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Identification of C-terminally and N-terminally truncated estrogen receptor α variants in the mouse

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ABSTRACT

We re-examined mouse ERα mRNA variants using rapid amplification of cDNA ends (RACE) and RT-PCR. Our analysis showed the presence of several mRNA variants containing unique 5′- or 3′-nucleotide sequences. We mapped the cDNA sequences on the mouse genome, and identified four novel 3′-terminal and 5′-leader exons in the intronic region between exons 4 and 5. RT-PCR analysis revealed that the expression patterns of the C-terminally truncated ERα products (CTERPs) were similar to that of Wild-type ERα and that the N-terminally truncated ERα products (NTERPs) appeared to have different expression profiles. Moreover, we constructed expression vectors and analyzed the subcellular localization and the transcriptional activation abilities of the variant proteins in transfected HEK293 cells using immunocytochemistry and luciferase reporter assay. The CTERP variants localized in the nuclei and constitutively activated estrogen response element (ERE)-driven promoters, while the NTERP variant was located in the extra-nuclear regions and had no ability to activate the ERE promoters in the presence or absence of 10 nM estradiol. Our results indicate that the mouse ERα gene is more complex than previously thought in terms of genomic organization and that alternative splicing and alternative usage of intronic promoters contribute to the remarkable diversity of ERα mRNAs and proteins.

1. Introduction

Ovarian steroid hormones, estrogens, are now recognized as pleiotropic hormones that have pivotal roles in a wide variety of physiological processes in reproductive and non-reproductive organs [1]. The physiological effects of estrogens often include changes in the expression patterns of specific target genes. This transcriptional regulation is mediated through nuclear estrogen receptors (ERs) that belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors. To date, two subtypes of nuclear ERs have been described in mammals: estrogen receptor α (ERα, also known as ESR1) and estrogen receptor β (ERβ, also known as ESR2) [2].

Alternative splicing of pre-mRNA contributes to the mRNA diversity of a single gene. Multiple splice variants are generated from both ER subtype genes [3–5]. Several mechanisms that generate splice variants are utilized by both ERs. ER splice variants are produced by the combination of exon-skipping, insertion of short exon(s) and alternative use of terminal exons [3]. Alternative promoter usage is one of the other mechanisms of variant generation. Transcription from ER genes is initiated from different promoters in the 5′-flanking regions, producing multiple variants with unique 5′-untranslated regions (5′-UTRs) although the encoded proteins are identical [3,6]. Furthermore, there are several promoters in the intronic regions of the genes and alternative utilization of intronic promoters is reported to yield N-terminally truncated ER variants [7–10].

The mouse is extensively used as a model animal to study ERα regulation and function in vivo. The mouse ERα gene consists of eight conventional coding exons. Most splice variants of mouse ERα are generated by exon-skipping. Human and rat ERα genes have intronic promoters and yield N-terminally truncated ERα variants [7–9]. In mouse, the existence of an N-terminally truncated variant, truncated ERα product-1 (TERP-1), was reported, although the detailed nucleotide sequence of mouse TERP-1 was not described [11,12]. In human ERα and ERβ, several terminal exons are utilized by alternative splicing, consequently generating variants with different C-termini [7,13–15], while alternative terminal exons are not reported in mouse ERα.

Recent advances in genomics and transcriptome research enable us to identify novel variants from a pool of expressed sequence tag (EST) clones and to determine the precise position of the mouse

Abbreviations: BLAST, basic local alignment Tool; BLAT, BLAST-like alignment tool; CTERP, C-terminally truncated estrogen receptor α product; DIG, digoxigenin; ER, estrogen receptor; ERα, estrogen receptor α; ERα46, 46 kDa estrogen receptor α; ERα66, 66 kDa estrogen receptor α; ERβ, estrogen receptor β; ERE, estrogen response element; EST, expressed sequence tag; NTERP, N-terminally truncated estrogen receptor α product; ORF, open reading frame; RACE, rapid amplification of cDNA ends; TERP, truncated estrogen receptor α product; UTR, untranslated.

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Table 1
Oligonucleotide primers used for RACE and ORF cloning.

	Target		Direction	Primer sequence (5′–3′)
3′-RACE	Universal	RT primer	Reverse	5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)18-3′
		1st PCR	Reverse	5′-GCTGTCAACGATACGCTACGTAACG-3′
		2nd PCR	Reverse	5′-CGCTACGTAACGGCATGACAGTG-3′
	Exon 4	1st PCR	Forward	5′-TGACTTGGAAGGCCGAAATGAAAT-3′
		2nd PCR	Forward	5′-AGAATAGCCCTGCCTTGTCC-3′
	Exon 4_L	2nd PCR	Forward	5′-GTGCCAGGTAAGGATGCTAAACTC-3′
	Exon i45b	2nd PCR	Forward	5′-TGCCCAAAAGCTCAGACAGATACG-3′
5′-RACE	Exon 6	RT primer	Reverse	5′-ACCATGCCTTCCACA-3′
	Universal	1st/2nd PCR	Forward	5′-GTCTACCAGGCATTCGCTTCAT-3′
	Exon 5	1st PCR	Reverse	5′-GGAGCGCCAGACGAGACCAATC-3′
		2nd PCR	Reverse	5′-AGCGCCAGACGAGACCAATCATCA-3′
	Exon i45c	2nd PCR	Reverse	5′-GCAGCCTTCAGAGATCAGCAACAA-3′
	Exon $i45d_S$, 5	2nd PCR	Reverse	5′-AGATTCAAGTCCCCAAAGCAGGTG-3′
	Exon $i45d_{M,L}$	2nd PCR	Reverse	5′-CTGATCTGGTCGATCCTCTC-3′
ORF cloning	Exon F1	PCR	Forward	5′-TGCCCCCTCCTCTGCCATTGTCTA-3′
	Exon C	PCR	Forward	5′-GATCATTCGAGCACATTCCTTCCT-3′
	Exon 1	PCR	Forward	5′-CGTGCGCGGGGAGCCAGTCT-3′
	Exon i45c	PCR	Forward	5′-CACCCCCTGCACTCCCAACCAT-3′
	Exon i45d	PCR	Forward	5′-GTCCTCTCCCTATGCCTCCTCAGCA-3′
	Exon 4_L	PCR	Reverse	5′-CCCAATACAGCCATCTCCCTAATC-3′
	Exon i45a	PCR	Reverse	5′-TAATTCCACCGAGTTTTCTTTGTA-3′
	Exon i45b	PCR	Reverse	5′-CCAGGACATGAGGGACCAAGAGGA-3′
	Exon 8	PCR	Reverse	5′-CATGCCACAGTGTACGCAGGAG-3′

ERα gene on the mouse genome. We, therefore, decided to identify mouse ERα splice variants and to re-examine the genomic organization of the mouse ERα gene.

The aims of the present study were to identify mouse ERα variants containing novel nucleotide sequences and to re-examine the genomic organization and structure of the coding region of the mouse ERα gene. To this end, we searched multiple mRNA variants with unique sequences in silico using the basic local alignment tool (BLAST) [16], then cloned several novel variants in vitro using rapid amplification of cDNA ends (RACE) and RT-PCR, and mapped the newly cloned and the previously reported cDNA sequences onto the mouse genome. Moreover, we characterized the subcellular localization and the transcriptional activation abilities of N-terminally and C-terminally truncated ERα variant proteins in transfected HEK293 cells.

