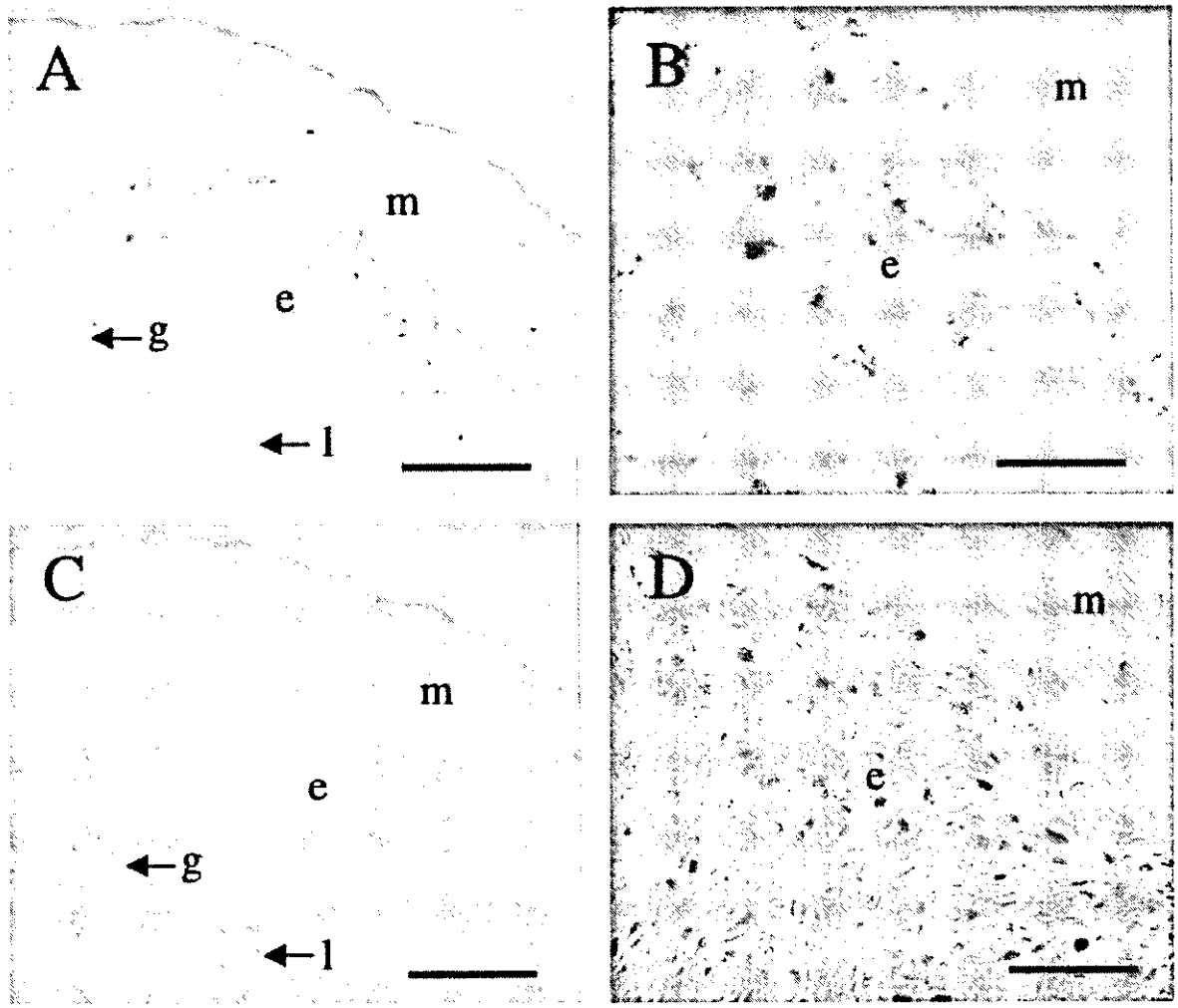

Figure 6 Distribution of sFRP4 mRNA and PCNA-positive cells in rat uteri at 48 h after estrogen treatment. *In situ* hybridization histochemistry of sFRP4 (A, B) and immunostaining of PCNA (C, D) were performed using ovariectomized rat uteri at 48 h after estrogen treatment. (A) sFRP4 mRNA was expressed abundantly in the endometrial stroma (e) whereas there was little in the myometrium (m) when hybridized with sFRP4-specific antisense RNA probe. No sFRP4 mRNA expression was detected in either the luminal epithelium (l) or glandular epithelium (g). (B) Higher magnification of (A). (C) PCNA-positive cells were detected in the stroma, luminal epithelium and glandular epithelium. (D) Higher magnification of (C). All experiments were performed three times. Bars represent (A, C) 200 μm and (B, D) 50 μm.

(Pepe & Albrecht 1995). Thus, we speculate the regulation of sFRP4 expression by estrogen in the uterus. To investigate this possibility, we used ovariectomized rats treated with estrogen. sFRP4 mRNA expression was detected 48 h after estrogen treatment. Unlike during pregnancy, the 2·0 kb sFRP4 transcript was observed whereas the 2·9 kb transcript was not. It is possible that several splice variants of sFRP4 may exist and only the 2·0 kb transcript can be especially up-regulated by estrogen. Alternatively, other transcripts were not detected in our Northern analysis because the expression levels were lower. In general, direct estrogen-responsive genes are up-regulated within several hours of estrogen treatment. For example, the level of progesterone receptor mRNA was elevated within 2 h after a single injection of 17β-estradiol (Shughrue *et al.* 1997). It was rather late that sFRP4 mRNA was detected in the uterus, at 48 h after estrogen injection, suggesting that up-regulation of sFRP4 may be an indirect effect of estrogen.

The expression of sFRP4 mRNA was observed in the proliferating endometrial stroma. It was reported that the maximal number of PCNA-positive cells was found after 36 h in the epithelium and stroma (Gunin 1997). Thus, the expression of sFRP4 mRNA might be accompanied by the late phase of proliferation after estrogen treatment.

In summary, we have identified sFRP4 as an up-regulated gene in the uterus during pregnancy. Our present data suggest that the expression of sFRP4 mRNA was accompanied by a late phase of proliferation in the decidual cells. It is possible that sFRP4 modulates signals of Wnt genes in the pregnant uterus.

Acknowledgements

We thank Ms M Kobayashi and Ms S Inada for expert technical assistance. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, and research grants from the Comprehensive Research on Aging and Health, the Ministry of Health and Welfare of Japan.

References

Abrahamsohn PA & Zorn TM 1993 Implantation and decidualization in rodents. *Journal of Experimental Zoology* **266** 603–628.

Abu-Jawdeh G, Comella N, Tomita Y, Brown LF, Tognazzi K, Sokol SY & Kocher O 1999 Differential expression of frpHE: a novel human stromal protein of the secreted frizzled gene family, during the endometrial cycle and malignancy. *Laboratory Investigation* **79** 439–447.

Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J & Nusse R 1996 A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* **382** 225–230.

Charnock JD, Sharkey AM, Fenwick P & Smith SK 1994 Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *Journal of Reproduction and Fertility* **101** 421–426.

Chen H, Chen CL & Chou JY 1994 Characterization of two promoters of a rat pregnancy-specific glycoprotein gene. *Biochemistry* **33** 9615–9626.

Chibbar R, Wong S, Miller FD & Mitchell BF 1995 Estrogen stimulates oxytocin gene expression in human chorio-decidua. *Journal of Clinical Endocrinology and Metabolism* **80** 567–572.

Danielian PS & McMahon AP 1996 Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature* **383** 332–334.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED & Siebert PD 1996 Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *PNAS* **93** 6025–6030.

Diatchenko L, Lukyanov S, Lau YF & Siebert PD 1999 Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. *Methods in Enzymology* **303** 349–380

Dowell RT & Kauer CD 1997 Maternal hemodynamics and uteroplacental blood flow throughout gestation in conscious rats. *Methods and Findings in Experimental and Clinical Pharmacology* **19** 613–625.

Faria TN, Deb S, Kwok SC, Talamantes F & Soares MJ 1990 Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta. *Developmental Biology* **141** 279–291.

Fletcher S, Thomas T, Schreiber G, Heinrich PC & Yeoh GC 1988 The development of rat α 2-macroglobulin. Studies *in vivo* and in cultured fetal rat hepatocytes. *European Journal of Biochemistry* **171** 703–709.

Fu X, Ulmsten U & Backstrom T 1994 Interaction of sex steroids and oxytocin on term human myometrial contractile activity *in vitro*. *Obstetrics and Gynecology* **84** 272–277.

Gu Y, Jow GM, Moulton BC, Lee C, Sensibar JA, Park-Sarge OK, Chen TJ & Gibori G 1994 Apoptosis in decidual tissue regression and reorganization. *Endocrinology* **135** 1272–1279.

Gunin AG 1997 Effect of heparin on oestradiol-induced cell growth and proliferation in the uterus of ovariectomized rats. *Journal of Endocrinology* **154** 441–448.

Guo K, Wolf V, Dharmarajan AM, Feng Z, Bielke W, Saurer S & Friis R 1998 Apoptosis-associated gene expression in the corpus luteum of the rat. *Biology of Reproduction* **58** 739–746.

Hansen PJ 1998 Regulation of uterine immune function by progesterone – lessons from the sheep. *Journal of Reproductive Immunology* **40** 63–79.

Hatthachote P & Gillespie JI 1999 Complex interactions between sex steroids and cytokines in the human pregnant myometrium: evidence for an autocrine signaling system at term. *Endocrinology* **140** 2533–2540.

Ishikawa K, Fujigasaki H, Saegusa H, Ohwada K, Fujita T, Iwamoto H, Komatsuzaki Y, Toru S, Toriyama H, Watanabe M, Ohkoshi N, Shoji S, Kanazawa I, Tanabe T & Mizusawa H 1999 Abundant expression and cytoplasmic aggregations of α 1A voltage-dependent calcium channel protein associated with neurodegeneration in spinocerebellar ataxia type 6. *Human Molecular Genetics* **8** 1185–1193.

Kelman Z & Hurwitz J 1998 Protein–PCNA interactions: a DNA-scanning mechanism? *Trends in Biochemical Science* **23** 236–238.

Koike S, Sakai M & Muramatsu M 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research* **15** 2499–2513.

Leimeister C, Bach A & Gessler M 1998 Developmental expression patterns of mouse sFRP genes encoding members of the secreted frizzled related protein family. *Mechanisms of Development* **75** 29–42.

Lim KJ, Odukoya OA, Ajjan RA, Li TC, Weetman AP & Cooke ID 1998 Profile of cytokine mRNA expression in peri-implantation human endometrium. *Molecular Human Reproduction* **4** 77–81.

Linguеglia E, Renard S, Voilley N, Waldmann R, Chassande O, Lazdunski M & Barbry P 1993 Molecular cloning and functional expression of different molecular forms of rat amiloride-binding proteins. *European Journal of Biochemistry* **216** 679–687.

Macphee DJ & Lye SJ 2000 Focal adhesion signaling in the rat myometrium is abruptly terminated with the onset of labor. *Endocrinology* **141** 274–283.

McMahon AP & Bradley A 1990 The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62** 1073–1085.

Miller C & Sassoon DA 1998 Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. *Development* **125** 3201–3211.

Miller C, Pavlova A & Sassoon DA 1998 Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle. *Mechanisms of Development* **76** 91–99.

Moulton BC 1994 Transforming growth factor-beta stimulates endometrial stromal apoptosis *in vitro*. *Endocrinology* **134** 1055–1060.

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998 Molecular cloning and characterization of human estrogen receptor β cx: a potential inhibitor of estrogen action in human. *Nucleic Acids Research* **26** 3505–3512.

Ogle TF, Mills TM & Costoff A 1990 Progesterone maintenance of the placental progesterone receptor and placental growth in ovariectomized rats. *Biology of Reproduction* **43** 276–284.

Ogle TF, George P & Dai D 1998 Progesterone and estrogen regulation of rat decidual cell expression of proliferating cell nuclear antigen. *Biology of Reproduction* **59** 444–450.

Ogle TF, Dai D & George P 1999 Progesterone-regulated determinants of stromal cell survival and death in uterine decidua are linked to protein kinase C activity. *Steroids* **64** 628–633.

Orimo A, Inoue S, Ikeda K, Noji S & Muramatsu M 1995 Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs. *Journal of Biological Chemistry* **270** 24406–24413.

Paria BC, Ma W, Tan J, Raja S, Das SK, Dey SK & Hogan BLM 2001 Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. *PNAS* **98** 1047–1052.

Parr BA & McMahon AP 1998 Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. *Nature* **395** 707–710.

Pepe GJ & Albrecht ED 1995 Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocrine Reviews* **16** 608–648.

