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Complex organization of the 5′-untranslated region of the mouse estrogen receptor α gene: Identification of numerous mRNA transcripts with distinct 5′-ends

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ABSTRACT

The 5′-untranslated region (5′-UTR) of the estrogen receptor α (ERα) gene plays an important role in determining its tissue-specific expression. We examined the 5′-UTRs of the mouse ERα mRNA variants in depth using the Basic Local Alignment Search Tool (BLAST), rapid amplification of 5′-cDNA ends (5′-RACE) and RT-PCR. We demonstrated the presence of multiple variants containing unique 5′-UTRs. We mapped the cDNA sequences onto the mouse genome, and found that both alternative splicing from four different leader exons (A, C, F1, and H) to exon 1, and combinations of 12 internal exons (X1, X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, X11, and B) generate multiple ERα transcripts. Mouse exon B, that has homologies with human exon B and rat exon 0T, was used as an internal exon, not as a leader exon. RT-PCR analysis revealed distinct expression patterns of the variants, suggesting that the alternative promoter usage and alternative splicing are regulated in a tissue-specific manner. Our results indicate that the genomic organization of the mouse ERα gene is complicated as previously shown in the rat ERα gene. In addition, both alternative promoter usage and alternative splicing contribute to the remarkable mRNA diversity of the mouse ERα gene.

1. Introduction

Ovarian steroids, estrogens, are now recognized as pleiotropic hormones [1]. Estrogen receptors are critical mediators of estrogen signaling and functions and play pivotal roles in a wide variety of physiological processes in reproductive and non-reproductive organs. To date, two subtypes of nuclear estrogen receptors have been described in mammals: estrogen receptor α (ESR1, also known as ERα) and estrogen receptor β (ESR2, also known as ERβ) [2].

Alternative promoter usage and alternative splicing contribute to the mRNA diversity of a single gene. Transcription from the ERα gene is initiated from multiple promoters [3,4]. Five (A, B, C, F1, and H), four (0/B, 0N, 0S, and 0U), and seven (A, B, C, D, E1, F, and T1) promoters are known for the mouse, rat, and human ERα genes, respectively [5–17]. These multiple promoter systems are involved in tissue-specific and temporal regulation of ERα gene expression. Furthermore, alternative splicing between the leader and untranslated internal exons results in diverse mRNA variants with distinct 5′-untranslated regions (5′-UTRs) [5,9,18–21]. However, there are several disparities in the genomic organization and the splicing patterns among species. Our recent studies demonstrated more complicated genomic structures and splicing profiles for the rat and human ERα genes than previously thought [5,21]. The rat ERα gene possesses four leader exons (exons 0/B, 0N, 0S, and 0U) and eleven untranslated internal exons (exons I1, I2, I3, I4, I5, C/I6, I7, I8, I9, I10, and 0T), and has a potential to generate more than a thousand of 5′-UTR variants with alternative usage of leader exons and differing combinations of internal exons. In contrast, the splicing patterns of the mouse ERα 5′-UTR variants have been reported to be relatively simple: this gene was thought to have only one untranslated internal exon (exon F2), alternative insertion of which between exons F1 and 1 would generate two 5′-UTR variants (F1-1 and F1-F2-1) [9]. To date, six 5′-UTR variants of the mouse ERα gene (A-1, B-1, C-1, F1-1, F1-F2-1, and H-1) have been reported [9,12]. It remains to be determined whether these disparities in the gene structures and the splicing patterns between mouse and rat are due to species-specific differences or to a paucity of information about the mouse ERα gene.

The aim of the present study was to determine the genomic organization and structure of the 5′-UTR of the mouse ERα gene. To this end, we searched *in silico* for expressed sequence tag (EST) clones encoding mouse ERα variants with novel sequences, then

Abbreviations: 5′-RACE, Rapid amplification of 5′-cDNA ends; BLAST, Basic Local Alignment Search Tool; BLAT, BLAST-like alignment tool; DIG, Digoxigenin; ERα, Estrogen receptor α; ERβ, Estrogen receptor β; EST, Expressed sequence tag; UTR, Untranslated region.