2. Materials and methods

2.1. Animals

All experiments were performed with the approval of the Nippon Medical School Animal Care Committee. 10-week-old male and female C57BL/6J mice were used in this study. The mice had free access to water and chow and were kept under a 14 h light and 10 h dark cycle.

2.2. Total RNA isolation

Mice were decapitated under ether anesthesia and organs were quickly removed and stored in liquid nitrogen until use. Total RNA was extracted using Isogen (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. Total RNA was treated with Turbo DNase (RNase-free DNase I; Ambion, Austin, TX, USA) and purified. RNA concentration was quantified by absorption at 260 nm.

2.3. RACE and open reading frame (ORF) cloning

3′-RACE cDNA was prepared using an adapter-oligo$(dT)_{18}$ primer. Total RNA was reverse-transcribed in RT reaction mixture [10 μg of total RNA, 1× RT buffer, 1 mM dNTP mixture, 20 U RNasin Plus (Promega, Madison, WI, USA) and 100 U RTase (ReverTra Ace, Toyobo, Osaka, Japan)] with the addition of an adapter-oligo$(dT)_{18}$ primer. The reaction was carried out at 42 °C for 60 min. 5′-RACE was performed using a CapFishing method. 5′-RACE cDNA was synthesized in RT reaction mixture with the addition of a gene-specific RT primer, BSA and $MnCl_2$. The reaction was carried out at 42 °C for 60 min, then the CapFishing adapter (Seegene, Seoul, Korea) and 100 U of fresh RTase were added, and incubated at 42 °C for 30 min. cDNA used for ORF cloning was synthesized in RT reaction mixture using an oligo$(dT)_{15}$ primer. The reaction was carried out at 42 °C for 60 min. The RT reactions were stopped by heating at 75 °C for 15 min and were then treated with RNase H (Takara bio, Shiga, Japan) at 37 °C for 30 min.

RACE products were amplified by two rounds of PCR with RACE primers and gene-specific primers. ORF fragments of C-terminally truncated ERα products (CTERPs) were amplified by one round of PCR with forward primers designed on exons C, F or 1 and reverse primers on respective terminal exons, and those of N-terminally truncated ERα products (NTERPs) were amplified using forward primers on respective leader exons and a reverse primer on exon 8. The conditions for the PCRs consisted of 28 cycles (for RACE) or 36 cycles (for ORF cloning) of 94 °C for 30 s, 60 °C for 20 s and 72 °C for 1 min, with an initial denaturing step of 94 °C for 2 min and a final elongation step of 72 °C for 5 min. The reaction was performed in 50 μl of PCR mixture comprising the cDNA corresponding to 1 μg of total RNA, 1× GC PCR buffer, 0.4 mM dNTP mixture, 0.4 μM each forward and reverse primers and 1.2 U LA Taq polymerase (Takara bio). The oligonucleotide primers used in RACE and ORF cloning reactions are listed in Table 1. PCR products were excised after agarose electrophoresis, purified, and cloned into pGEM-T-Easy vectors (Promega).

2.4. RT-PCR

Total RNA was reverse-transcribed into first-strand cDNA using an oligo$(dT)_{15}$ primer. Reaction solution (50 μl) contained 20 μg of total RNA, 1× RT buffer, 1 mM dNTP mixture, 2 μg oligo$(dT)_{15}$ (Promega), 40 U RNasin Plus, and 200 U RTase. The reaction was carried out at 42 °C for 60 min and stopped by heating. cDNA was treated with RNase H and stored at −20 °C until use.

Table 2
Oligonucleotide primers used for RT-PCR.

Gene	Target	Direction	Exon(s)	Primer sequence (5′–3′)	Product size (bp)
Esr1	ORF region	Forward	4	5′-AGACCGCCGAGGAGGGAGAATGTT-3′	415
		Reverse	5	5′-GGAGCGCCAGACGAGACCAATC-3′	
	ORF region	Forward	6	5′-TGCTGGCTACGTCAAGTCGGTTCC-3′	419
		Reverse	8	5′-CTGGGTCTGGCTGGGCTCCTCTG-3′	
	CTERP-1	Forward	3	5′-GCTGCGCAAGTGTTACGA-3′	491
		Reverse	4_L	5′-CCCAATACAGCCATCTCCCTAATC-3′	
	CTERP-2, 3	Forward	3, 4	5′-TGAAAGGCGGCATACGGAAAGACC-3′	586, 691, 840
		Reverse	i45b	5′-CCAGGACATGAGGGACCAAGAGGA-3′	
	NTERP-1	Forward	i45c	5′-CACCCCCTGCACTCCCAACCAT-3′	300
		Reverse	5	5′-AGCGCCAGACGAGACCAATCATCA-3′	
	NTERP-2	Forward	i45d	5′-GTCCTCTCCCTATGCCTCCTCAGCA-3′	637, 685, 687
		Reverse	8	5′-CTGGGTCTGGCTGGGCTCCTCTG-3′	
Actb	β-Actin	Forward	3	5′-CCTAAGGCCAACCGTGAAAAGATG-3′	430
		Reverse	4	5′-ACCGCTCGTTGCCAATAGTGATG-3′	

PCR was performed in 25 μl of PCR mixture comprising cDNA corresponding to 200 ng of total RNA, 1× PCR buffer, 0.2 mM dNTP mixture, 0.2 μM each forward and reverse primers, and 0.63 U Blend Taq polymerase (Toyobo). PCR was performed with cycle reactions of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s, with an initial denaturing step of 95 °C for 2 min and a final elongation step of 72 °C for 5 min. The sequences of oligonucleotide primers used in RT-PCR are shown in Table 2.

2.5. Electrophoresis

PCR products (5 μl) were separated by electrophoresis on 2% agarose gels, and visualized by ethidium bromide staining under UV irradiation. Gel images were captured using a FAS-III system (Toyobo).

2.6. DNA sequencing

PCR products were extracted from agarose gels using a Wizard SV Gel and PCR Clean-up System (Promega) and then cloned into pGEM-T-Easy vectors. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Fluorescent signals were detected using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

2.7. Plasmid vectors

A reporter plasmid vector containing three tandem repeats of a consensus estrogen response element (ERE) upstream of a minimum TATA promoter sequence (pERE-Luc) and an ERE-less control plasmid vector (pControl-Luc) were purchased from Panomics/Affymetrix (Santa Clara, CA, USA). pRT-TK vector was purchased from Promega.

The ORFs of mouse Wild-type ERα (ERα66), Δexon 1 ERα variant (ERα46) [17], CTERPs and NTERP were cloned into pcDNA3.1/Hygro(+) (Invitrogen) and confirmed by DNA sequencing.

2.8. Cell culture and transfection

HEK293 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 1 mM Na pyruvate, 24 mM $NaHCO_3$, 4 mM L-glutamine, 10% FBS (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin without phenol red. The cultures were maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2. Cells were routinely passaged every 3–4 days.

