find any significant change of ERβ expression in both organs (data not shown).

Expression of ER in trabecular and cortical bone

The rates of ERα and ERβ mRNA expression were analyzed in trabecular and cortical bone. Trabecular bone was surgically collected from the distal metaphysis, and cortical bone was collected from the distal diaphysis. Both ERα and ERβ were expressed in the trabecular bone [$(3.2 \pm 0.56) \times 10^3$ copy/μg of RNA] and [$(2.8 \pm 0.21) \times 10^3$ copy/μg of RNA], respectively. However, they were not detected in the cortical bone. After ovariectomy, both ERα and ERβ were hardly detected in the trabecular bone.

Expression of ER in cultured marrow stromal cells

ERα and ERβ mRNA were not detected after a 24-h bone marrow stromal cell culture; however, the rates of mRNA expression of ERα and ERβ reached ~10^5 copies/μg of total RNA and ~10^2 copies/μg of total RNA, respec-

TABLE 2. mRNA EXPRESSION OF ERα AND ERβ IN CULTURED MARROW STROMAL CELLS

	ER	
Culture period	*Alpha*	*Beta*
24 h	—	—
9 days	$(0.7 \pm 0.13) \times 10^5$	$(1.1 \pm 0.25) \times 10^2$
11 days	$(1.2 \pm 0.24) \times 10^5$	$(1.5 \pm 0.32) \times 10^2$
13 days	$(1.3 \pm 0.32) \times 10^5$	$(1.1 \pm 0.14) \times 10^2$

Copies/μg of total RNA.

tively, after 9-, 11-, and 13-day cultures (Table 2). To make certain whether they were not expressed at all after a 24-h culture, we repeated RT-PCR several times without adding any internal standard mRNA.

DISCUSSION

The second estrogen receptor (ERβ) was cloned from a rat prostate cDNA library; however, it has been hard to find major differences between ERα and ERβ.[2,15,16] The formation of a heterodimeric complex between ERα and ERβ, recently observed by Pettersson et al. might constitute a hitherto unrecognized mechanism involved in the tissue type– and cell type–specific effects of estrogens and certain antiestrogens.[17] Therefore, it is crucial to study the degree of expression of each ER and the changes of ER expression before and after ovariectomy in organs involved in the regulation of calcium metabolism to understand the potential physiologic implications of this effect on bone. Since antibodies against ERβ are not yet available, we studied the expression of ERα and ERβ mRNA in each organ and we also studied the change of expression of both mRNAs in these organs 3 weeks after ovariectomy by competitive RT-PCR. In this study, we showed clearly that there was a relative abundance of ERα mRNA expression in the bone marrow and renal cortex, and its expression decreased abruptly after ovariectomy. Our system was quantitative, and it was clear that the relative distribution of both ER subtypes was quite different.

ERα and ERβ were expressed abundantly in the ovary as reported previously.[14,18] An interesting finding was that bone marrow was one of the important target organs of estrogen; however, the degree of ERβ expression was much lower than that of ERα. A previous immunoelectron microscopic study also revealed that most of the electron-dense reaction was observed in many mononuclear cells located in the marrow stroma[19] and that some ER-positive cells were situated in the hematopoietic tissue.[18] There was a change of time course in the mRNA expression of both ERα and ERβ in the primary culture of bone marrow stromal cells. Both ERα and ERβ were not detected after a 24-h culture; however, the rates of mRNA expression of ERα and ERβ reached ~10^5 copies/μg of total RNA and ~10^2 copies/μg of total RNA, respectively, after 9-, 11-, and 13-day cultures. Marrow stromal cells, osteoblast and osteoclast precursor cells were mixed up in this culture system. Onoe et al. also reported that there was time course of change in mRNA expression of ERα and ERβ in primary osteoblastic cells during differentiation into mature osteoblasts.[20] Therefore, antiresorptive effects of estrogen could be mediated by indirect effect through binding to ERα on bone marrow stromal cells and by the direct effects through binding to both ERα and ERβ on differentiated osteoblast and osteoclast cells. Numbers of pre-B cells changed dramatically and reciprocally in response to estrogen levels in mice, suggesting that normal lymphophoiesis might be under hormonal control.[21] Increased proliferation of osteoclast precursor cells after ovariectomy[22] or clonal expansion of B-cell precursors followed by bone loss after estrogen deficiency in mice, which could be mimicked by repletion of interleukin (IL)-7 in cultured B cell precursors, may possibly be the crucial evidence of our story.[23]

ERα was expressed in the renal cortex, which is another important organ for regulating calcium homeostasis, and the degree of expression was very high [$(2.3 \pm 0.24) \times 10^5$ copies/μg of RNA]. However, ERβ was not expressed at all in contrast to the abundant expression of ERα. A previous study performed by immunohistochemical technique showed that ER was expressed not in tubular epithelial cells but in interstitial cells, capillaries, arteries, and renal capsules.[24] However, because calcium is reabsorbed through tubular epithelial cells and immunohistochemical technique is not a sensitive method if the rate of expression is low, it is therefore necessary that further studies be undertaken to confirm whether ERα is expressed in tubular epithelial cells or not. Parathyroid hormone performs the central role in the regulation of calcium homeostasis in mammals; however, the parathyroid gland was not a target organ of estrogen because both ERα and ERβ were not expressed in this study.