Saito S, Ibaraki T, Enomoto M, Ichijo M & Motoyoshi K 1994 Macrophage colony-stimulating factor induces the growth and differentiation of normal pregnancy human cytotrophoblast cells and hydatidiform moles but does not induce the growth and differentiation of choriocarcinoma cells. *Japanese Journal of Cancer Research* **85** 245–252.

Shughrue PJ, Lane MV & Merchenthaler I 1997 Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an *in situ* hybridization study. *Endocrinology* **138** 5476–5484.

Stewart EA, Floor AE, Jain P & Nowak RA 1995 Increased expression of messenger RNA for collagen type I, collagen type III, and fibronectin in myometrium of pregnancy. *Obstetrics and Gynecology* **86** 417–422.

Takahashi K, Akasaka M, Yamamoto Y, Kobayashi C, Mizoguchi J & Koyama J 1990 Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. *Journal of Biochemistry* **108** 145–148.

Thomas KR & Capecchi MR 1990 Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346** 847–850.

Thomas T 1993 Distribution of α-2 macroglobulin and alpha 1-acid glycoprotein mRNA shows regional specialization in rat decidua. *Placenta* **14** 417–428.

Thordarson G, Southard JN & Talamantes F 1991 Purification and characterization of mouse decidual calcyclin: a novel stimulator of mouse placental lactogen-II secretion. *Endocrinology* **129** 1257–1265.

Tsurimoto T 1998 PCNA, a multifunctional ring on DNA. *Biochimica et Biophysica Acta* **1443** 23–39.

Vogiagis D & Salamonsen LA 1999 Review: the role of leukaemia inhibitory factor in the establishment of pregnancy. *Journal of Endocrinology* **160** 181–190.

Waterhouse P, Parhar RS, Guo X, Lala PK & Denhardt DT 1992 Regulated temporal and spatial expression of the calcium-binding proteins calcyclin and OPN (osteopontin) in mouse tissues during pregnancy. *Molecular Reproduction and Development* **32** 315–323.

Welsh AO & Enders AC 1985 Light and electron microscopic examination of the mature decidual cells of the rat with emphasis on the antimesometrial decidua and its degeneration. *American Journal of Anatomy* **172** 1–29.

Wolf V, Ke G, Dharmarajan AM, Bielke W, Artuso L, Saurer S & Friis R 1997 DDC-4, an apoptosis-associated gene, is a secreted frizzled relative. *FEBS Letters* **417** 385–389.

de Ziegler D, Fanchin R, de Moustier B & Bulletti C 1998 The hormonal control of endometrial receptivity: estrogen (E2) and progesterone. *Journal of Reproductive Immunology* **39** 149–166.

Received 25 February 2002
Accepted 25 March 2002

Journal of Neuroendocrinology, 2002, Vol. 14, 836–842

Isoform Specificity for Oestrogen Receptor and Thyroid Hormone Receptor Genes and Their Interactions on the NR2D Gene Promoter

N. Vasudevan,*[1] H. K. Kia,*[1] S. Inoue,† M. Muramatsu‡ and D. Pfaff*
*Laboratory of Neurobiology and Behavior, The Rockefeller University, New York, NY, USA.
†Department of Geriatric Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan.
‡Future Program Research Division, Saitama Medical School, Moroyama, Iruma-gun, Saitama, Japan.

Key words: NMDA-2D, thyroid hormone, oestrogen, transfection, oestrogen response element, crosstalk.

Abstract

Oestrogens are critical for the display of lordosis behaviour and, in recent years, have also been shown to be involved in synaptic plasticity. In the brain, the regulation of ionotropic glutamate receptors has consequences for excitatory neurotransmission. Oestrogen regulation of the *N*-methyl-D-aspartate receptor subunit 2D (NR2D) has generated considerable interest as a possible molecular mechanism by which synaptic plasticity can be modulated. Since more than one isoform of the oestrogen receptor (ER) exists in mammals, it is possible that oestrogen regulation via the ERα and ERβ isoforms on the NR2D oestrogen response element (ERE) is not equivalent. In the kidney fibroblast (CV1) cell line, we show that in response to 17β-oestradiol, only ERα, not ERβ, could upregulate transcription from the ERE which is in the 3′ untranslated region of the NR2D gene. When this ERE is in the 5′ position, neither ERα nor ERβ showed transactivation capacity. Thyroid hormone receptor (TR) modulation of ER mediated induction has been shown for other ER target genes, such as the preproenkephalin and oxytocin receptor genes. Since the various TR isoforms exhibit distinct roles, we hypothesized that TR modulation of ER induction may also be isoform specific. This is indeed the case. The TRα1 isoform stimulated ERα mediated induction from the 3′-ERE whereas the TRβ1 isoform inhibited this induction. This study shows that isoforms of both the ER and TR have different transactivation properties. Such flexible regulation and crosstalk by nuclear receptor isoforms leads to different transcriptional outcomes and the combinatorial logic may aid neuroendocrine integration.

Oestrogen and thyroid hormones play critical roles in reproduction and growth in mammals. They are small molecules that bind to intracellular nuclear receptors, the oestrogen (ER) and thyroid hormone (TR) receptors (1). These nuclear receptors are ligand-activated transcription factors, which regulate the transcription of target genes (2).

The brain is an important target for both oestrogen (3, 4) and thyroid hormones (5). In the brain, oestrogen, via the ER, exerts a central neuroendocrine control on gonadotropin production, thereby controlling reproduction and lordosis behaviour (6–9). Thyroid hormone is important for growth, development and thermoregulation in adult homeotherms (10). Indeed, neonatal hypothyroidism leads to cretinism in humans, a syndrome characterized by mental retardation and growth defects (11). Despite such important physiological roles, the downstream target genes of either oestrogen or thyroid hormone in the brain remain poorly understood.

N-methyl-D-asparate (NMDA) acting via the ionotropic glutamate receptor, the NMDA receptor, has diverse and important functions in synapse plasticity, excitotoxicity (12) and reproduction (13) and long-term potentiation (14, 15). Functional NMDA receptor channels are composed of a NMDA1 subunit (NR1) and one of four NMDA2 subunits (NR2A-2D) (16, 17). Expression of the NR2D (16, 18) and ERα mRNA (19–21) has been demonstrated in the hypothalamus and amygdala of the rat brain. The NR2D mRNA has also been shown to be upregulated by oestrogen via ERα and β through four half-palindromic oestrogen response

Correspondence to: Dr Nandini Vasudevan, 208 Mueller Laboratories, Pennsylvania State University, University Park, PA 16802, USA (e-mail: nuv1@psu.edu).
[1]These authors contributed equally to this study.

elements (EREs) in the 3′ untranslated region (UTR) (22). A decrease in glutamineragic transmission inhibits lordosis while NMDA can facilitate lordosis in female rat (23) via stimulation of gonadotropin-releasing hormone release (23, 24).

More than one isoform of the ER and TR exists in mammals. There are two ER isoforms, termed ERα and ERβ (25, 26) and four isoforms of the TR (27–29). Three of the four TR isoforms, TRα1, TRβ1 and TRβ2, can bind ligand; the fourth isoform TRα2 cannot do so due to the loss of 40 amino acids in the *C*-terminal ligand-binding domain (29). The ability of TR to modulate oestrogen dependent transcription also demonstrates that the interactions between different ER and TR isoforms lead to distinct transcriptional outcomes. For example, on the behaviourally relevant pre-proenkephalin (PPE) promoter, TRα1 inhibits the ERα-mediated induction while stimulating the ERβ-mediated induction in kidney fibroblast cells (30). However, on the same promoter, the TRβ1 and TRβ2 isoforms have no effect on either ERα or ERβ-induced transcription (30). Therefore, the crosstalk between the different ER and TR isoforms shows considerable promoter and isoform specificity.

It has been hypothesized that the thyroid hormone elevation that occurs at cold temperatures may signal unfavourable environmental conditions for reproduction (31). It is therefore possible that the NR2D gene, as an oestrogen regulated gene that facilitates lordosis, is modulated by thyroid hormone. However, among the ligand-binding TR isoforms, the TRβ2 has an extremely restricted distribution in the anterior pituitary while the TRα1 and TRβ1 isoforms are expressed in the ventromedial hypothalamus (VMH) and in the amygdala, both areas involved in reproductive behaviour. Hence, we restricted our choice of TR isoforms to ligand-binding TR isoforms expressed in the brain regions of interest. The aim of the present study was to investigate the interactions between these different ligand-binding TR isoforms and the ER isoforms on a physiologically and behaviourally relevant NR2D promoter containing four half-palindromic EREs in the 3′ UTR, with the hypothesis that TR isoforms may inhibit ERα-mediated transactivation.

Materials and methods

Tissue culture

CV-1, kidney fibroblast cells, were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Bioreclamation, Inc., New York, NY, USA), 100 U/ml penicillin and 50 μg/ml streptomycin. For transfections, cells were cultured in DMEM without phenol red and 10% fetal bovine serum, which was charcoal stripped (Bioreclamation, Inc.) to render it free of steroids, as described in previous studies (30, 32).

Plasmids/constructs

The 3′ NR2D and 5′ NR2D reporter constructs were a gift from Dr Muramatsu (22). They contain a 174-bp insert which comprises the four half-palindromic EREs from the 3′ UTR of the rat NR2D sequence inserted into the pBLCAT vector upstream of the chloramphenicol acetyl transferase (CAT) sequence (5′ NR2D-CAT construct) or inserted into the ptkCAT2 vector as a 3′ UTR insertion (3′ NR2D-CAT construct). In both cases, the minimal thymidine kinase promoter is used to drive expression of the reporter gene (22). The pSG-hERα and the pcDNAI/Amp expression vectors containing the rat TRα1, β1 and β2 isoforms have been described previously (32, 33). The CMV-hERβ is the ERβ expression vector and was provided by E. Enmark (34). The TRα P box mutant is a DNA binding mutant of rat TRα1 expressed from pSG5X vector (35).

Transfections

CV-1 cells were plated at a density of 0.1×10^6/well in six-well plates (Falcon, Lincoln Park, NJ, USA) and transfected using Effectene reagent as per the manufacturer's instructions (Qiagen, GmbH, Germany). The cells were approximately 60–70% confluent at the time of transfection. Briefly, 24 h after plating, 0.2 μg of the reporter plasmid, 3′ NR2D-CAT or 5′ NR2D-CAT, and 0.04 μg of the expression constructs (the pCDNAI-TR vectors, the ER vectors) were transfected into cells along with 0.08 μg of pSV-βgal plasmid (Promega; Madison, WI, USA) and pBSSKII+ (Stratagene, La Jolla, CA, USA) to a final DNA concentration of 0.4 μg per well. The pSV-βgal plasmid was used as an internal control to normalize for transfection efficiency and lysate preparation. After exposure to Effectene for 16 h, the cells were washed with phosphate buffered saline. Then, DMEM media without phenol red containing stripped sera and 17β-oestradiol (Sigma, St Louis, MO, USA) (10^{-7} M) and triiodothyronine (T_3) (Sigma) (10^{-6} M) dissolved in ethanol were added to the media. Ethanol in equal amounts was added to the media as the vehicle control. Forty-eight hours after hormone treatment, cells were lysed using reporter lysis buffer as per the manufacturer's instructions (Promega). The lysates so obtained were used for both CAT and β-galactosidase (βgal) assays (Promega). The CAT activity was normalized with the βgal activity for every sample.

Statistical analysis

The results are presented as the mean ± SEM of samples in a treatment group from replicate experiments. The results were plotted as fold over control vehicle levels using GraphPad Prism statistical software (GraphPad Prism, San Diego, CA, USA). Statistical analysis on raw data was performed using ANOVAs followed by the Student–Newman–Keuls post-hoc test for comparison between treatment groups.

Results

Oestrogen regulation of the rat NR2D gene

The rat NR2D gene has been reported to be regulated in response to 17β-oestradiol in MCF-7 cells (22). In this study, a kidney fibroblast cell line, devoid of endogenous ER and TR (36) was used to check for (i) oestrogen induction of the NR2D gene via either the 5′ enhancer or the 3′ enhancer ERE and (ii) oestrogen induction via either transfected ERα or transfected ERβ. The ERα acting via the ERE enhancer at the 3′ UTR position (Fig. 1A) but not via the 5′ enhancer (Fig. 1c) could induce the rat NMDA gene in this cell line. The oestrogen induction at 10^{-7} M concentration is small but highly reproducible. In other studies using oestrogen-responsive reporters in this cell line, this concentration of oestrogen was used (30, 32, 37), and therefore this concentration was also used in subsequent experiments. Under the same conditions used to test for oestrogen induction via ERα, the ERβ could not induce the reporter either when the ERE was in its native 3′ UTR configuration or in the artificial 5′ position at any of the concentrations of 17β-oestradiol studied (Figs 1B,D, respectively). Therefore, there is isoform specificity in oestrogen regulation of the NR2D gene in this cell line.