Reporter plasmid vectors and/or recombinant plasmid constructs (pcDNA-ERα66, pcDNA-ERα46, pcDNA-NTERP, pcDNA-CTERP-1, pcDNA-CTERP-2, or pcDNA-CTERP-3) were introduced into HEK293 cells using the TransFast Transfection reagent (Promega), according to the manufacturer's instructions. For Western blot analysis, cells were harvested 48 h after transfection of recombinant plasmid constructs and lysed with lysis buffer [50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% SDS and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)]. In immunocytochemical experiments, cells were washed with serum-free DMEM 24 h after transfection of recombinant plasmid constructs, and then treated with 10 nM 17β-estradiol (E2) or 0.1% EtOH as vehicle control for 24 h in DMEM containing 10% charcoal-stripped FBS (Invitrogen). For luciferase reporter assay, cells were seeded into 24-well plates to achieve approximately 40–60% confluence on the day of transfection. The cells were transfected with 1 μg of the reporter plasmid (pERE-Luc or pControl-Luc), 200 ng of the appropriate expression constructs (pcDNA-ERα66, pcDNA-ERα46, pcDNA-NTERP, pcDNA-CTERP-1, pcDNA-CTERP-2, pcDNA-CTERP-3 or empty pcDNA3.1) and 50 ng of pRL-TK vector in serum-free medium for 1 h, and then cultured in DMEM/2.5% charcoal-stripped FBS. 24 h after transfection, the cells were washed with serum-free DMEM and then treated with 10 nM E2 or 0.1% EtOH in fresh DMEM containing 2.5% charcoal-stripped FBS for 24 h. The cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl and 1.47 mM KH_2PO_4) and then dissolved in 200 μl of 1× passive lysis buffer (Promega).

2.9. Western blot analysis

Protein samples were denatured at 95 °C for 5 min in gel-loading buffer, then resolved on polyacrylamide gels and transferred onto PDVF membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). The membranes were blocked in blocking buffer (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20 and 0.3% skimmed milk powder). The membranes were incubated in blocking buffer with MC-20 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was raised against a peptide mapping to the C-terminus of mouse ERα (The dilution ratio is 1:3000.) or H-184 rabbit polyclonal antibody (Santa Cruz Biotechnology), which was raised against amino acids 2–185 of human ERα (1:1000). The membranes were then incubated with the appropriate secondary antibody coupled with horseradish peroxidase (1:2500) (Cell Signaling Technology, Beverly, MA, USA). The results were visualized using a chemiluminescent substrate (ECL Plus; GE Healthcare) and a light-capture system.

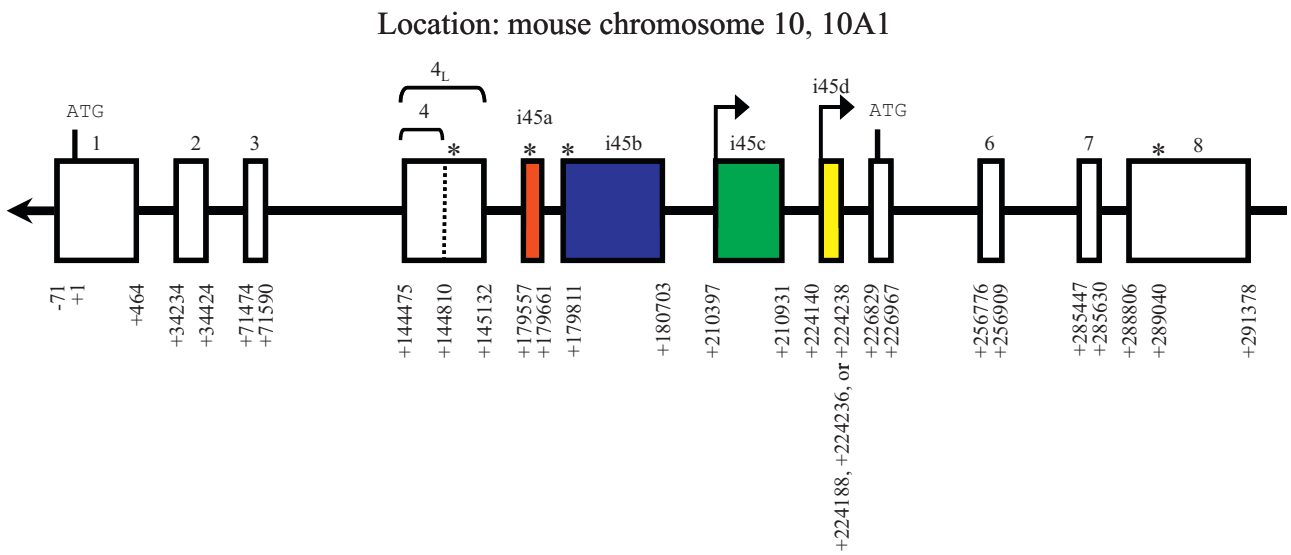

Fig. 1. Genomic organization of the mouse ERα gene. Schematic representation of the mouse ERα gene structure determined in the present study. The image is constructed with reference to Swope et al. [19]. cDNA sequences of multiple mouse ERα variants are mapped on the mouse genome assembly (Build 37). The mouse ERα gene is located at 10A1 on mouse chromosome 10. The numbers below exons correspond to the distance from the adenine of the first start codon, which is located at nucleotide position 5634157 on mouse chromosome 10 and is defined as nucleotide position +1 in the image. Exon i45d has three alternative splice donor sites. The open and filled boxes represent the conventional coding exons and the newly identified exons, respectively. The arrow indicates the orientation of the chromosome. The bent arrows symbolize the putative transcription start sites. The asterisks represent the terminal codons. Nucleotide sequences are shown in detail in Supplemental Fig. 1. The image is not to scale.

2.10. Immunocytochemistry

Transfected and mock-transfected cells were washed three times with PBS, fixed with 10% formalin for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 10 min. Cells were incubated in PBST/BSA (PBS with 0.1% Tween 20 and 5% BSA) for 1 h at room temperature and then MC-20 (1:1000) or H-184 (1:500) primary antibodies were added and cells were incubated for 16 h at 4 °C. After several washes in PBST, Alexa Fluor 488-conjugated secondary antibody (1:500) (Invitrogen) was added and cells were incubated for 1 h. Nuclei were counterstained with 0.2% propidium iodide (Invitrogen). Fluorescence images were digitized using a BZ-9000 fluorescence microscopy system (Keyence, Osaka, Japan).

2.11. Luciferase reporter assay

Luciferase activity was measured using a Lumat LB 9507 luminometer (Berthold Technologies, Wildbad, Germany) and a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was measured after adding 10 μl of cell lysate with 50 μl of luciferase assay reagent II (Promega). The activity of Renilla luciferase was subsequently measured by adding 50 μl Stop & Glo Reagent (Promega). Each sample was analyzed in triplicate and the firefly luciferase activity was compensated to the Renilla luciferase activity. Statistical differences between E2-treated and untreated groups were examined using Student's *t*-test.

3. Results

3.1. Identification of novel ERα mRNA variants

Extensive homology searches were performed with a BLAST program and the DNA Data Bank of Japan (DDBJ) and to look for EST clones containing mouse ERα cDNA sequences and novel sequences. We identified four EST clones (accession numbers: AK136228, AK139745, BY720287 and CF539762). The nucleotide sequence of BY720287 overlapped with that of AK136228. The clone, AK139745, contained novel 3′-nucleotide sequence, and the others possessed unique 5′-sequences. To confirm the authenticity of novel sequences and to determine their positions in the mouse ERα gene, we mapped the EST clones on the mouse genome (Genome assembly, Build 37) using the BLAST-like alignment tool (BLAT) [18]. BLAT analysis revealed that the novel sequences consisted of 1 or 2 exons localized in the intronic region between exons 4 and 5. We named the exons identified here as exons i45 and indexed them with a–d according to their 5′–3′ order in the gene (Fig. 1). The structures of the EST clones identified were i45d$_S$-5-6-7-8 for AK136228, F1-F2-1-2-3-4-i45a-i45b for AK139745, i45d$_S$-5-6-7 for BY720287 and i45c-5-6-7 for CF539762. The subscript letter, "S", indicates a short form of exon i45d generated by an alternative usage of splice donor sites. Further homology searches were performed using the novel exonic sequences as queries. We found one EST clone (accession number: CN700530), which covered the upstream region of exon i45c.