The degree of ERβ expression was very low in the jejunum [$(1.1 \pm 0.24)10^2$ copies/μg of RNA]. However, both receptors were not detected in the duodenum and ileum in our system. ERα was not detectable in the jejunum, which differed from other findings. We extracted total RNA from the whole layer of jejunum, not just from the mucosal layer. Therefore, if the total copy number of ERα was extremely low, it may not have been detected in our study. However, we could compare the relative abundance of ERβ to that of ERα in the jejunum. Our results also reconfirmed that epithelial cells in the jejunum, which is an important site for calcium absorption, contained ER capable of regulating gene transcription for stimulating intestinal calcium absorption or enhancing the uptake of calcium after treatment of 17β-estradiol in vitro.[25] The thyroid gland was one of the target organs of estrogen as ERβ, not ERα, was expressed in low abundance. Given the higher incidence and better prognosis of thyroid cancer in women, there were suggestions that this lesion may be influenced by sex hormones without any supporting evidence about the presence of ER in thyroid cancer.[26] Here we found that ERβ was expressed in the thyroid gland and its expression was not affected by ovariectomy at all. This may indicate that estrogen regulates the proliferation or differentiation of thy-

roid cells or performs some other potential roles providing a better prognosis of thyroid cancer through ERβ.

Both ERα and ERβ were expressed in the trabecular bone; however, the rate of ERβ expression was weak, [$(3.2 \pm 0.56) \times 10^3$ copy/μg of RNA] and [$(0.8 \pm 0.21) \times 10^2$ copy/μg of RNA], respectively. Recently, a lack of ER was reported to lead to a failure of growth plate to close, as well as to osteoporosis.[9,27,28] These estrogen receptor-alpha knockout (ERKO) human and mice models brought us in agreement with our findings that ERα was the main ER in the bone marrow, kidney, and even bone.[27,28] If ERβ was expressed sufficiently or as the main ER in these organs, ERβ should compensate for the lack of ERα and bone mass should be preserved in ERKO human and mice. Therefore, it can be further evidence that ERα is the main ER in these organs.

The change of ERα expression after ovariectomy was interesting. Most receptors are up-regulated after withdrawal of ligands, with some exceptions.[29] The expression of ERα in bone marrow was undetectable and its expression in the renal cortex dropped markedly 3 weeks after ovariectomy. Recently, the molecular basis of ER regulation of the IL-6 promotor has been determined.[30] Osteoclast formation was decreased by the inhibition of IL-6, and estrogen withdrawal stimulated the proliferation of hematopoietic osteoclast precursors originating from cells of the granulocyte macrophage colony stimulating unit or macrophage colony stimulating unit lineage.[22] Therefore, the effects of estrogen deficiency in bone marrow should be amplified by the near-complete suppression of ERα at the transcription level following ovariectomy, and it explains why bone loss is accelerated just after surgical menopause or the early natural postmenopausal period. Meanwhile, the expression of both receptors in the uterus was low, and we failed to provide data demonstrating the effectiveness of ovariectomy on ER expression in the uterus. We could not explain these results clearly. One plausible explanation might be that the expression of ER was measured in the whole uterus, not in endometrium in this study, and the complete down-regulation of ER after ovariectomy might not be biologically relevant in the uterus. We were unable to observe the changes of ERβ expression in the jejunum and thyroid gland shortly after ovariectomy. To clarify the biologic significance of such changes of mRNA expression of ER after ovariectomy in different target tissues, further studies about the transcriptional regulation of the ERα and ERβ gene in different tissues should be undertaken. In addition, the long-term changes of the degree of ER expression after ovariectomy should be also studied in various tissues.

In conclusion, ERα was the main ER in organs important for calcium homeostasis, except in the jejunum; the bone marrow and renal cortex highly expressing ERα were the most important target organs for estrogen; and the expression of ERα was undetectable in the bone marrow, and its expression in the renal cortex dropped markedly 3 weeks after ovariectomy. Our findings may partially explain the reason why the rate of bone loss, especially in trabecular bone, is accelerated and the bony effect of estrogen deficiency is amplified after surgical or natural menopause.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Henry M. Kronenberg, M.D. for valuable discussion of this work and to Mr. Robert Ross for English language revision.