Antagonistic effects of the oestrogen-ligand ERβ isoform towards the ERα isoform have been noted on the consensus ERE in HeLa cells (38). Recently, a study from our laboratory has also noted the antagonistic effect of ERβ toward ERα induction of the physiological OTR promoter in a neuroblastoma cell line (32). Upon cotransfection of

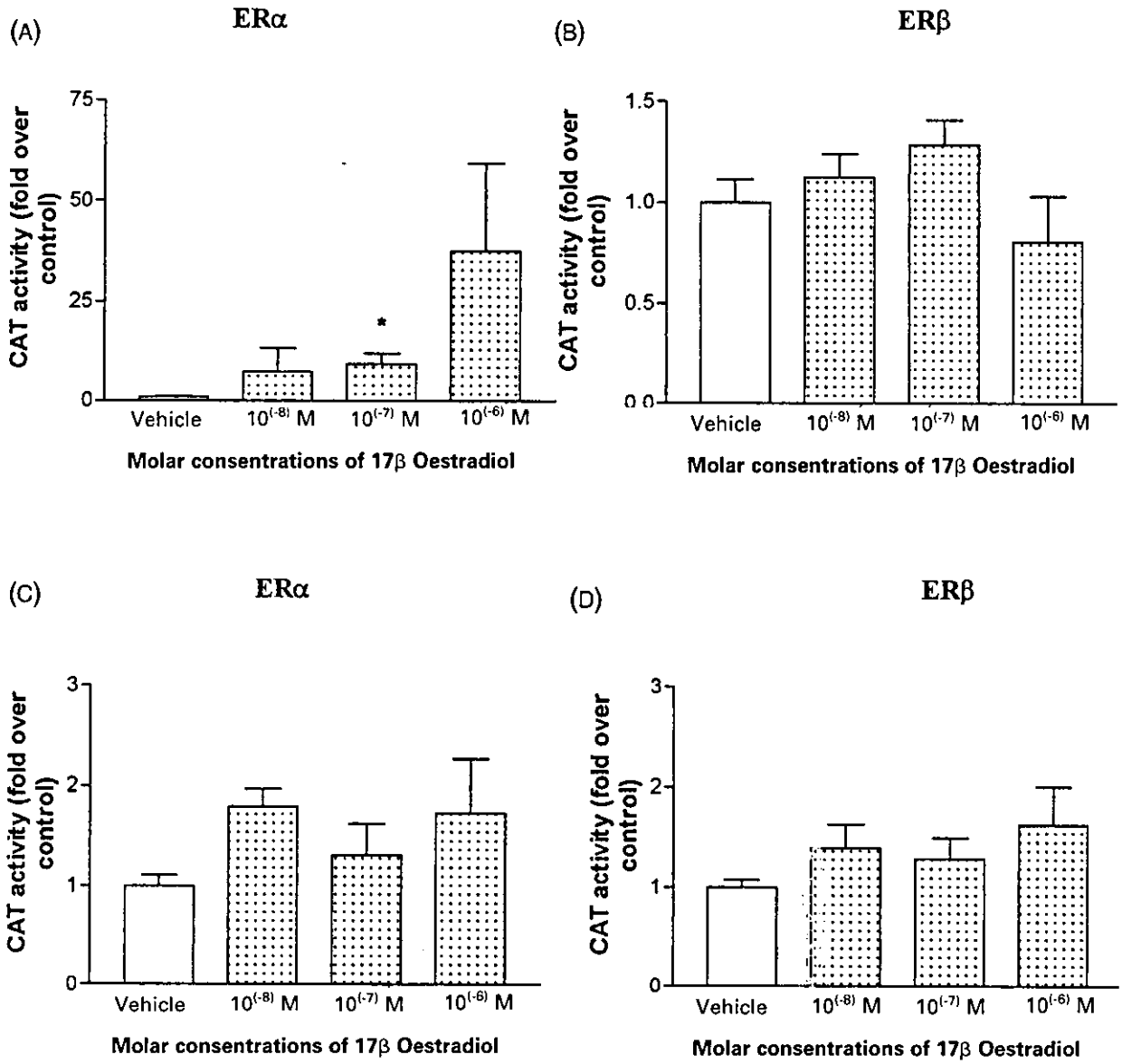

FIG. 1. (A–D) Dose–response curve for 17-β-oestradiol using oestrogen receptor (ER)α (A,C) and ERβ (B,D) on the 3′ and 5′ NR2D gene promoters. Transfections were performed as described in the Methods section. Briefly, CV-1 cells were cotransfected with 3′ NR2D (A,B) or 5′ NR2D (C,D) constructs and pSG-hERα or pCMV ERβ expression vectors as indicated. 17-β-Oestradiol dissolved in ethanol was added at various concentrations to the cells. Ethanol in equal measure was added to the control (vehicle) wells. Chloramphenicol acetyl transferase (CAT) and β galactosidase (βgal) enzyme assays were performed on the cell lysates 48 h after hormone treatment. CAT values were normalized to βgal values for every sample in each of the treatment groups (A,C: n = 4 per treatment group; B,D: n = 7 per treatment group). The results are presented as mean ± SEM from replicate experiments. The results (fold over control) were analysed statistically using ANOVA to compare between treatment groups. (A) *$P < 0.05$ compared to vehicle treatment.

ERα and ERβ and in response to 10^{-7} M 17β-oestradiol, the ERβ isoform also inhibited the ERα induction from the 3′ NR2D gene promoter (Fig. 2).

Thyroid hormone (T_3) modulation of ERα-mediated induction from the 3′ NR2D gene

Since the ERα could induce the 3′ NR2D construct in response to 10^{-7} M 17β-oestradiol, this was chosen to investigate thyroid hormone modulation of oestrogen-mediated induction. The TRα1 isoform stimulated ERα-mediated induction from the 3′ NR2D gene promoter (Fig. 3). In contrast, the TRβ1 isoform inhibited ERα-mediated induction from the 3′ NR2D promoter (Fig. 4). The TR P box mutant is a mutant that cannot bind DNA (35) and was used to investigate if DNA binding is necessary for the stimulatory effect. The TR P box mutant was unable to stimulate the ERα-mediated induction (Fig. 5) and even led to an inhibition,

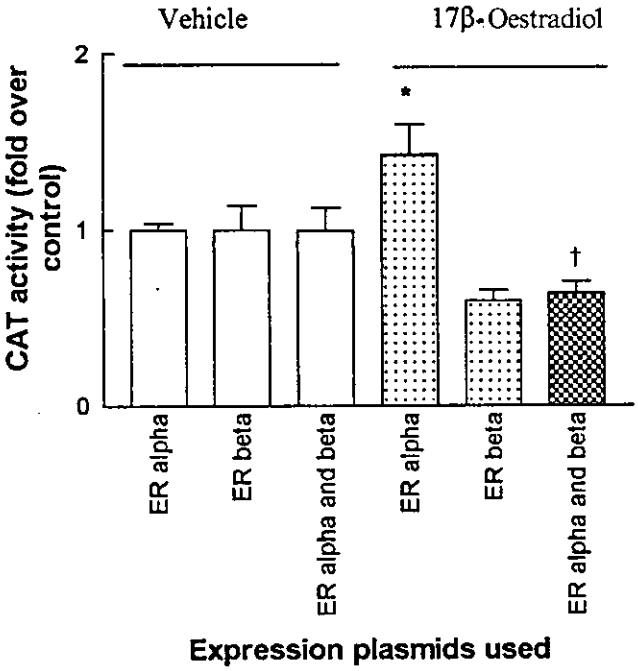

FIG. 2. Coexpression of oestrogen receptor (ER)β with ERα reduces ERα-mediated induction from the 3' NR2D enhancer. CV-1 cells were cotransfected with ERα alone, ERβ alone or with ERα and ERβ and 3' NR2D reporter construct as detailed in the Methods section. A day after transfection, the cells were treated with either ethanol or 10^{-7} M 17β-oestradiol (n=8 per treatment group). 48 h after hormone treatment, cells were lysed and chloramphenicol acetyl transferase (CAT) and β galactosidase (βgal) assays performed on every sample. The results are presented as mean±SEM and were analysed statistically using ANOVA followed by the Student–Newman–Keuls post-hoc test to compare between treatment groups. *P<0.01 compared to vehicle treatment of ERα transfected group. †P<0.001 compared to oestrogen-treated ERα group.

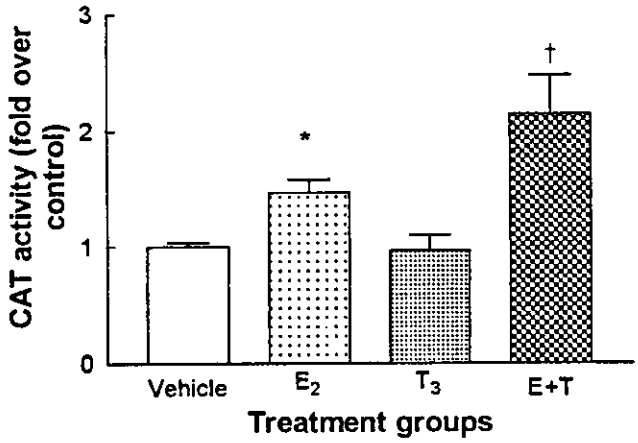

FIG. 3. Thyroid hormone receptor (TR)α1 increases the oestrogen receptor (ER)α-mediated induction from the 3' NR2D enhancer. CV-1 cells were cotransfected with 3' NR2D-CAT, pCDNA-rTRα1 and pSG-hERα as detailed in the Methods section. Oestrogen (E_2, 10^{-7} M), triiodothyronine (T_3, 10^{-6} M) or both (E+T) was added to the samples (n=8 per treatment group) for 48 h. Ethanol in equal amounts was added to a set of eight plates as the vehicle control. The cells were then lysed and chloramphenicol acetyl transferase (CAT) activity normalized to the β galactosidase (βgal) activity for every sample. The values are presented as mean±SEM from replicate experiments. The results (fold over vehicle control) were analysed using ANOVA followed by the Student–Newman–Keuls post-hoc comparison test between treatment groups. *P<0.05 compared to vehicle-treated group. †P<0.05 compared to oestrogen-treated group.

suggesting that DNA binding may be necessary for TRα1-mediated stimulation from this enhancer element.

Discussion

Oestrogen regulation of the NR2D gene has been proposed to be important in the regulation of sexual behaviour and in synaptic plasticity (13, 39). Oestrogen regulates dendritic spine density in CA1 pyramidal cells via an NMDA receptor dependent mechanism (40). However, the regulation of NR2D by the different ER isoforms remains poorly characterized.

In this study, we provide evidence that the NR2D gene is regulated principally by ERα. Long-term potentiation sensitivity peaks during proestrous in intact female rats at a time when excitatory synapse density is at its peak (41). NMDA receptor activation in the hippocampus CA1 layer aids excitatory neurotransmission (42) and hence, synaptogenesis (40, 43). Also, ERα and ERβ mRNA has been detected by *in situ* hybridization in adult CA1 cells (20, 21, 44) and ERα protein detected in the inhibitory interneurones in the hippocampus (43). In the adult rat, NR2D is expressed to

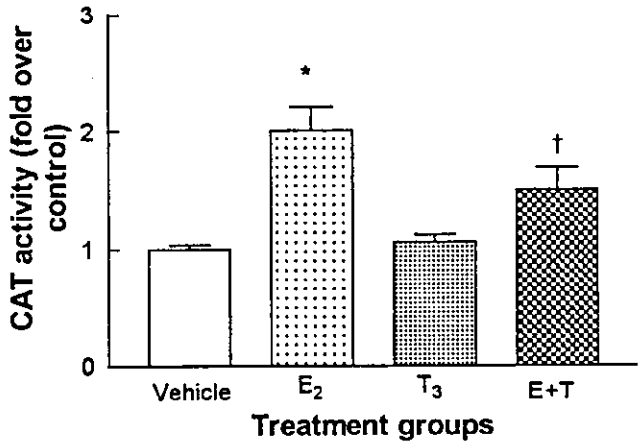

FIG. 4. Expression of thyroid hormone receptor (TR)β1 inhibits oestrogen receptor (ER)α-mediated induction from the 3' NR2D enhancer. CV-1 cells were cotransfected with 3' NR2D-CAT, pCDNA-rTRβ1 and pSG-hERα as detailed in the Methods section. Oestrogen (E_2, 10^{-7} M), triiodothyronine (T_3, 10^{-6} M) or both (E+T) was added to the samples (n=8 per treatment group) for 48 h. The cells were then lysed and chloramphenicol acetyl transferase (CAT) activity normalized to the β galactosidase (βgal) activity for every sample. The results (fold over vehicle control) are the mean±SEM from replicate experiments. They were analysed using ANOVA followed by the Student–Newman–Keuls post-hoc comparison test between treatment groups. *P<0.001 compared to vehicle-treated group. †P<0.05 compared to oestrogen-treated group.