To further isolate mRNA variants with novel sequences and to determine ORFs and the precise 5′- or 3′-ends of novel leader and terminal exons, RACE and RT-PCR were performed. 3′-RACE with primers designed on exon 4 amplified two mRNA variants; one was a 4$_L$ variant reported by Swope et al. [19] and the other was a 4-i45b variant. We could not identify further novel variants using 5′-RACE. RT-PCR cloning revealed a novel alternative splice event, 4-i45a-intron-i45b, and the use of alternative splice donor sites of exon i45d (i45d$_L$, i45d$_M$, and i45d$_S$; the subscript letters, "L", "M", and "S", indicate forms of exon i45d with long, middle, and short lengths, respectively). The ORFs of the variants were amplified and cloned using ORF cloning primers. Nucleotide sequences of four novel exons are shown in detail in Supplemental Fig. 1. All splice boundaries of introns in the mouse ERα gene obey the GT/AG rule. The terminal exons, 4$_L$ and i45b, contained two and five putative polyadenylation signals (ATTAAA and AATAAA), respectively.

In total, we identified four C-terminally truncated ERα mRNA variants, 1-2-3-4$_L$ (CTERP-1), 1-2-3-4-i45a-intron-i45b (CTERP-2a), 1-2-3-4-i45a-i45b (CTERP-2b) and 1-2-3-4-i45b (CTERP-3) and four N-terminally truncated variants, i45c-5-6-7-8 (NTERP-1), i45d$_L$-5-6-7-8 (NTERP-2a), i45d$_M$-5-6-7-8 (NTERP-2b) and i45d$_S$-5-6-7-8 (NTERP-2c) (Fig. 2). The nucleotide sequences determined in this study are registered in DDBJ/EMBL/Genbank with acces-

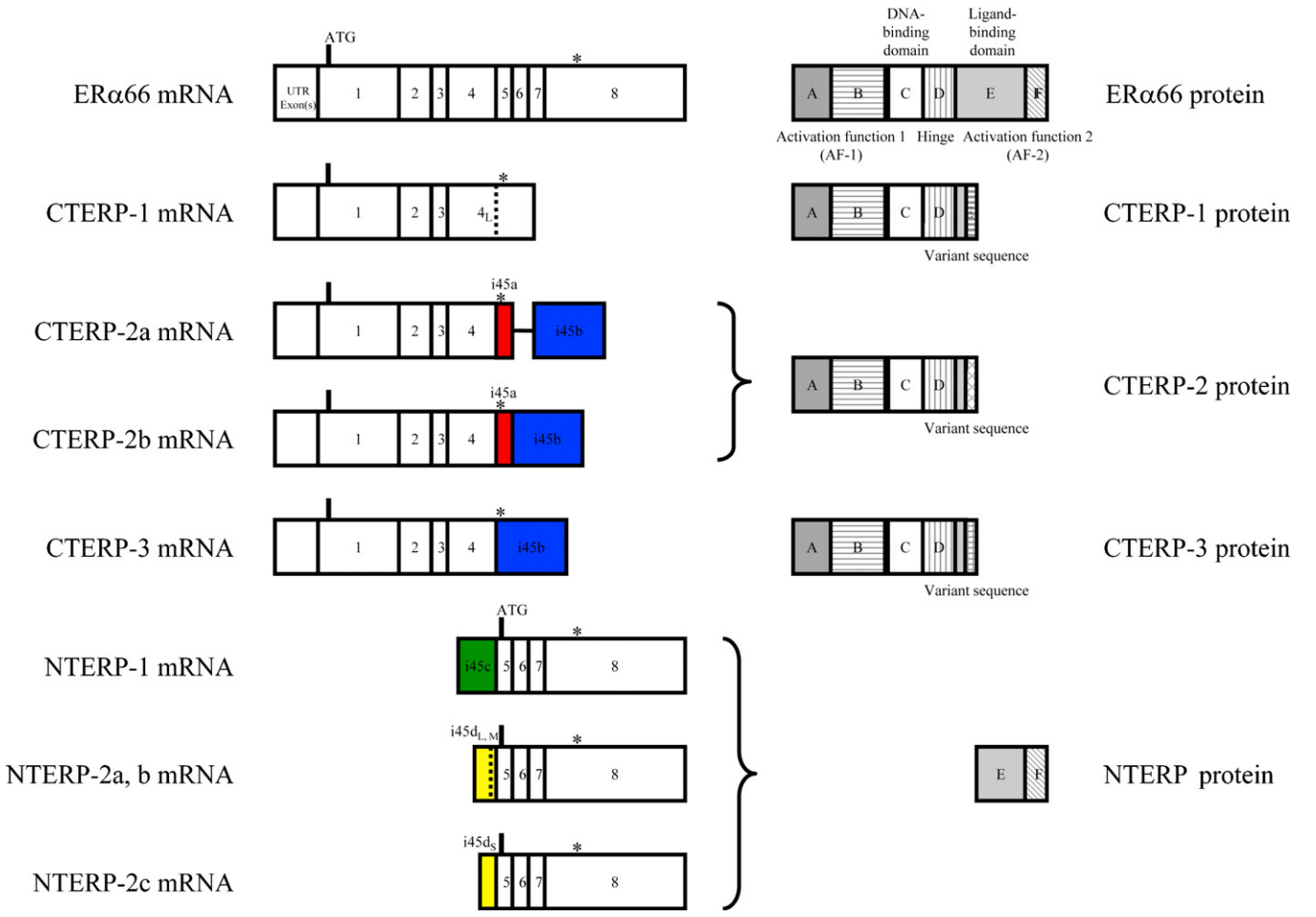

Fig. 2. Depiction of mRNA and protein structures of mouse Wild-type and truncated ERα. The mRNA and protein structures of Wild-type ERα (ERα66), CTERP-1, CTERP-2 s, CTERP-3 and NTERPs are indicated. The asterisks represent the terminal codons.

sion numbers: AB560753 for CTERP-1, AB560754 for CTERP-2a, AB560755 for CTERP-2b, AB560756 for CTERP-3, AB560757 for NTERP-1, AB560758 for NTERP-2a, AB560759 for NTERP-2b and AB560760 for NTERP-2c.

The encoded proteins for CTERP mRNA variants lack most of the ligand-binding domains (Fig. 2). The NTERP mRNA variants encode identical proteins lacking the A/B, DNA-binding and hinge domains, as well as a small portion of the ligand-binding domain (Fig. 2). Detailed amino acid sequences are shown in Supplemental Fig. 2.