REFERENCES

1. Milgrom E, Savouret JF, Mantel A, Perrot-Applanat M, Delabre K, Lescop P 1997 Promotor-and cell-specific responses to sex steroids. Osteoporos Int **7** (Suppl 1)**:**S23–S28.
2. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA **93:**5925–5930.
3. Mosselman S, Polman J, Dijkema R 1996 ERβ: Identification and characterization of a novel human estrogen receptor. FEBS Lett **392:**49–53.
4. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol **11:**353–365.
5. Pennisi E 1997 Differing roles found for estrogen's two receptors. Science **277:**1439.
6. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y, Muramastsu M 1997 Molecular cloning and functional analysis of human estrogen receptor β2. J Bone Miner Res **12** (Suppl 1)**:**S165.
7. Lindsay R, Cosman F 1997 Skeletal effects of estrogen analogs. Osteoporos Int **7** (Suppl 1)**:**S40–S42.
8. Kangas L 1992 Agonistic and antagonistic effects of antiestrogens in different target organs. Acta Oncol **31:**143–146.
9. Pan LC, Ke HZ, Simmons HA, Crawford DT, Chidsey-Frink KL, McCurdy SP, Schafer JR, Kimbro KS, Taki M, Korach KS, Thomson DD 1997 Estrogen receptor-alpha knockout (ERKO) mice lose trabecular and cortical bone following ovariectomy. J Bone Miner Res **12** (Suppl 1)**:**S134.
10. Paech K, Webb P, Kuipper GGJM, Nilsson S, Gustafsson J-A, Kushner PJ, Scanlan TS 1997 Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science **277:**1508–1510.
11. Beekman JM, Allan GF, Tsai SY, Tsai M-J, O'Malley BW 1993 Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. Mol Endocrinol **7:**1266–1274.
12. Cowley SM, Hoare S, Mosselman S, Parker MG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem **272:**19858–19862.
13. Kim GS, Kim CH, Choi CS, Park JY, Lee KU 1997 Involvement of different second messengers in parathyroid hormone–and interleukin-1–induced interleukin-6 and interleukin-11 production in human bone marrow stromal cells. J Bone Miner Res **12:**896–902.
14. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β. Endocrinology **138:**863–870.
15. Grandien KF, Berkenstam A, Nilsson S, Gustafsson JA 1993 Localization of DNAse I hypersensitive sites in the human estrogen receptor gene correlates with the transcriptional activity of two differentially used promotors. J Mol Endocrinol **10:**269–277.
16. Stewart PJ, Stern PH 1986 Effects of the antiestrogens tamoxifen and clomiphene on bone resorption in vitro. Endocrinology **118:**125–131.
17. Pettersson K, Grandien K, Kuipper GGJM, Gustafsson J-A 1997 Mouse estrogen receptor βs forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol **11:**1486–1496.
18. Enmark E, Pelto-huikko M, Grandien K, Lagercrantz S, La-

gercrantz J, Fried G, Nordenskjold M, Gustanfsson J-A 1998 Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab **82:**4258–4265.
19. Ohashi T, Kusuhara S 1993 Immunoelectron microscopic detection of estrogen target cells in the bone marrow of estrogen treated male Japanese quail. Bone Miner **20:**31–39.
20. Onoe Y, Miyaura C, Ohata H, Nozawa S, Suda T 1997 Expression of estrogen receptor β in rat bone. Endocrinology **138:**4509–4512.
21. Smithson G, Medina K, Ponting I, Kincade PW 1995 Estrogen suppresses stromal cell-dependent lymphopoiesis in culture. J Immunol **155:**3409–3417.
22. Pacifici R 1996 Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res **11:**1043–1051.
23. Miyaura C, Onoe Y, Inada M, Maki K, Ikuta K, Ito M, Suda T 1997 Increased B-lymphopoiesis by IL-7 treatment induces bone loss in mice with intact ovarian function: Similarity to estrogen deficiency. J Bone Miner Res **12** (Suppl 1)**:**S126.
24. Bhat HK, Hacker HJ, Bannasch P, Thompson EA, Liehr JG 1993 Localization of estrogen receptors in interstitial cells of hamster kidney and in estradiol-induced renal tumors as evidence of the mesenchymal origin of this neoplasm. Cancer Res **53:**5447–5551.
25. Salih MA, Sim SH, Kalu DN 1996 Putative intestinal estrogen receptor: Evidence for regional differences. Mol Cell Endocrinol **121:**47–55.
26. Jaklic BR, Rushin J, Ghosh BC 1995 Estrogen and progesterone receptors in thyroid lesions. Ann Surg Oncol **2:**429–434.
27. Korach KS 1994 Insights from the study of animals lacking functional estrogen receptor. Science **266:**1524–1527.
28. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen receptor gene in men. N Engl J Med **331:**1056–1061.
29. Delvin EE, Lopez V, Levy E, Menard D 1996 Calcitriol differentially modulates mRNA encoding calcitriol receptors and calcium-binding protein 9 kDa in human fetal jejunum. Biochem Biophys Res Commun **224:**544–548.
30. Stein B, Yang MX 1995 Repression of the interleukin-6 promotor by estrogen receptor is mediated by NF-*k*B and C/EBPβs. Mol Cell Biol **15:**4971–4979.

Address reprint requests to:
Dr. Sung-Kil Lim
College of Medicine
Yonsei University
Department of Internal Medicine
134 Shichon Dong Sue Dae Mon Gu
Seoul 120-749, Korea

Received in original form April 29, 1998; in revised form October 20, 1998; accepted March 16, 1999.