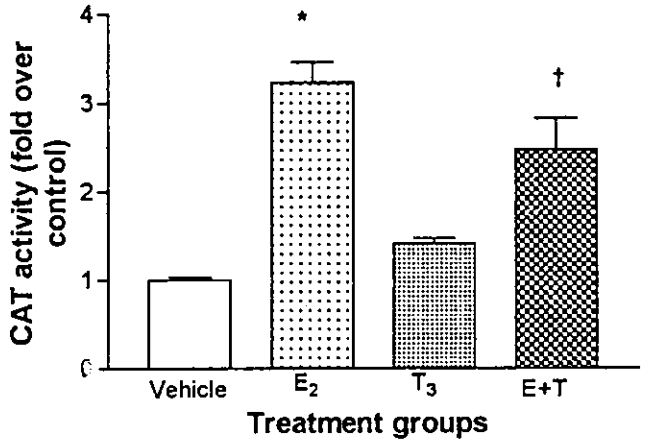

FIG. 5. A DNA binding mutant, TR P box mutant, is unable to stimulate oestrogen receptor (ER)α-mediated induction from the 3′ NR2D enhancer. CV-1 cells were cotransfected with 3′ NR2D-CAT, pSG-hERα and a non DNA binding mutant of the rat TRα1 (TR P box mutant). After hormone treatment for 48 h, β galactosidase (βgal) and chloramphenicol acetyl transferase (CAT) assays were carried out on every sample in all treatment groups (n = 4 per treatment group). The CAT activity was normalized to βgal activity for every sample. The values are presented as mean ± SEM. The results (fold over vehicle control) were analysed using ANOVA followed by Student–Newman–Keuls post-hoc test to compare between groups. *$P < 0.001$ compared to vehicle-treated group. †$P < 0.05$ compared to oestrogen-treated group.

a moderate level in these interneurones (18). Therefore, regulation of NR2D by oestrogen via ERα may provide a mechanism for improved synapse formation during proestrous in rodents.

Position dependence of the enhancer in the NR2D gene promoter

In a previous study using both 3′ and 5′-ERE constructs in the MCF-7 cell line, 17β-oestradiol could upregulate the NR2D gene no matter the position of the enhancer (45). However, the role of ERα and ERβ remained unclear since the MCF-7 cell line possesses both ERα and ERβ. In this study in the CV-1 cell line, the ERα regulates the NR2D gene only via the physiological 3′-ERE. This shows position dependence of the enhancer element and demonstrates that this cell line is a good model system to study oestrogen actions. The noninducibility of the 3′ construct by ERβ under similar conditions to ERα is not due to unequal amounts of ERα and ERβ proteins. Using the ^{3}H-oestradiol binding technique and this transfection model, we have shown previously that both ERα and ERβ proteins are equivalently expressed from these plasmids in CV-1 cells (30). The ability of ERβ to induce gene expression in response to 10^{-7} M 17β-oestradiol under the same transfection conditions has also been previously demonstrated on the physiological PPE promoter containing two putative EREs where the fold transactivation obtained with ERβ is equivalent to ERα, demonstrating that ERβ is functionally active as a transactivator in this cell line (30). However, on promoters and in cell lines which require both AF-1 and AF-2 activity, ERβ appears to be a poorer transcriptional activator than ERα (46). Therefore, ERβ induction of gene promoters containing EREs shows promoter specificity.

TR modulation of ERα-mediated induction from the 3′ NR2D gene

Since the consensus hormone response elements bound by the TR and ER are similar, competition for the ERE by the TR isoforms with consequent inhibition of ER action has been shown in previous studies (47). Indeed, the TRα1 is an inhibitor of ERα-mediated transactivation of the physiological PPE (30), OTR (32) and simple consensus ERE (37) promoters in the CV-1 cell line. In all these promoters, the ERE functions as a 5′ gene enhancer. This is the first study that reports TR modulation via a 3′-ERE.

In contrast, with the 3′ NR2D construct, surprisingly, TRα1 stimulates the ERα-mediated transactivation to a small but reproducible extent. It is possible that the position of the ERE enhancer (in this case, in the 3′ UTR) may influence TRα1 modulation. Is DNA binding necessary for such stimulation? The TR P box mutant is an artificial TRα1 mutant with a deletion of three amino acids in the conserved DNA binding domain. This leads to a loss of DNA binding ability (35). The use of the TR P box mutant shows that DNA binding is critical in the ability of the TRα1 isoform to stimulate transcription. This is in contrast to the PPE (30) and OTR (32) promoters where the loss of DNA binding ability does not result in loss of TRα1-mediated inhibition. The TRβ1 isoform inhibited the ERα-mediated induction from this 3′ ERE. This shows that the interaction of the TRα1 and TRβ1 isoform with the ERα isoform on the NR2D enhancer is not equivalent.

Isoform specificity: ex-vivo *and* in-vivo *studies*

The *ex-vivo* data clearly show that the different TR isoforms interact differently with the ERα isoform and suggest that the TRα and TRβ isoforms may have unique physiological roles. The TRα and TRβ isoforms also play distinct roles in the facilitation of lordosis in female mice. Deletion of the TRα1 isoform resulted in decreased lordosis behaviour in female mice while loss of the TRβ isoforms resulted in increased lordosis (48). The data obtained from both cell culture and knockout mice studies therefore reinforce the idea that TR isoforms have distinct roles.

Colocalization studies show that, at a rostral level in the VMH and Arc, 57% and 68% of the neurones which possess ERα mRNA also possess NMDA-2D, respectively. In the amygdala, 52% and 45% of ERα positive cells expressed NMDA-2D receptor mRNA in the cortical and medial nuclei, respectively (49). Therefore, the data obtained in such *ex-vivo* cell culture studies may mirror ERα upregulation of NR2D mRNA in the amygdala and VMH *in vivo*. A caveat to these studies is that it is not clear if the upregulation of a single NR2 subunit would allow for an increase in glutaminergic transmission and higher lordosis. Further studies on oestrogen regulation of other NR2 subunits would be neccessary.

Role of differential crosstalk in neuroendocrine integration

Thyroid hormone elevation has been shown to have an adverse effect of reproduction in several species, such as starlings (50, 51), sheep (52–56) and rodents (57, 58). Administration of thyroxine to ovariectomized oestrogen-treated rats (58) and mice (57) reduces lordosis compared to ovariectomized rodents that received oestrogen alone. Female ovariectomized and oestrogen-treated mice deleted for TRβ isoforms demonstrated higher lordosis than the βTRWT, suggesting that TRβ may exert an inhibitory influence on ER controlled reproduction (48).

Downstream ER target genes, such as OT, OTR and PPE and NR2D, might comprise the causal routes through which such inhibition could be mediated (31, 59). In fact, the inhibition of ERα-mediated induction of NR2D by TRβ1 could be one of the factors that results in the lower lordosis behaviour of the female βTRWT mice. The existence of several isoforms of a single gene therefore could provide for different transcriptional outcomes which, in turn, subserve neuroendocrine integration and homeostasis.

Acknowledgements

The authors thank Dr Noriyuki Koibuchi and Dr Bill Chin for the kind gift of the TR expression vectors. We thank Dr Pierre Chambon for the pSG-hERα construct and Dr Enmark for the ERβ construct. We also thank Ms Jenny Schroeder and Ms Elena Won for technical assistance. This work was supported by HD-05751 to Donald Pfaff.

Accepted 22 July 2002

References

1 Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schultz G, Umeseno K, Blumber B, Kastner P, Mark M, Chambon P, Evans RM. Overview: the nuclear receptor superfamily: the second decade. *Cell* 1995; **83:** 835–840.

2 Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988; **240:** 889–895.

3 Pfaff DW. *Estrogens and Brain Function: Neural Analysis of a Hormone Controlled Mammalian Reproductive Behavior.* New York: Springer Verlag, 1980.

4 Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and non-reproductive functions? *Biochem Biophys Res Comms* 2000; **270:** 1–10.

5 Oppenheimer JH, Schwartz HL. Molecular basis of thyroid hormone-dependent brain development. *Endocrine Rev* 1997; **18:** 462–475.

6 Knobil E, Neill J. *The Physiology of Reproduction*, 2nd edn. New York: Raven Press Ltd, 1994.

7 Knobil E, Neill J. *Encyclopedia of Reproduction.* San Diego: Academic Press, 1999.

8 Pfaff DW, McCarthy M, Schwartz-Giblin S, Kow LM. Female reproductive behavior. In: Knobil E, Neill J, eds. *The Physiology of Reproduction.* New York: Raven Press Ltd, 1994: 107–220.

9 Pfaff D. Lordosis. In: Knobil E, Neill J, eds. *Encyclopedia of Reproduction.* San Diego: Academic Press, 1999: 1074–1075.

10 Oppenheimer JH, Schwartz HL, Strait KA. An integrated view of thyroid hormone actions in vivo. In: Weintraub B, ed. *Molecular Endocrinology: Basic Concepts and Clincal Correlations.* New York: Raven Press Ltd, 1995: 249–268.

11 Delange FM, Ermans AM. Endemic goiter and cretinism. In: Hershman JM, Bray GA, eds. *The Thyroid: Physiology and Treatment of Disease.* New York: Pergamon Press, 1979: 417–451.

12 Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 1990; **13:** 171–182.

13 Nakanishi S, Masu M. Molecular diversity and functions of glutamate receptors. *Annu Rev Biophys Biomol Struct* 1994; **23:** 319–348.

14 Katagiri H, Tanaka K, Manabe T. Requirement of appropriate glutamate concentrations in the synaptic cleft for hippocampal LTP induction. *Eur J Neurosci* 2001; **14:** 547–553.

15 Zakharenko SS, Zablow L, Siegelbaum SA. Visualization of changes in presynaptic function during long-term synaptic plasticity. *Nature Neurosci* 2001; **4:** 711–717.

16 Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J Biol Chem* 1993; **268:** 2836–2843.

17 Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M. Molecular diversity of the NMDA receptor channel. *Nature* 1992; **358:** 36–41.

18 Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 1994; **12:** 529–540.

19 Laflamme N, Nappi RE, Drolet G, Labrie C, Rivest S. Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. *J Neurobiol* 1998; **36:** 357–378.

20 Shughrue P, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol* 1997; **388:** 507–525.

21 Simerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *J Comp Neurol* 1990; **294:** 76–95.

22 Watanabe T, Inoue S, Hiroi H, Orimo A, Muramatsu M. NMDA receptor type 2D gene as target for estrogen receptor in the brain. *Brain Res Mol Brain Res* 1999; **63:** 375–379.

23 Hsu C, Lee JN, Ho ML, Cheng BH, Li PH, Yu JY. The facilitatory effect of N-methyl-D-aspartate on sexual receptivity in female rats through GnRH release. *Acta Endocrinol* 1993; **128:** 385–388.

24 Brann DW, Mahesh VB. Excitatory amino acid regulation of gonadotropin secretion: modulation by steroid hormones. *J Steroid Biochem Mol Biol* 1992; **41:** 847–850.

25 Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* 1997; **11:** 353–365.

26 Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; **93:** 5925–5930.

27 Lazar M. Thyroid hormone receptors: multiple forms, multiple possiblilites. *Endocrine Rev* 1993; **14:** 184–193.

28 Zhang J, Lazar MA. The mechanism of action of thyroid hormones. *Annu Rev Physiol* 2000; **62:** 439–466.

29 Chin WW. Nuclear thyroid hormone receptors. In: Parker M, ed. *Nuclear Hormone Receptors.* New York: Academic Press, 1991: 79–102.

30 Vasudevan N, Zhu YS, Daniel S, Koibuchi N, Chin WW, Pfaff DW. Crosstalk between estrogen receptors and thyroid hormone receptor isoforms results in differential regulation of the preproenkephalin gene. *J Neuroendocrinol* 2001; **13:** 229–790.

31 Zhu Y, Dellovade T, Pfaff D. Interactions between hormonal and environmental signals on hypothalamic neurons: molecular mechanisms signaling environmental events. *Trends Endocrinol Metab* 1997; **8:** 111–115.

32 Vasudevan N, Davidkova G, Zhu YS, Koibuchi N, Chin WW, Pfaff DW. Differential interaction of estrogen receptor and thyroid hormone receptor isoforms on the rat oxytocin receptor promoter leads to differences in transcriptional regulation. *Neuroendocrinology* 2001; **74:** 309–324.

33 Zhu YS, Yen PM, Chin WW, Pfaff DW. Estrogen and thyroid hormone interaction on regulation of gene expression. *Proc Natl Acad Sci USA* 1996; **93:** 12587–12592.

34 Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997; **277:** 1508–1510.