3.2. Expression profiles of mouse ERα variants

Expression patterns of truncated ERα mRNA variants were analyzed using RT-PCR with gene-specific primers located on ORF exons and variant-specific primers located on respective leader/terminal exons (Fig. 3). To assess the expression of the mouse ERα gene, the coding regions between exons 4 and 5, and exons 6 and 8 were amplified (Fig. 3A and B). The ERα mRNA was widely distributed. The levels of ERα mRNA were high in the reproductive organs, adrenal gland, kidney, liver and pituitary, moderate in the brain, lung and spleen and low in the intestine and stomach. Although a larger number of PCR cycles (33 cycles) was required to detect the expression of CTERP-1 mRNA, its pattern of expression was essentially similar to that of ERα mRNA (Fig. 3C). The expression of CTERP-2 and CTERP-3 mRNAs was analyzed using a forward primer located on the splice boundary of exons 3 and 4 and a reverse primer on exon i45b (Fig. 3D). Three amplicons corresponding to CTERP-2a (840 bp), CTERP-2b (691 bp), and CTERP-3 (586 bp) were observed. The total signal intensity patterns of the amplicons were similar to those of ERα mRNA. The expression of NTERP-1 mRNA was observed in the reproductive organs, brain, kidney and pituitary (Fig. 3E). The NTERP-2 mRNAs were predominantly expressed in the pituitary and faintly in the brain and ovary (Fig. 3F).

Although the structure and distribution of NTERP-2 mRNAs are similar to those of mouse TERP-1 mRNAs, which were previously reported by Schreihofer et al. [11,12], other properties of NTERP-2 mRNAs are different from those of mouse TERP-1 mRNAs. RT-PCR with primers used in the report of Schreihofer et al. clearly amplified TERP-1 mRNAs from rat pituitary, while these primers did not produce amplicons corresponding to mouse TERP-1 mRNAs from mouse pituitary (Supplemental Fig. 3A). The other rat TERP variant, TERP-2, is generated by alternative splicing of exon "TERP-2" (Supplemental Fig. 3B and C), whereas mouse NTERP-2a, b, and c are produced by alternative use of splice donor sites of exon i45d.

3.3. Subcellular localization of mouse ERα variants

We constructed expression vectors encoding mouse ERα66, ERα46, CTERP-1, CTERP-2, CTERP-3 and NTERP and analyzed the subcellular localization of the variant proteins in transfected HEK293 cells. To confirm the successful construction of vectors and the appropriate expression of variant proteins in transfected

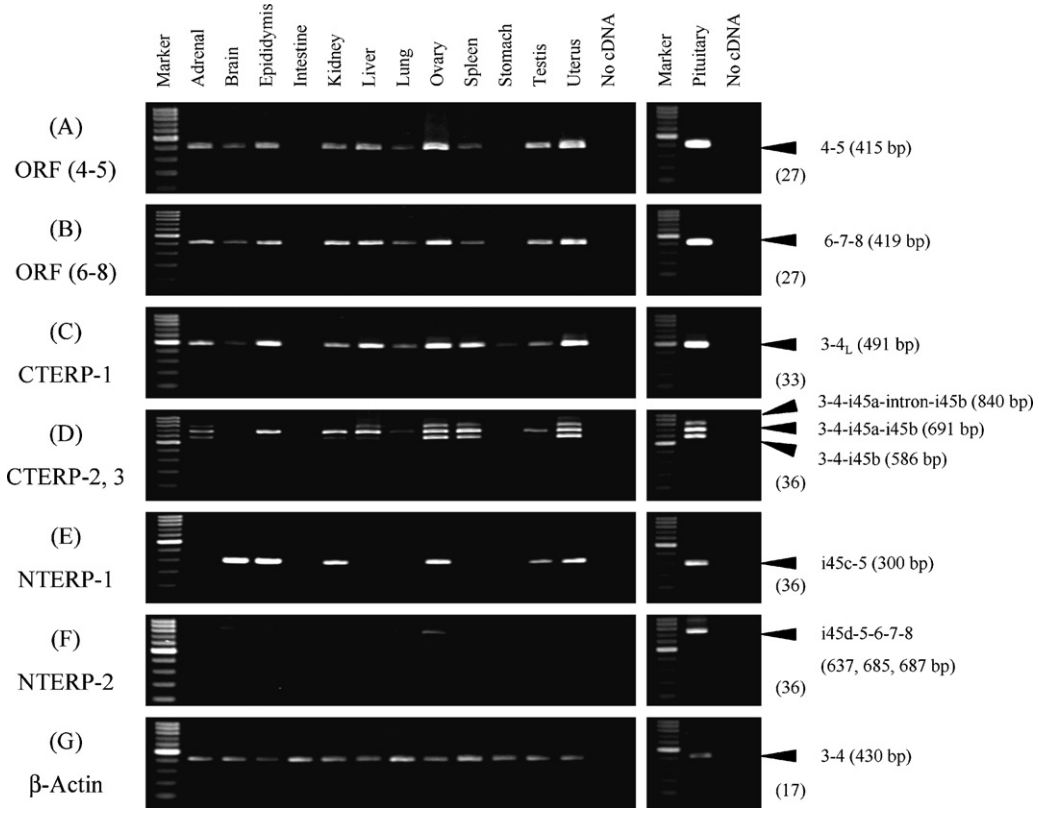

Fig. 3. Expression patterns of mouse ERα variants. Total RNAs isolated from mouse tissues were subjected to RT-PCR using primers designed from respective variant-specific exons (exons 4_L, i45a, i45b, i45c and i45d) and ORF exons. To assess overall ERα expression, the ORF regions between exons 4 and 5 [ORF (4–5)] and exons 6 and 8 [ORF (6–8)] were amplified. From upper to lower panels, the expressions of ERα ORF (4–5) (A), ERα ORF (6–8) (B), CTERP-1 (C), CTERP-2 and CTERP-3 (D), NTERP-1 (E), NTERP-2 (F) and β-actin (G) are shown. β-Actin was used as an internal control. The number of PCR cycles is indicated on the lower right of each panel. The same results were observed in four separately prepared samples.