35 Yen PM, Ikeda M, Wilcox EC, Brubaker JH, Spanjaard RA, Sugawara A, Chin WW. Half-site arrangement of hybrid glucocorticoid and thyroid hormone response elements specifies thyroid hormone

receptor complex binding to DNA and transcriptional activity. *J Biol Chem* 1994; **269:** 12704–12709.
36 Zhu XG, McPhie P, Lin KH, Cheng SY. The differential hormone dependent transcriptional activation of thyroid hormone receptor isoforms is mediated by interplay of their domains. *J Biol Chem* 1997; **272:** 9048–9054.
37 Vasudevan N, Koibuchi N, Chin WW, Pfaff DW. Differential crosstalk between estrogen receptor (ER) alpha and ER beta and the thyroid hormone receptor isoforms results in flexible regulation of the consensus ERE. *Mol Brain Res* 2001; **95:** 9–17.
38 Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999; **140:** 5566–5578.
39 Chung S, Pfaff D, Cohen R. Estrogen-inducted alterations in synaptic morphology in the midbrain central gray. *Exp Brain Res* 1988; **69:** 522–530.
40 Woolley CS, McEwen BS. Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. *J Neurosci* 1994; **14:** 7680–7687.
41 Warren SG, Humphreys AG, Juraska JM, Greenough WT. LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. *Brain Res* 1995; **703:** 26–30.
42 Woolley CS, Weiland NG, McEwen BS, Schwartzkroin PA. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. *J Neurosci* 1997; **17:** 1848–1859.
43 McEwen BS, Akama K, Alves S, Brake WG, Bulloch K, Lee S, Li C, Yen G, Milner T. Tracking the estrogen receptor in neurons: implications for estrogen induced synapse formation. *Proc Natl Acad Sci USA* 2001; **98:** 7093–7100.
44 Shughrue P, Scrimo P, Lane M, Askew R, Merchenthaler I. The distribution of estrogen receptor-beta mRNA in forebrain regions of the estrogen receptor-alpha knockout mouse. *Endocrinology* 1997; **138:** 5649–5652.
45 Watanabe T, Inoue S, Hiroi H, Orimo A. NMDA receptor type 2D gene as target for estrogen receptor in the brain. *Brain Res Mol Brain Res* 1999; **63:** 375–379.
46 Cowley SM, Parker MG. A comparison of the transcriptional activation by ER alpha and ER beta. *J Steroid Biochem Mol Biol* 1999; **69:** 165–175.
47 Glass CK, Holloway J, Devary OV, Rosenfeld MG. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* 1988; **54:** 313–323.
48 Dellovade TL, Chan J, Vennstrom B, Forrest D, Pfaff DW. The two thyroid hormone receptor genes have opposite effects on estrogen stimulated sex behaviors. *Nature Neurosci* 2000; **3:** 472–475.
49 Kia HK, Yen G, Krebs CJ, Pfaff DW. Colocalization of ER alpha and NMDA-2D mRNAs in amygdaloid and hypothalamic nuclei of the mouse brain. *Brain Res Mol Brain Res* 2002; **104:** 47–54.
50 Goldsmith AR, Nichols TJ. Thyroidectomy prevents the development of photorefractoriness and the associated rise in plasma prolactin in starlings. *Gen Comp Endocrinol* 1984; **54:** 256–263.
51 Goldsmith AR, Nichols TJ. Thyroxine induces photorefractoriness and stimulates prolactin secretion in European starlings (*Sturnus vulgaris*). *J Endocrinol* 1984; **101:** 1–3.
52 Webster JR, Moenter SM, Woodfill CJI, Karsch JF. Role of the thyroid gland in seasonal reproduction. II. Thyroxine allows a season-specific suppression of gonadotropin secretion in sheep. *Endocrinology* 1991; **129:** 176–183.
53 Webster JR, Moenter SJ, Barrell GK, Lehman MN, Karsch JF. Role of the thyroid gland in seasonal reproduction. III. Thyroidectomy blocks seasonal suppression of gonadotropin-releasing hormone secretion in sheep. *Endocrinology* 1991; **129:** 1635–1643.
54 Moenter SM, Woodfill CJI, Karsch FJ. Role of the thyroid gland in seasonal reproduction: thyroidectomy blocks seasonal suppression of reproductive neuroendocrine activity in ewes. *Endocrinology* 1991; **128:** 1337–1344.
55 Dahl GE, Evans NP, Thrun LA, Karsch FJ. Thyroxine is permissive to seasonal transitions in reproductive neuroendocrine activity in the ewe. *Biol Reprod* 1995; **52:** 690–696.
56 Thrun LA, Dahl GE, Evans NP, Karsch FJ. A critical period for thyroid hormone action on seasonal changes in reproductive neuroendocrine function in the ewe. *Endocrinology* 1997; **138:** 3402–3409.
57 Morgan MA, Dellovade TL, Pfaff DW. Effect of thyroid hormone administration on estrogen-induced sex behavior in female mice. *Horm Behav* 2000; **37:** 15–22.
58 Dellovade TL, Zhu YS, Krey L, Pfaff DW. Thyroid hormone and estrogen interact to regulate behavior. *Proc Natl Acad Sci USA* 1996; **93:** 12581–12586.
59 Pfaff DW, Kow LM, Zhu YS, Scott REM, Wu-Peng SX, Dellovade T. Hypothalamic cellular and molecular mechanisms helping to satisfy axiomatic requirements for reproduction. *J Neuroendocrinol* 1996; **8:** 325–336.

Geriatrics and Gerontology International 2002; **02:** *138–142*

ORIGINAL ARTICLE

Association of amino acid variation (Trp64Arg) in the beta3-adrenergic receptor gene with bone mineral density

Sumito Ogawa,[1] Mitsuru Emi,[2] Masataka Shiraki,[3] Takayuki Hosoi,[1,4] Hajime Orimo,[4] Yasuyoshi Ouchi[1] and Satoshi Inoue[1,5,6]

[1]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, [2]Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, Kawasaki, [3]Research Institute and Practice for Involutional Diseases, Nagano, [4]Tokyo Metropolitan Geriatric Hospital, Tokyo, [5]Research Center for Genomic Medicine, Saitama Medical School, Saitama, and [6]CREST, Japan Science and Technology Corporation, Saitama, Japan

Background: Recent studies have revealed that a mis-sense mutation replacing tryptophan with arginine at codon 64 (Trp64Arg) of the beta3-adrenergic receptor (*β3-AR*) gene was associated with insulin resistance in non-diabetic subjects and to earlier onset of non-insulin dependent diabetes mellitus. To analyze a possible involvement of *β3-AR* in bone metabolism, we investigated the association between bone mineral density (BMD) and the Trp64Arg polymorphism.

Methods: A large cohort of Japanese postmenopausal women comprised the study population. The genotypic frequencies in this cohort were Trp/Trp, 61.8%; Trp/Arg, 33.2%; and Arg/Arg, 5.0%.

Results: When the subjects were separated into two groups, one bearing at least one Trp allele at codon 64 (Trp/Trp and Trp/Arg) and the other with none (Arg/Arg), the former subjects had significantly higher Z scores for total-body BMD (mean±SD, 0.432±0.93 versus −0.135±0.93; $P=0.033$).

Conclusions: These data suggested an association between this single-nucleotide polymorphism (SNP) in the *β3-AR* gene and BMD, and therefore a possible involvement of the Arg allele (or the absence of Trp64) in postmenopausal osteoporosis among Japanese women.

Keywords: beta3-adrenergic receptor, genetics, osteoporosis, polymorphism.

Introduction

The beta3-adrenergic receptor (β3-AR), a G-protein linked receptor with seven membrane-spanning domains,[1] is expressed predominantly in brown adipose tissue in neonates and in visceral adipose tissue in adults.[2] Cumulative evidence indicates that β3-AR mediates adrenergic regulation of metabolism of adipose tissues, playing important roles in the control of lipolysis and thermogenesis. Dysfunction of β3-AR may cause obesity and insulin resistance, a hypothesis supported on the basis of phenotypes of knockout mice.[3] In humans, a Trp64Arg mis-sense mutation in the first exon has been associated with increased body mass index,[4] insulin resistance[5] and earlier onset of type II diabetes mellitus.[6] Adrenergic receptors are present in osteoblastic cells, and pharmacological activation of β-AR has caused bone resorption in an organ-culture system.[7] Moreover, β-adrenergic stimulation induces expression of osteoclast differentiation factor (ODF) in osteoblastic cells in a process mediated by β-AR.[8]

Accepted for publication 24 July 2002.

Correspondence: Mitsuru Emi, MD, PhD, Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1–396 Kosugi-cho, Nakahara-ku, Kawasaki 211–8533, Japan. Email: memi@nms.ac.jp.

Osteoporosis is characterized by low bone-mineral density (BMD) and by deterioration of the microarchitecture of bone tissue with a consequent increase in fragility and susceptibility to fracture.[9] Bone-mineral density, the most important predictor of fracture, is determined by genetic as well as environmental factors. The importance of genetic factors has been well supported from twin[10-12] and family studies.[13,14] People carrying genetic risk are considered more susceptible to life-style factors. Therefore genetic risk factors need to be clarified for adequate diagnosis, prevention and early treatment of osteoporosis and, from a scientific point of view, if the relevant genes were identified the pathogenesis of osteoporosis could be explained by variations in those genes or in loci adjacent to them.

After an association of BMD with vitamin D receptor (VDR) genotypes was reported,[15] polymorphisms in several other genes were investigated as potential risk factors.[16] These genes include the estrogen receptor (*ER*),[17,18] collagen type Ia1 (*COLIA1*),[19] and parathyroid hormone (*PTH*).[20] Considering the polygenic nature of BMD distribution and the multiplicity of endocrine factors known to regulate bone mass and bone turnover, it is important that the panel of candidate genes be expanded, to elucidate the whole genetic background of osteoporosis.

For the present study we chose the *β3-AR* gene as a candidate genetic marker for osteoporosis, and investigated a possible association between the Trp64Arg polymorphism in this gene and BMD in postmenopausal Japanese women.

Materials and methods

Subjects

Genotypes were analyzed in DNA samples obtained from 280 healthy postmenopausal Japanese women (mean age ± SD; 65.4 ± 8.9 years) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin), or unusual gynecologic history. Diagnostic criteria of diabetes mellitus for exclusion was as follows: (i) past history of diabetes mellitus; (ii) medication of antidiabetic drugs; (iii) fasting venous plasma blood sugar levels above 110 mg/dL (see online at http://www.jds.or.jp/shindankijyun/tables/Table3_e.html). All were non-related volunteers and provided informed consent before the study.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total-body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (AL-P), intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), 1,25 $(OH)_2D_3$, parathyroid hormone (PTH), and calcitonin (CT). We also measured the urinary calcium/creatinine and phosphate/creatinine ratios, pyridinoline (Pyr, HPLC method) and deoxypyridinoline (Dpyr, HPLC method). The BMD data were recorded as 'Z scores'; that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Polymerase chain reaction-restriction fragment length polymorphism

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) experiments were performed as previously described[18] using oligonucleotide PCR primers designed to amplify part of exon 1 of *β3-AR*. The reaction was carried out in a final volume of 25 μL containing 100 ng of genomic DNA obtained from peripheral white blood cells, 10 pmol of each primer (primer 1: 5′ – CGC CCA ATA CCG CCA ACA – 3′; primer 2: 5′ – CCA CCA GGA GTC CCA TCA – 3′), 200 mmol/L each dNTP, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.001% gelatin and 0.1 U Taq DNA polymerase (Takara, Kyoto, Japan). Each PCR was performed in 30 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C. The PCR product was electrophoresed in a 2% agarose gel to verify the reaction; then the amplified product was digested with *Bst*NI and electrophoresed in a 3.0% agarose gel. DNA was visualized by staining with ethidium bromide.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two alleles of the Trp genotype and the group with only Arg encoded at that locus were subjected to non-parametric analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied the Student's *t*-test, using the InStat 3 software package for Windows (GraphPad software, San Diego, CA). Bonferroni's correction was proposed for use in case-control association studies using normal and case populations in which allelic association derives from linkage disequilibrium between alleles of certain loci and affected state (i.e. when disease-bearing chromosomes are descended from one or few ancestral chromosomes). In allelic asso-

ciation studies, Bonferroni's school considers that this correction would avoid type-1 errors (false positives). However, most other statisticians believe that this correction is too stringent, because when each hypothesis is tested independently, there is no reason to change the significance level according to the number of hypotheses tested in a single study. For instance, in a genome-wide scan for allelic association using 300 markers, only extraordinarily strong associations would be taken as significant when the Bonferroni correction is applied (i.e. $P<0.00017$, instead of the ordinary level of $P<0.05$). In other words, the more loci being analyzed in an association study, the more difficult it would be to obtain significant results under such a hypothesis.[21]

Results

We used the PCR–RFLP method to detect the Trp → Arg substitution in exon 1 of the *β3-AR* gene in DNA from Japanese women (Fig. 1). As predicted, digestion of the 161-bp PCR product with *Bst*-NI produced three distinct patterns. A single 161-bp product was derived from Arg/Arg homozygotes (lane 2); 161-, 99- and 62-bp products were derived from Trp/Arg heterozygotes (lane 3); and 99- and 62-bp products were derived from Trp/Trp homozygotes (lane 4).

Among the 280 healthy postmenopausal volunteers who were not taking any medication, 178 were Trp/Trp homozygotes, 89 were Trp/Arg heterozygotes, and 13 were Arg/Arg homozygotes. Allelic frequencies were

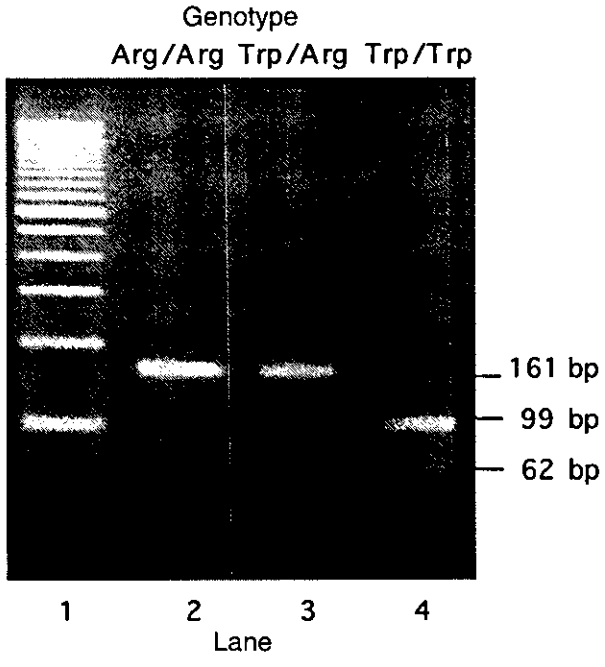

Figure 1 Electrophoresis of PCR products revealing polymorphism in *β3-AR*, after digestion of DNA with *Bst*NI. In recombinant DNA samples, a single 161-bp product was derived from Arg/Arg homozygotes (lane 2); 161-, 99-, and 62-bp products were derived from Trp/Arg heterozygotes (lane 3); and 99- and 62-bp products were derived from Trp/Trp homozygotes (lane 4). Lane 1, molecular-weight marker.

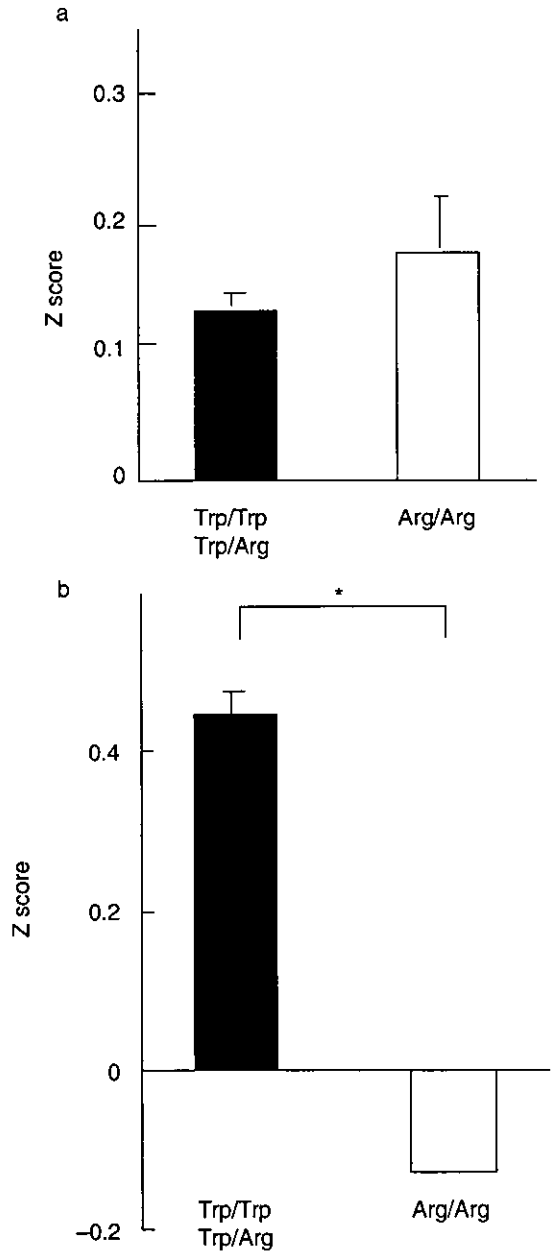

Figure 2 Z scores for (a) lumbar-spine bone mass density and (b) total-body bone mass density in postmenopausal women with each genotype of the *β3-AR* restriction fragment length polymorphism. (■) Women with genotypes having one or two Trp alleles (Trp/Trp and Trp/Arg), $n=267$; (□) women with Arg/Arg genotypes, $n=13$. Values are expressed as mean ± SE. $*P<0.05$.

0.795 for the wild-type Trp allele and 0.205 for the Arg allele in this test population.

We compared Z scores for BMD of lumbar spine and total body between subjects bearing at least one Trp allele (Trp/Trp + Trp/Arg) and subjects with no Trp alleles (Arg/Arg). Z scores for lumbar BMD between the groups with and without Trp alleles showed no statistically significant difference (Fig. 2a). In contrast, Z scores for total-body BMD in the Arg/Arg homozygote group were significantly lower than in women carrying at least one Trp allele (Fig. 2b). As shown in Table 1, there were no differences in the background data from one group to the other; that is, mean age, height, weight, years since menopause, and biochemical markers (AL-P, I-OC, Pyr, and Dpyr). Thus, apart from whole-body BMD, we found no statistically significant association between the *β3-AR* polymorphism and any other profile.

Discussion

This study is the first to investigate the influence of a genetic variation of the *β3-AR* gene on bone-mineral properties. The allelic frequencies of the Trp → Arg substitution in exon 1 in Japanese postmenopausal women were in Hardy–Weinberg equilibrium. We demonstrated that the women who carried two alleles of the silent transition to Arg at that locus showed lower total-body BMD. Lowered BMD in postmenopausal women can reflect abnormally rapid bone loss and/or lower peak bone mass (bone mass of the young adult). The data presented here showed no association between markers of bone metabolism and the *β3-AR* polymorphism, suggesting that the effects of this locus on BMD may be related to metabolism of bone in an earlier period of life, for example in the growing phase. Studies in different age groups could assist our understanding the mechanism involved.

In the present study, significant correlation was observed between total body BMD and the allelic status at the *β3-AR* locus; however, such correlation was not found with lumbar spine BMD. It is known that lumber spine BMD mainly reflects cancerous bone volume, whereas total bone volume reflects both cortical bone thickness and cancerous bone volume equally. The present data implies that the *β3-AR* gene variation might affect mainly cortical bone thickness and, to a lesser extent, cancerous bone volume. Alternatively, site-specific effects of the gene variation might influence

Table 1 Comparison of background and biochemical data for all subjects in the two genotypic groups

Items	Genotype (mean ± SD)		*P*-value
	Trp/Trp + Trp/Arg	Arg/Arg	
No. subjects	267	13	–
Age (years)	65.4 ± 8.8	66.2 ± 11.0	NS
Height (kg)	150.5 ± 6.4	148.4 ± 6.8	NS
Body weight (kg)	50.9 ± 8.1	47.7 ± 11.0	NS
Time since menopause (years)	15.6 ± 9.6	18.6 ± 9.7	NS
Lumber spine BMD (Z score)	0.145 ± 1.36	0.179 ± 2.11	NS
Total body BMD (Z score)	0.432 ± 0.93	−0.135 ± 0.93	0.033
Ca (mg/dL)	9.14 ± 0.39	8.99 ± 0.42	NS
P (mgtdl)	3.51 ± 0.43	3.35 ± 0.50	NS
AL-P (IU/1)	173.7 ± 56.2	166.1 ± 64.1	NS
I-OC (ng/mL)	7.6 ± 3.7	6.7 ± 3.0	NS
PD (pmol/umol of Cr)	33.5 ± 10.5	32.2 ± 9.8	NS
DPD (pmol/umol of Cr)	7.28 ± 2.33	6.95 ± 1.69	NS
Intact PTH (pg/mL)	37.2 ± 13.6	41.0 ± 13.1	NS
Calcitonin (pg/mL)	22.5 ± 9.3	21.1 ± 9.1	NS
1,25 $(OH)_2D_3$ (pg/mL)	33.8 ± 12.4	30.8 ± 10.2	NS
TC (mg/dL)	195.7 ± 33.4	201.3 ± 37.2	NS
TG (mg/dL)	144.7 ± 80.3	126.5 ± 74.6	NS
% fat	32.1 ± 7.9	28.0 ± 11.0	NS
BMI	22.5 ± 3.2	21.2 ± 4.2	NS

BMD, bone mass density; Ca, calcium; P, phosphate; AL-P, alkaline phosphatase; I-OC, intact-osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant.
Statistical analysis was performed according to the method described in the text.

regulation of cancerous bone volume in regions other than vertebral bone.

The Trp64Arg mis-sense mutation in exon 1 of the *β3-AR* gene has already been associated with increased body-mass index[4] and insulin resistance[5] as well as earlier onset[6] and high prevalence[22] of type II diabetes mellitus. If the polymorphism contributes to the observed variability in body-mass index and insulin sensitivity, those factors in turn may influence bone mineralization. Moreover, recent studies have revealed that physiological responses of osteoblastic cells to adrenergic stimulation are mediated by β-AR as well as by α-AR;[8,23] the expression of ODF and its decoy receptor, osteoclastogenesis inhibitory factor (OCIF), are regulated by b-AR and a-AR, respectively.[8] It is possible that allelic variation at the (Trp64Arg) site in the *β3-AR* gene can disturb the physiological balance between ODF and OCIF expression, thus modifying bone-mineral density.

Acknowledgments

We thank Ms Koayashi M Kobayashi for technical assistance.

This work was partly supported by the 'Yokoyama' Foundation, Funds for Comprehensive Research on Aging and Health; the 'Gakujutsu-Frontier' program; a grant for Strategic Research from the Ministry of Education, Science, Sports and Culture of Japan; by a Research Grant for Research from the Ministry of Health and Welfare of Japan; and by a Research for the Future Program Grant of The Japan Society for the Promotion of Science.

References

1 Emorine LJ, Marullo S, Briend-Sutren MM *et al.* Molecular characterization of the human beta 3-adrenergic receptor. *Science* 1989; **245**: 1118–1121.
2 Krief S, Lonnqvist F, Raimbault S *et al.* Tissue distribution of beta 3-adrenergic receptor mRNA in man. *J Clin Invest* 1993; **91**: 344–349.
3 Susulic VS, Frederich RC, Lawitts J *et al.* Targeted disruption of the beta 3-adrenergic receptor gene. *J Biol Chem* 1995; **270**: 29483–29492.
4 Clement K, Vaisse C, Manning BS *et al.* Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N Engl J Med* 1995; **333**: 352–354.
5 Widen E, Lehto M, Kanninen T, Walston J, Shuldiner AR, Groop LC. Association of a polymorphism in the beta 3-adrenergic-receptor gene with features of the insulin resistance syndrome in Finns. *N Engl J Med* 1995; **333**: 348–351.
6 Walston J, Silver K, Bogardus C *et al.* Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the beta3-adrenergic receptor gene. *N Engl J Med* 1995; **333**: 343–347.
7 Moore RE, Smith 2nd CK, Bailey CS, Voelkel EF, Tashjian Jr AH. Characterization of beta-adrenergic receptors on rat and human osteoblast-like cells and demonstration that beta-receptor agonists can stimulate bone resorption in organ culture. *Bone Miner* 1993; **23**: 301–315.
8 Takeuchi T, Tsuboi T, Arai M, Togari A. Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differentiation factor in MC3T3-E1 osteoblast-like cells. *Biochemistry* 2001; **61**: 579–586.
9 Kanis JA, Melton 3rd LJ, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res* 1994; **9**: 1137–1141.
10 Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Eberl S. Genetic determinants of bone mass in adults. A twin study. *J Clin Invest* 1987; **80**: 706–710.
11 Slemenda CW, Christian JC, Williams CJ, Norton JA, Johnston Jr CC. Genetic determinants of bone mass in adult women: a reevaluation of the twin model and the potential importance of gene interaction on heritability estimates. *J Bone Miner Res* 1991; **6**: 561–567.
12 Kelly PJ, Nguyen T, Hopper J, Pocock N, Sambrook P, Eisman J. Changes in axial bone density with age: a twin study. *J Bone Miner Res* 1993; **8**: 11–17.
13 Seeman E, Hopper JL, Bach LA *et al.* Reduced bone mass in daughters of women with osteoporosis. *N Engl J Med* 1989; **320**: 554–558.
14 Krall EA, Dawson-Hughes B. Heritable and life-style determinants of bone mineral density. *J Bone Miner Res* 1993; **8**: 1–9.
15 Morrison NA, Qi JC, Tokita A *et al.* Prediction of bone density from vitamin D receptor alleles. *Nature* 1994; **367**: 284–287.
16 Nelson DA, Kleerekoper M. The search for the osteoporosis gene. *J Clin Endocrinol Metab* 1997; **82**: 989–990.
17 Sano M, Inoue S, Hosoi T *et al.* Association of estrogen receptor dinucleotide repeat polymorphism with osteoporosis. *Biochem Biophys Res Commun* 1995; **217**: 378–383.
18 Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H. Association of bone mineral density with polymorphism of the estrogen receptor gene. *J Bone Miner Res* 1996; **11**: 306–311.
19 Uitterlinden AG, Burger H, Huang Q *et al.* Relation of alleles of the collagen type Ialpha1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. *N Engl J Med* 1998; **338**: 1016–1021.
20 Hosoi T, Miyao M, Inoue S *et al.* Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women. *Calcif Tissue Int* 1999; **64**: 205–208.
21 Hulley S, Cummings S (Eds). *Designing Clinical Research: an Epidemiological Approach.* Baltimore, MD: Williams & Wilkins, 1988.
22 Fujisawa T, Ikegami H, Yamato E *et al.* Association of Trp64Arg mutation of the beta 3-adrenergic-receptor with NIDDM and body weight gain. *Diabetologia* 1996; **39**: 349–352.
23 Suzuki A, Palmer G, Bonjour JP, Caverzasio J. Catecholamines stimulate the proliferation and alkaline phosphatase activity of MC3T3-E1 osteoblast-like cells. *Bone* 1998; **23**: 197–203.

JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 10, 2002

Klotho Gene Polymorphisms Associated With Bone Density of Aged Postmenopausal Women

KEN-ICHI KAWANO,[1] NAOSHI OGATA,[1] MATHIAS CHIANO,[2] HELEN MOLLOY,[2] PATRICK KLEYN,[2] TIM D. SPECTOR,[3] MOTOYUKI UCHIDA,[1] TAKAYUKI HOSOI,[4] TAKAO SUZUKI,[4] HAJIME ORIMO,[4] SATOSHI INOUE,[5] YOICHI NABESHIMA,[6] KOZO NAKAMURA,[1] MAKOTO KURO-O,[7] and HIROSHI KAWAGUCHI[1]

ABSTRACT

Because mice deficient in *klotho* gene expression exhibit multiple aging phenotypes including osteopenia, we explored the possibility that the *klotho* gene may contribute to age-related bone loss in humans by examining the association between *klotho* gene polymorphisms and bone density in two genetically distinct racial populations: the white and the Japanese. Screening of single-nucleotide polymorphisms (SNPs) in the human *klotho* gene identified 11 polymorphisms, and three of them were common in both populations. Associations of the common SNPs with bone density were investigated in populations of 1187 white women and of 215 Japanese postmenopausal women. In the white population, one in the promoter region (G-395A, $p = 0.001$) and one in exon 4 (C1818T, $p = 0.010$) and their haplotypes ($p < 0.0001$) were significantly associated with bone density in aged postmenopausal women (≥65 years), but not in premenopausal or younger postmenopausal women. These associations were also seen in Japanese postmenopausal women. An electrophoretic mobility shift analysis revealed that the G–A substitution in the promoter region affected DNA-protein interaction in cultured human kidney 293 cells. These results indicate that the *klotho* gene may be involved in the pathophysiology of bone loss with aging in humans. (J Bone Miner Res 2002;17:1744–1751)

Key words: osteoporosis, aging, pathophysiology, genetics, association

INTRODUCTION

OSTEOPOROSIS IS a systemic bone disorder characterized by decreased bone density and disturbed skeletal architecture, which results in an increased risk for bone fractures with consecutively increased morbidity and mortality. Accumulating evidence has shown the involvement of genetic factors in the decrease of bone density.[1–3] Twin and sibling studies have revealed that 50–90% of the variation in bone density is accounted for by genetic factors.[4–9] In fact, some loci, such as the vitamin D and estrogen receptor genes, as well as the collagen type Iα1 gene, have been reported as promising genetic determinants of bone density.[10–15] However, this is controversial and the molecular basis of osteoporosis remains largely undefined.[16–21] Considering that the effect of each candidate gene is expected to be modest, discrepancies between allelic association studies may have arisen because different populations carry different genetic backgrounds.

We recently established a mouse model for human aging termed *klotho*.[22] The mouse was serendipitously generated by insertional mutation in a transgenic mouse, which dis-

The authors have no conflict of interest.

[1]Department of Orthopedic Surgery, University of Tokyo, Tokyo, Japan.
[2]Gemini Genomics plc, Cambridge, United Kingdom.
[3]Twin Research and Genetic Epidemiology Unit, St. Thomas' Hospital, London, United Kingdom.
[4]Tokyo Metropolitan Geriatric Center, Tokyo, Japan.
[5]Department of Geriatric Medicine, University of Tokyo, Tokyo, Japan.
[6]Department of Pathology and Tumor Biology, Kyoto University, Kyoto, Japan.
[7]Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA.

rupted the *klotho* gene encoding a novel single-pass membrane protein (KL protein). Function of the KL protein remains to be determined; however, this may be involved in the suppression of aging because a defect in *klotho* gene expression leads to multiple aging phenotypes and age-related disorders. These include such maladies as a shortened lifespan, arteriosclerosis, decreased spontaneous activity, infertility, skin atrophy, premature thymic involution, pulmonary emphysema, lipodystrophy, ectopic calcification, and osteopenia. Osteopenia observed in the *klotho*-deficient mouse is accompanied by low turnover during bone metabolism, in which a decrease in bone formation that exceeds a decrease in bone resorption results in a net bone loss.[23] Because this state resembles bone loss by aging in humans, osteopenia observed in the *klotho*-deficient mouse can be regarded as one of the manifestations of generalized aging.

A human homologue of the *klotho* gene was isolated and its gene structure was determined.[24] The human *klotho* gene is composed of five exons and ranges over 50 kb on chromosome 13q12. To examine a possible contribution of the *klotho* gene to the pathophysiology of osteoporosis in humans, this study screened for single-nucleotide polymorphisms (SNPs) in and around the coding regions of the human *klotho* gene that could modify KL protein expression or function and examined the association of these SNPs with bone density. To avoid influence of the difference in genetic backgrounds, we analyzed two genetically distant populations: white and Japanese women.

MATERIALS AND METHODS

SNP screening

For the screening of SNPs in the *klotho* gene, DNA samples were extracted from peripheral blood obtained with written informed consent from 16 unrelated white women taking part in Gemini Genomics clinical genetics programs and 115 unrelated Japanese subjects (56 men and 59 women) who visited the orthopedic clinic of Tokyo University Hospital. All exons (exons 1–5) with their flanking sequences and ~2.0 kb of the promoter region were directly sequenced with DNA sequencer (ABI PRISM 310; Perkin Elmer, Foster City, CA, USA) using 17 sets of primers (the information of primers and polymerase chain reaction [PCR] conditions are available on request). The allelic frequency of each SNP in the Japanese population was calculated based on the results obtained from this direct sequencing. To determine the allelic frequency of each SNP in the white population, 288 unrelated white female samples were analyzed further in several ways as follows. For the G-395A, G1110C, C1818T, and C2298T SNPs, Taqman allelic discrimination assays were used (see the following paragraphs for details). The G-959C, -744delA, and IVS 4+22A->T SNPs were analyzed by allele-specific PCR, and the G1204A SNP was analyzed by PCR restriction fragment length polymorphism (RFLP) by ApoI endonuclease (details available on request).

Association study

For the study on the white population, DNA samples were obtained from 1187 unrelated white women recruited nationwide from the United Kingdom via media campaigns, as part of the St. Thomas' UK Adult Twin Registry, and written informed consent was obtained before investigation. No participant had medical complications known to affect bone metabolism, and no participant was receiving therapy for osteoporosis. Genotyping was performed for the three common SNPs (G-395A, C1818T, and C2298T) by Taqman allelic discrimination assay using primers and probes as follows (the polymorphic base in each probe is underlined):

G-395A—forward primer, TAGGGCCCGGCAGGAT; reverse primer, CCTGGAGCGGCTTCGTC; FAM-labeled probe, CCCCAAGTCGGG<u>A</u>AAAGTTGGTC; TET-labeled probe, CCCCCAAGTCGGG<u>G</u>AAAGTTGGTC

C1818T—forward primer, GCCATCCAGCCCCAGATC; reverse primer, GGGCCCAGTCCAGGGA; FAM-labeled probe, TTTACTCCAGGAAATGCA<u>T</u>GTTACACATTTT; TET-labeled probe, TTACTCCAGGAAATGCA<u>C</u>GTTACACATTTT

C2298T—forward primer, CCTGCCCTTTCTCCCAAAA; reverse primer, AATCTCCAGAGCCGAAAATGG; FAM-labeled probe, CCAAAACTCTCTC<u>A</u>GCCACCTCTTTGT; TET-labeled probe, CCAAAACTCTCTC<u>G</u>GCCACCTCTT.

Primer and probe concentrations were optimized according to the manufacturer's recommendations so that each reaction contained 50 nM of FAM-labeled probe, 200 nM of TET-labeled probe for assays G-395A and C1818T, and 350 nM of TET-labeled probe for C2298T, 300 nM of reverse primer, and 50, 300, or 900 mM of forward primer for G-395A, C1818T, and C2298T assay, respectively. Taqman reactions were thermocycled as follows: 50°C for 2 minutes, 95°C for 10 minutes; 40 cycles of 95°C for 15 s followed by 60°C for 1 minute. The completed reactions were analyzed on an ABI Prism 7200 sequence detection platform (Perkin Elmer). Bone mineral density (BMD), g/cm^2) of the whole body was measured by DXA (QDR 4500/w; Hologic, Inc. Waltham, MA, USA). This parameter was also recorded as a Z score that is a deviation from the weight-adjusted average BMD of each age based on data installed in the densitometer.

For the study on the Japanese population, DNA samples were obtained from the peripheral blood of 215 Japanese postmenopausal women living in a rural area of Akita prefecture on the mainland of Japan. All were unrelated volunteers and gave their written informed consent before the study. The exclusion criteria were the same as those of the white population described previously. Genotyping for the three common SNPs was also performed by Taqman allelic discrimination. BMD and its Z score of the distal one-third of the radius were measured by DXA using a bone mineral analyzer (DTX-200; Osteometer Co., Ltd., Hoersholm, Denmark).

Electrophoretic mobility shift assay

Two hundred ninety-three cells established from a human primary embryonal kidney were confirmed to express the

TABLE 1. SNPs DETECTED IN THE *KLOTHO* GENE OF THE WHITE AND JAPANESE POPULATIONS

	Location	*Nucleotide change*	*Amino acid substitution*	*Allelic frequency*
White population	Promoter	−959 (G → C)	—	0.003
(n = 288)	Promoter	−744 (del A)	—	0.212
	Promoter	−395 (G → A)	—	0.196
	Exon 2	1110 (G → C)	Cys → Ser	0.154
	Exon 2	1204 (G → A)	Lys → Lys	0.170
	Exon 4	1818 (C → T)	His → His	0.411
	Exon 4	2298 (C → T)	Ala → Ala	0.132
	Intron 4	IVS 4 + 22 (A → T)	—	0.121
Japanese population	Promoter	−395 (G → A)	—	0.143
(n = 115)	Exon 1	44 (A → C)	Gly → Pro	0.025
	Exon 1	234 (C → G)	Ala → Gly	0.031
	Exon 3	1541 (C → T)	Ser → Ser	0.043
	Exon 4	1818 (C → T)	His → His	0.247
	Exon 4	2298 (C → T)	Ala → Ala	0.270

Allelic frequency indicates the frequency of the minor allele in each SNP.

klotho transcript by reverse-transcription (RT)–PCR. Two hundred ninety cells were cultured in DMEM supplemented with 10% FBS and lysed to obtain nuclear extracts. Complementary single-stranded oligonucleotides were synthesized as follows (variant nucleotides underlined): 5′-TCGACAAGTCGGGG/AAAAGTTGGTG-3′. Complementary strands were annealed by combining 200 pmol of each oligonucleotide and 36 μl of annealing buffer (10 mM of Tris-HCL, 1 mM of EDTA, and 0.1 M of NaCl, pH 8.0) in a 40-μl reaction, incubating at 100°C for 5 minutes and allowing to cool to room temperature. The DNA-protein binding reaction was conducted in an 18-μl volume containing 2.5 μg of nuclear extract, 1 μg of poly (dI-dC), 4 μl of 5× binding buffer (Boehringer Mannheim, Mannheim, Germany), and 5.0×10^5 cpm of [^{32}P]-labeled oligonucleotide probe. For the competition experiment, various concentrations (X1–X100 of the labeled probe) of unlabeled probes with G- and A-bearing alleles were added to the solution. The reaction mixture was incubated at room temperature for 30 minutes and then was fractionated by 5% polyacrylamide gel. The DNA-protein complex was detected by exposing to X-ray film.