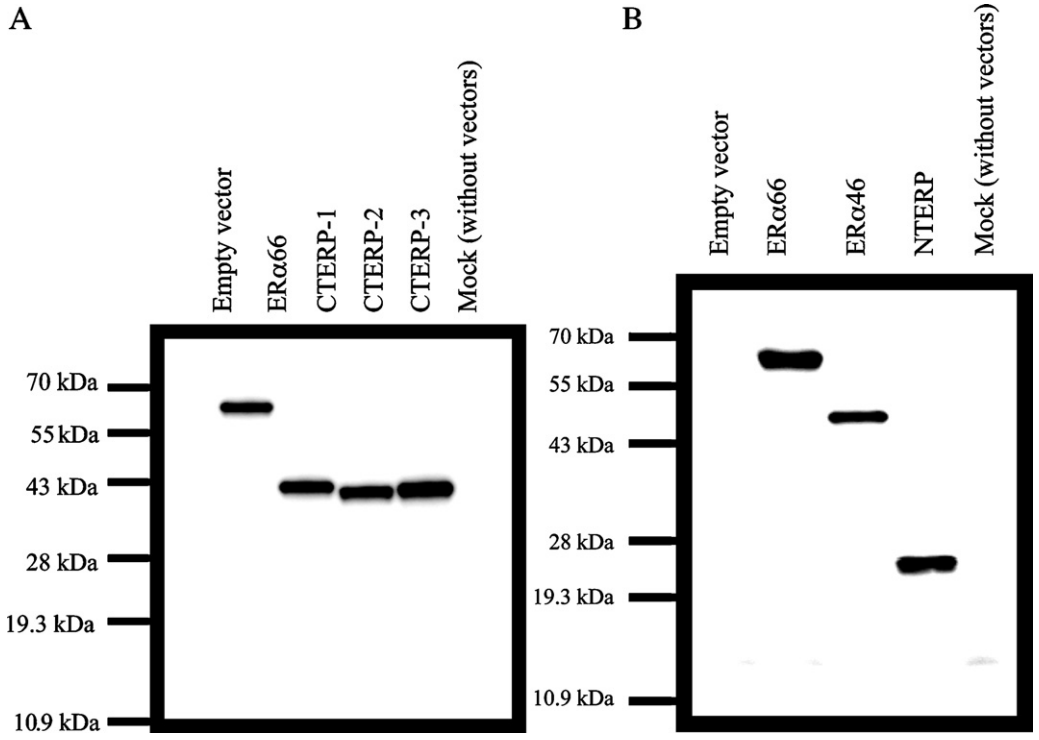

Fig. 4. Expression of mouse Wild-type and truncated ERα proteins in transfected HEK293 cells. Appropriate expression of Wild-type and truncated ERα proteins in transfected HEK293 cells was confirmed by Western blot analysis. The Wild-type (ERα66) and C-terminally truncated variants (CTERP-1, CTERP-2 and CTERP-3) were detected with H-184 antibody raised against amino acids 2–185, which map at the N-terminus of human ERα (A). ERα66, Δexon 1-type ERα (ERα46) and NTERP were detected with MC-20 antibody against the C-terminus of mouse ERα (B). 0.1 μg of whole cell lysate protein prepared from transfected HEK 293 cells was loaded on each lane. Samples prepared from mock-transfected HEK293 cells (Mock) and transfected with empty pcDNA3.1 vector (empty vector) were used as negative controls. The same results were observed in four separately prepared samples.

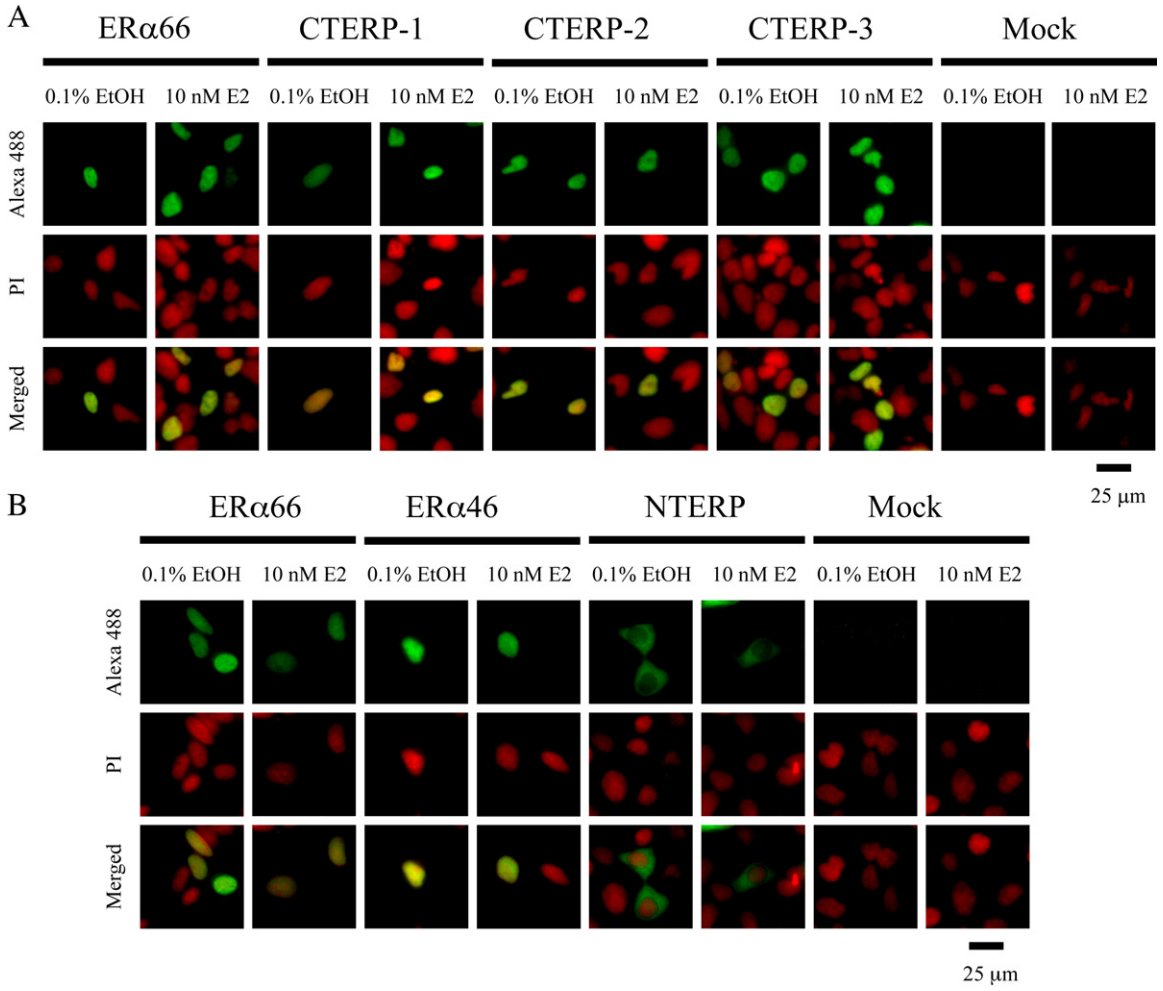

Fig. 5. Subcellular localization of mouse Wild-type and truncated ERα proteins in transfected HEK293 cells. Subcellular localization of mouse Wild-type and truncated ERα proteins in transfected HEK293 cells was determined by fluorescence immunocytochemistry. Transfected cells were treated with 10 nM E2 or 0.1% EtOH as vehicle control for 24 h in DMEM/10% charcoal stripped FBS. The Wild-type (ERα66) and C-terminally truncated variants (CTERP-1, CTERP-2 and CTERP-3) were analyzed with H-184 antibody (A). ERα66, Δexon 1-type ERα (ERα46) and NTERP were detected with MC-20 antibody (B). Primary antibodies were detected with Alexa 488-conjugated secondary antibody and the signals were observed using fluorescence microscopy. The nuclei were stained with propidium iodide (PI). The scale bar at the right bottom of each panel indicates 25 μm. The same results were observed in four separately prepared samples.

HEK293 cells, Western blot analysis was performed (Fig. 4). The bands with appropriate molecular weights were observed in transfected HEK293 cells. The H-184 antibody specifically recognized mouse ERα66, CTERP-1, CTERP-2 and CTERP-3 proteins (Fig. 4A), and the MC-20 antibody appropriately bound to mouse ERα66, ERα46 and NTERP proteins (Fig. 4B).

Fluorescence immunocytochemistry in transfected HEK293 cells revealed the nuclear localization of mouse ERα66, ERα46 and CTERP proteins and the extra-nuclear localization of NTERP proteins (Fig. 5). Treatment of cells with 10 nM E2 did not alter these localizations.

3.4. Transcriptional activation activities of mouse ERα variants

The abilities of mouse ERα variants to activate transcription in HEK293 cells was tested by cotransfection with an ERE luciferase reporter gene (Fig. 6A). Mouse ERα66 and ERα46 stimulated luciferase activities in response to E2. In contrast, CTERPs constitutively activated transcriptions, while NTERP alone did not exhibited any transcriptional activations in the presence or absence of E2. The ERα variants did not induce transcriptional initiation of an ERE-less control promoter (Fig. 6B), indicating that these transcriptional activation activities of ERα66, ERα46 and CTERPs were ERE-dependent.