Statistical analysis

The χ^2 test was used for the Hardy-Weinberg equilibrium and the distribution of allelic frequencies. The difference in BMD between the major and minor alleles was determined by nonparametric analysis (Student-Newman–Keuls). The differences in BMD, body height, weight, body mass index (BMI) [BMI = (weight; kg)/(height; m)^2] among genotypes, and haplotypic analysis were performed using nonparametric analysis (Kruskal-Wallis). This test indicates whether there are differences among the population means of the groups being compared, but it does not pinpoint which groups, if any, differ from the others. All statistical analyses were performed using the statistical package Stat View version J-5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Identification of polymorphisms in the klotho *gene in white and Japanese populations*

In total, eight SNPs in the white popultion and six SNPs in the Japanese population were identified (Table 1). Among the 11 distinct SNPs identified in the two populations, three of them, one in the promoter region (G-395A) and two in exon 4 (C1818T and C2298T), were common in both populations. The SNPs in exon 4 were not accompanied by amino acid substitutions. Allelic frequencies of minor alleles in these SNPs were fairly frequent in both populations but were significantly different between populations.

Characteristics of the common polymorphisms in white and Japanese women

These three SNPs commonly identified in the two populations were used to study the association of the *klotho* gene with bone density in women. Unrelated white women (n = 1187, 18–72 years, 47.1 ± 12.0 years, mean ± SD) and unrelated Japanese postmenopausal women (n = 215, 66–92 years, 72.9 ± 5.5 years) were analyzed for association. Because menopause is known to be a major factor for bone loss in women, we divided the white population into three subgroups according to their menopausal status: definite premenopausal women (n = 506, 18–58 years, 36.8 ± 8.6 years), definite postmenopausal women (n = 364, 48–72 years, 57.9 ± 6.7 years), and others whose menopausal status was unclear. Aging is also known to be another major factor affecting bone loss; therefore, we further divided the white postmenopausal women into three age groups: those ≤54 years, 55–64 years, and ≥65 years old (Table 2). The allelic frequency of minor alleles was not

TABLE 2. ASSOCIATION OF COMMON SNPS WITH MENOPAUSAL STATUS, AGE, AND BMI

	G-395A				C1818T				C2298T			
		BMI				BMI				BMI		
	Allelic frequency	G/G	G/A	A/A	Allelic frequency	C/C	C/T	T/T	Allelic frequency	C/C	C/T	T/T
White population												
All (n = 1187)	0.215	24.75 ± 0.16	25.03 ± 0.23	25.03 ± 0.70	0.421	24.62 ± 0.21	25.03 ± 0.19	24.79 ± 0.30	0.124	24.90 ± 0.15	24.84 ± 0.26	22.58 ± 0.88
Premenopausal (n = 506)	0.225	24.57 ± 0.25	24.40 ± 0.36	24.54 ± 1.35	0.448	24.14 ± 0.35	24.72 ± 0.30	24.50 ± 0.45	0.121	24.62 ± 0.23	24.22 ± 0.45	22.91 ± 1.35
Postmenopausal (n = 364)	0.213	25.08 ± 0.29	25.75 ± 0.42	24.79 ± 0.99	0.416	25.65 ± 0.41	25.22 ± 0.32	24.78 ± 0.53	0.109	25.15 ± 0.26	25.91 ± 0.51	24.55 ± 0.30
≤54 years (n = 112)	0.204	23.95 ± 0.57	25.16 ± 0.66	22.69 ± 1.18	0.426	24.77 ± 0.80	24.23 ± 0.59	23.64 ± 0.79	0.120	24.04 ± 0.47	25.30 ± 1.06	24.56 ± 0.52
55–64 years (n = 197)	0.210	25.50 ± 0.36	26.11 ± 0.59	25.05 ± 1.66	0.417	25.99 ± 0.52	25.67 ± 0.44	25.13 ± 0.75	0.093	25.64 ± 0.35	25.93 ± 0.59	24.53 ± 0.32
≥65 years (n = 55)	0.236	25.93 ± 0.72	25.65 ± 0.23	26.36 ± 1.76	0.382	25.84 ± 1.23	25.99 ± 0.78	25.36 ± 0.89	0.136	25.52 ± 0.66	26.77 ± 1.30	—
Japanese population												
All: Postmenopausal (n = 215, > 65 years)	0.128	23.96 ± 0.34	22.73 ± 1.45	23.45 ± 0.53	0.248	23.46 ± 0.48	23.34 ± 0.46	21.02 ± 0.83	0.256	23.21 ± 0.28	22.89 ± 0.28	23.46 ± 2.24

Allelic frequency indicates the frequency of the minor allele in each SNP. BMI data are mean ± SEM. There was no significant difference of BMI among genotypes of each SNP (all p > 0.05).

significantly different among subpopulations in each population and was similar to that obtained from the SNP screening study shown in Table 1. No significant difference in height, weight (data not shown), or BMI (Table 2) was seen among genotypes of these SNPs in any subpopulation (all $p > 0.05$). These results indicate that these SNPs are not associated with menopausal status, age, height, or weight in each population.

The genotypic frequencies for these SNPs in any subpopulations were not significantly different from those expected for populations in Hardy-Weinberg equilibrium (all $p > 0.05$, data not shown). Linkage disequilibrium among these SNPs was evaluated by calculating haplotype frequencies according to the method by Hill[25] and Thompson et al.[26] None of the disequilibrium values for marker pairs differed significantly from zero (the maximum-likelihood estimate of $D = -0.047$–0.058, all $p > 0.05$, data not shown), indicating there was no significant linkage disequilibrium among these SNPs.

Association of the common polymorphisms with bone density in white women

In all white women (n = 1187, 2374 alleles), there were no significant differences in the whole body BMD between major and minor alleles of these SNPs (Fig. 1A). We then investigated the association between BMD and the allele types in definite premenopausal women (n = 506, 1012 alleles) and definite postmenopausal women (n = 364, 728 alleles), respectively. No difference in BMD was seen between allele types of any SNPs in premenopausal women. However, there was a weak but significant association between BMD and C1818T SNP in postmenopausal women: the minor T allele was associated with lower BMD than the C allele ($p = 0.029$, Fig. 1A). These results were unchanged when we repeated the analysis using the Z score that was adjusted by age and weight. The T allele at the C1818T site was still associated with a lower Z score in postmenopausal women (P=0.004), although no association was found in the overall population or in the premenopausal subpopulation.

We performed further analysis by dividing the white postmenopausal women into three age groups (Fig. 1B). No significant association was seen between any of the SNPs and BMDs in the two younger subpopulations. However, in the oldest subpopulation (≥65 years), the association was stronger than that seen in the overall postmenopausal women and was detected not only with the C1818T SNP ($p = 0.010$) but also with the G-395A SNP ($p = 0.001$; Fig. 1B). Association between the Z score and allele types was also not seen in the two younger subpopulations but was observed in the oldest subpopulation: G-395A SNP ($p = 0.001$) and C1818T SNP ($p = 0.018$). Association analysis based on three genotypes was also performed (Fig. 1C). Again, both G-395A and C1818T SNPs showed a significant association with BMD in the oldest subpopulation ($p = 0.003$ and 0.014, respectively), and BMD was decreased dose dependently of the minor alleles.

Furthermore, G-395A and C1818T SNPs were examined jointly by haplotypic analysis (Table 3). Here again, the minor alleles were significantly associated with lower BMD

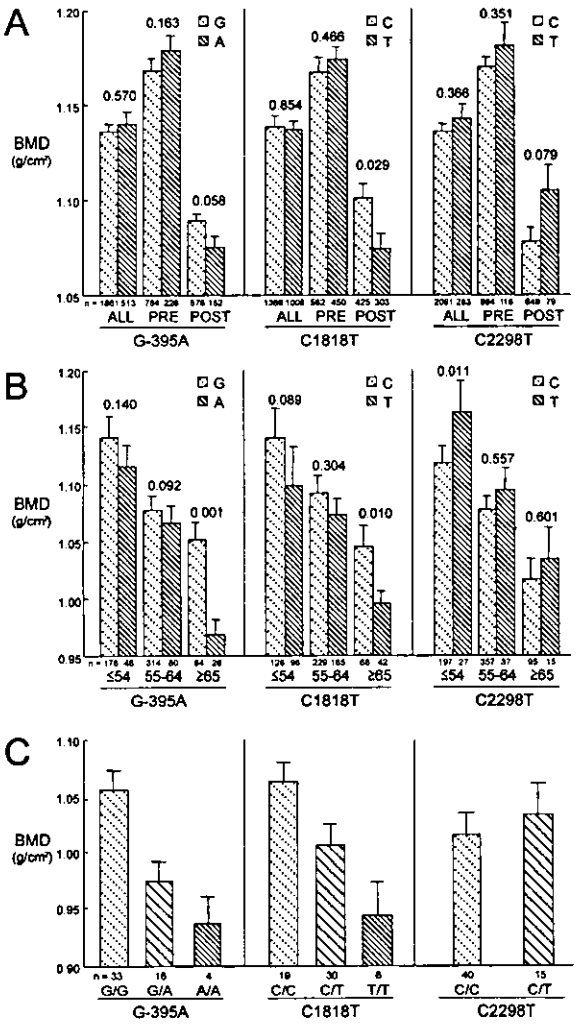

FIG. 1. Association of the three common SNPs with BMD in white women. BMD of the whole body was compared (A and B) between the major and minor alleles and (C) among genotypes. (A) Association of allele types with BMD in the three different subgroups classified according to their menopausal status: all women (ALL; n = 1187, 2374 alleles), definite premenopausal women (PRE; n = 506, 1012 alleles), and definite postmenopausal women (POST; n = 364, 728 alleles). (B) Association of allele types with BMD in the three different age groups of definite postmenopausal women (n = 364): those ≤54 years (n = 112, 224 alleles), 55–64 years (n = 197, 394 alleles), and ≥ 65 years (n = 55, 110 alleles). (C) Association of three genotypes with BMD in the oldest subpopulation (≥65 years old, n = 55). Data are expressed as means (bars) ± SEMs (error bars) for the (A and B) number of alleles and (C) women shown under each bar. The p values of the difference in the mean BMD between major and minor alleles in panels A and B are shown as the numbers above the bars (Student–Newman–Keuls test) and those among genotypes in panel C are 0.003, 0.014, and 0.573 for G-395, C1818T, and C2298T, respectively (Kruskal-Wallis test).

in postmenopausal women (p = 0.007), especially in aged women (≥65 years, $p < 0.0001$), but not in all premenopausal or younger postmenopausal women.

TABLE 3. BMD OF EACH HAPLOTYPE OF G-395A AND C1818T SNPS IN THE WHITE POPULATION

		Premenopausal	*Postmenopausal*			
					55-64 years	
Haplotype	*All* (n = *1187*)	(n = *506*)	*All* (n = *364*)	≤*54 years* (n = *112*)	(n = *197*)	≥*65 years* (n = *55*)
H1 (− −)	1.138 ± 0.002 (301)	1.169 ± 0.003 (120)	1.113 ± 0.004 (87)	1.139 ± 0.009(21)	1.116 ± 0.006(51)	1.052 ± 0.010(15)
H2 (− +)	1.135 ± 0.003 (423)	1.170 ± 0.004 (181)	1.098 ± 0.006 (140)	1.112 ± 0.009(49)	1.115 ± 0.009(73)	1.009 ± 0.014(18)
H3 (+ −)	1.138 ± 0.006 (82)	1.173 ± 0.007 (30)	1.081 ± 0.010 (30)	1.101 ± 0.014 (9)	1.097 ± 0.014(17)	0.985 ± 0.020 (4)
H4 (+ +)	1.142 ± 0.006 (381)	1.186 ± 0.006 (175)	1.069 ± 0.012 (107)	1.111 ± 0.014(33)	1.080 ± 0.019(56)	0.944 ± 0.015(18)
p Value	0.644	0.140	0.007	0.066	0.054	<0.0001

(−) Denotes women without the minor allele (G/G for G-395A and C/C for C1818T) and (+) denotes those with the minor allele (G/A or A/A for G-395A, and C/T or T/T for C1818T). Data are means ± SEM for the number of women in the parenthesis. p Values were determined by nonparametric analysis (Kruskal-Wallis).