4. Discussion

The present study shows that the coding region of the mouse ERα gene is a more complex unit than previously thought and that alternative usage of intronic promoters and alternative splicing generate multiple mRNA transcripts encoding C-terminally and N-terminally truncated ERα proteins. Previously, Swope et al. [19] reported the genomic organization of mouse ERα gene and the existence of a C-terminally truncated mRNA variant (CTERP-1 in the present study), generated by termination in the downstream region of exon 4 (exon 4_L). We confirmed their results and further identified novel 3′-terminal and 5′-leader exons (exons i45a, i45b, i45c and i45d) in the intronic region between exons 4 and 5. By alternative splicing among exon i45a, intron and exon i45b, mRNA variants encoding novel C-terminally truncated ERα products (CTERP-2 and CTERP-3) are generated. Transcription of NTERP mRNAs is initiated from intronic promoters located between exons 4 and 5. Further-

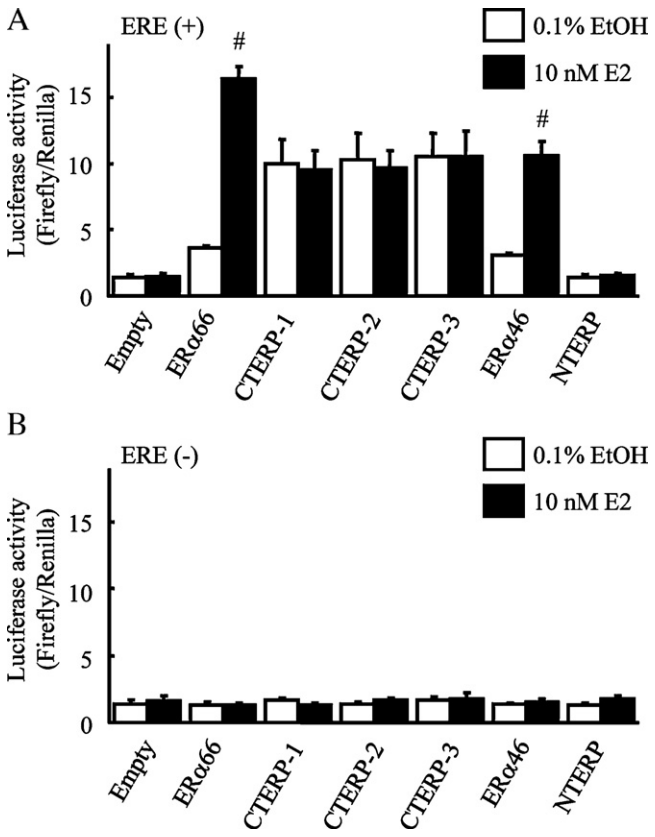

Fig. 6. Transcriptional activation by mouse ERα variants. HEK293 cells were transiently transfected with 1 μg of the reporter vector (pERE-Luc or pControl-Luc) and 200 ng of respective ERα constructs. The cells were treated with or without 10 nM E2 for 24 h before being assayed for luciferase activities. (A) The effects of the ERα variants on transcriptional activation of ERE-driven promoters were estimated from the relative luciferase activities in transfected HEK293 cells. (B) The effects of the ERα variants on transcriptional activation of ERE-less control promoters were estimated from the relative luciferase activities. The firefly luciferase values were corrected for transfection efficiency using Renilla luciferase activities. Data are expressed as mean ± SEM of three independent experiments. The hash symbols indicate significant differences between E2-treated and untreated groups, $^{\#}P < 0.01$.

more, alternative usage of splice donor sites in exon i45d produces three mRNA variants (NTERP-2a, b and c) with different lengths.

Each CTERP protein consists of the A/B domain, the DNA-binding domain, the hinge domain, a small portion of the ligand-binding domain and a short variant-specific sequence. The encoded proteins of NTERP mRNAs are identical and the proteins are composed of two thirds of the ligand-binding domain and the F domain. Mouse ERα66, ERα46 and CTERP proteins localized to the nuclei of transfected cells, while mouse NTERP protein was located in the extra-nuclear cytoplasmic regions. Furthermore, CTERPs constitutively activated the transcriptional activities of ERE-driven promoters, whereas NTERP alone could not induce the activities in the presence or absence of E2. These results are consistent with the previous findings. The nuclear localization signal of ERα is located between the DNA-binding and hinge domains [20]. An ERα mutant lacking the ligand-binding domain loses its ligand-dependent functions, but can exert constitutive DNA-binding and transcriptional activation abilities [21,22]. The structure of the NTERP protein is the same as that of rat TERP-1 protein, and TERP-1 alone cannot modulate transcriptional activations [23,24].

The mouse ERα gene has a multiple promoter system [6]. In mouse multiple promoters, promoters F1 and C are predominantly active in various mouse tissues [25]. Using RT-PCR with forward primers designed on exons F1 and C, we confirmed that the transcription of CTERP mRNAs was initiated from promoters F1 and C (data not shown). The distribution profiles of CTERP mRNAs are essentially similar to that of total ERα mRNA. This evidence suggests that the splice failure of exon 4_L and the splicing from exon 4 to exons i45a and i45b are "constitutive", that is, the extents of the splicing events are at the same ratios in all or most cell types. In contrast, the expression patterns of NTERP mRNAs appear to be distinctive, suggesting that the intronic promoters might be independently regulated.

The expression of the N-terminally truncated ERα variant, TERP-1, has been reported in mouse and rat and results from alternative use of intronic promoters located between exons 4 and 5 [9,11,12]. Schreihofer et al. [11,12] detected the expression of mouse TERP-1 mRNA in mouse pituitary and in mouse pituitary-derived cell lines using RT-PCR with a forward primer designed on the rat TERP-1 leader exon and a reverse primer on a common region of mouse and rat exon 7, but they did not describe any detailed nucleotide sequences of mouse TERP-1 mRNA. Thus, we cannot compare in detail the sequences of NTERP mRNAs with that of TERP-1 mRNA. The distribution and structures of NTERP-2 mRNAs are similar to those of rat TERP-1 mRNA. However, we surmise that NTERP-2 mRNAs are completely different from mouse TERP-1 mRNA and that Schreihofer et al. amplified contaminants from rat samples for the following reasons: (1) mouse exon i45d has no homology with rat exon "TERP-1", (2) NTERP-2 splice variants, NTERP-2a, b, and c, are generated by alternative usage of splice donor sites, while rat TERP variants, TERP-1 and TERP-2, are produced by alternative insertion of exon "TERP-2", (3) RT-PCR with the primers that Schreihofer et al. used did not amplify any products from mouse pituitary cDNAs, although NTERP-2 variants were properly amplified from the same samples, and (4) analysis of the mouse genome indicated that there are no homologous sequences with rat exon "TERP-1" between exons 4 and 5 of mouse ERα.

Several analyses of ERα proteins in the rodent brain using electron microscopy have demonstrated that ERα(-like) immunoreactivities localize in the extra-nuclear regions including axons, cell bodies, dendrites, and pre- and post-synapses [26,27]. These studies used polyclonal antibodies raised against C-terminal regions of ERα [27,28], suggesting that the extra-nuclear ERα(-like) proteins in the rodent brain possess the C-terminal region of the ERα protein. The NTERP protein has the C-terminus of ERα and localizes in the extra-nuclear cytoplasmic regions. Furthermore, NTERP-1 mRNA is preferentially expressed in the brain. We, therefore, speculate that at least one part of the ERα(-like) immunoreactivities in the brain is due to NTERP proteins.

In conclusion, we have determined the genomic organization and structure of the mouse ERα gene and have identified N-terminally and C-terminally truncated ERα transcripts generated by alternative usage of intronic promoters and alternative splicing. In addition, we have characterized the subcellular localization and the transcriptional activation abilities of the truncated ERα variant proteins. These findings provide opportunities for further investigation into the regulatory mechanisms and actions of mouse ERα variants.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.01.003.

References

[1] J.F. Couse, K.S. Korach, Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 20 (3) (1999) 358–417.
[2] J. Matthews, J.A. Gustafsson, Estrogen signaling: a subtle balance between ERα and ERβ, Mol. Interv. 3 (5) (2003) 281–292.
[3] S. Hirata, T. Shoda, J. Kato, K. Hoshi, Isoform/variant mRNAs for sex steroid hormone receptors in humans, Trends Endocrinol. Metab. 14 (3) (2003) 124–129.
[4] S.E. Taylor, P.L. Martin-Hirsch, F.L. Martin, Oestrogen receptor splice variants in the pathogenesis of disease, Cancer Lett. 288 (2) (2010) 133–148.
[5] M.A. Shupnik, Oestrogen receptors, receptor variants and oestrogen actions in the hypothalamic–pituitary axis, J. Neuroendocrinol. 14 (2) (2002) 85–94.
[6] M. Kos, G. Reid, S. Denger, F. Gannon, Minireview: genomic organization of the human ERα gene promoter region, Mol. Endocrinol. 15 (12) (2001) 2057–2063.
[7] Z. Wang, X. Zhang, P. Shen, B.W. Loggie, Y. Chang, T.F. Deuel, Identification, cloning, and expression of human estrogen receptor-α36, a novel variant of human estrogen receptor-α66, Biochem. Biophys. Res. Commun. 336 (4) (2005) 1023–1027.
[8] S. Hirata, T. Shoda, J. Kato, K. Hoshi, The novel isoform of the estrogen receptor-α cDNA (ERα isoform S cDNA) in the human testis, J. Steroid Biochem. Mol. Biol. 80 (3) (2002) 299–305.
[9] K.E. Friend, L.W. Ang, M.A. Shupnik, Estrogen regulates the expression of several different estrogen receptor mRNA isoforms in rat pituitary, Proc. Natl. Acad. Sci. U. S. A. 92 (10) (1995) 4367–4371.
[10] T. Shoda, S. Hirata, J. Kato, K. Hoshi, Cloning of the novel isoform of the estrogen receptor beta cDNA (ERα isoform M cDNA) from the human testicular cDNA library, J. Steroid Biochem. Mol. Biol. 82 (2–3) (2002) 201–208.
[11] D.A. Schreihofer, M.H. Stoler, M.A. Shupnik, Differential expression and regulation of estrogen receptors (ERs) in rat pituitary and cell lines: estrogen decreases ERα protein and estrogen responsiveness, Endocrinology 141 (6) (2000) 2174–2184.
[12] D.A. Schreihofer, D.F. Rowe, E.F. Rissman, E.M. Scordalakes, J.J. Gustafsson, M.A. Shupnik, Estrogen receptor-α (ERα), but not ERα, modulates estrogen stimulation of the ERα-truncated variant, TERP-1, Endocrinology 143 (11) (2002) 4196–4202.
[13] S Ogawa, S. Inoue, T. Watanabe, A. Orimo, T. Hosoi, Y. Ouchi, M. Muramatsu, Molecular cloning and characterization of human estrogen receptor αcx: a potential inhibitor of estrogen action in human, Nucleic Acids Res. 26 (15) (1998) 3505–3512.
[14] J.T. Moore, D.D. McKee, K. Slentz-Kesler, L.B. Moore, S.A. Jones, E.L. Horne, J.L. Su, S.A. Kliewer, J.M. Lehmann, T.M. Willson, Cloning and characterization of human estrogen receptor α isoforms, Biochem. Biophys. Res. Commun. 247 (1) (1998) 75–78.
[15] S. Lewandowski, K. Kalita, L. Kaczmarek, Estrogen receptor α. Potential functional significance of a variety of mRNA isoforms, FEBS Lett. 524 (1–3) (2002) 1–5.
[16] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410.
[17] A. Sanyal, B.L. Riggs, T.C. Spelsberg, S. Khosla, Bone marrow stromal cells express two distinct splice variants of ER-α that are regulated by estrogen, J. Cell. Biochem. 94 (1) (2005) 88–97.
[18] W.J. Kent, BLAT—the BLAST-like alignment tool, Genome Res. 12 (4) (2002) 656–664.
[19] D. Swope, J.C. Harrell, D. Mahato, K.S. Korach, Genomic structure and identification of a truncated variant message of the mouse estrogen receptor α gene, Gene 294 (1–2) (2002) 239–247.
[20] D. Picard, V. Kumar, P. Chambon, K.R. Yamamoto, Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors, Cell Regul. 1 (3) (1990) 291–299.
[21] V. Kumar, P. Chambon, The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer, Cell 55 (1) (1988) 145–156.
[22] S.A. Onate, V. Boonyaratanakornkit, T.E. Spencer, S.Y. Tsai, M.J. Tsai, D.P. Edwards, B.W. O'Malley, The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors, J. Biol. Chem. 273 (20) (1998) 12101–12108.
[23] D.A. Schreihofer, E.M. Resnick, A.Y. Soh, M.A. Shupnik, Transcriptional regulation by a naturally occurring truncated rat estrogen receptor (ER), truncated ER product-1 (TERP-1), Mol Endocrinol. 13 (2) (1999) 320–329.
[24] N.A. Mitchner, C. Garlick, R.W. Steinmetz, N. Ben-Jonathan, Differential regulation and action of estrogen receptors α and α in GH_3 cells, Endocrinology 140 (6) (1999) 2651–2658.
[25] M. Kos, S. O'Brien, G. Flouriot, F. Gannon, Tissue-specific expression of multiple mRNA variants of the mouse estrogen receptor α gene, FEBS Lett. 477 (1–2) (2000) 15–20.
[26] T.A. Milner, B.S. McEwen, S. Hayashi, C.J. Li, L.P. Reagan, S.E. Alves, Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites, J. Comp. Neurol. 429 (3) (2001) 355–371.
[27] H. Mukai, T. Tsurugizawa, G. Murakami, S. Kominami, H. Ishii, M. Ogiue-Ikeda, N. Takata, N. Tanabe, A. Furukawa, Y. Hojo, Y. Ooishi, J.H. Morrison, W.G. Janssen, J.A. Rose, P. Chambon, S. Kato, S. Izumi, T. Yamazaki, T. Kimoto, S. Kawato, Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons, J. Neurochem. 100 (4) (2007) 950–967.
[28] H. Okamura, K. Yamamoto, S. Hayashi, A. Kuroiwa, M. Muramatsu, A polyclonal antibody to the rat oestrogen receptor expressed in *Escherichia coli*: characterization and application to immunohistochemistry, J. Endocrinol. 135 (2) (1992) 333–341.