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Table 1
Primer pairs used for RT-PCR cloning and identified amplicons.

Forward primer	Reverse primer	Identified amplicons
ExF1_F3	Ex1_R1	F1-1, F1-X2-1, F1-X4-1, F1-X11-1, F1-F2/X5-1, F1-X2-F2/X5-1, F1-X4-F2/X5-1, F1-F2/X5-X11-1, F1-X2-X4-F2/X5-1
ExX1_F	Ex1_R4	X1-1, X1-X2-1, X1-F2/X5-1, X1-X2-X4-1, X1-F2/X5-X11-1, X1-X2-F2/X5-1
ExX2_F	Ex1_R2	X2-1, X2-F2/X5-1, X2-X4-F2/X5-1, X2-X3$_S$-F2/X5-1, X2-F2/X5-X11-1
ExX3_F	Ex1_R4	X3-1, X3-X4-1, X3-F2/X5-1, X3-X4-F2/X5-1, X3$_S$-X4-F2/X5-X6-1
ExX4_F	Ex1_R2	X4-1, X4-F2/X5-1, X4-X10-1, X4-F2/X5-X11-1
ExF2/X5_F	Ex1_R1	F2/X5-1, F2/X5-X6-1, F2/X5-X7-1, F2/X5-X8-1, F2/X5-X10-1, F2/X5-X11-1, F2/X5-X6-X11-1
ExX6_F	Ex1_R2	X6-1, X6-X7-1, X6-X11-1, X6-X7-X11-1
ExH_F	Ex1_R1	H-1, H-X7-1, H-X11-1, H-X7-X11-1, H-X9-X10-1, H-X9-X10-X11-1
ExX7_F	Ex1_R3	X7-1, X7-X8-1, X7-X11-1, X7-X8-X11-1
ExX8_F	Ex1_R1	X8-1, X8-X11-1
ExX9_F	Ex1_R4	X9-1, X9-X10-1, X9-X11-1, X9-X10-X11-1
ExX10_F	Ex1_R4	X10-1, X10-X11-1
ExX11_F	Ex1_R4	X11-1
ExC_F2	Ex1_R1	C-1
ExB_F	Ex2_R	B-1-2[a]
ExA_F	Ex2_R	A-1-2[a]
ExF1_F3	ExX1_R	F1-X1
ExF1_F1	ExX2_R	F1-X2, F1-X1-X2
ExF1_F2	ExX3_R	F1-X3, F1-X2-X3, F1-X1-X2-X3
ExF1_F3	ExX4_R	F1-X4, F1-X2-X4, F1-X3$_S$-X4, F1-X2-X3$_S$-X4
ExF1_F1	ExF2/X5_R	F1-F2/X5, F1-X2-F2/X5, F1-X4-F2/X5, F1-X2-X4-F2/X5
ExF1_F3	ExX6_R	F1-F2/X5-X6, F1-X4-F2/X5-X6, F1-X3$_S$-X4-F2/X5-X6
ExF1_F1, 2, 3	ExH_R	Not detectable
ExF1_F3	ExX7_R	F1-X7, F1-F2/X5-X7, F1-X2-F2/X5-X7
ExF1_F1	ExX8_R	F1-F2/X5-X8, F1-F2/X5-X7-X8, F1-X3$_S$-X4-F2/X5-X7-X8, F1-X4-F2/X5-X6-X7-X8
ExF1_F3	ExX9_R	F1-X9, F1-F2/X5-X9
ExF1_F3	ExX10_R	F1-X10, F1-F2/X5-X10, F1-F2/X5-X9-X10
ExF1_F2	ExX11_R	F1-X11, F1-F2/X5-X11, F1-X4-F2/X5-X11
ExC_F1	ExB_R	C-B[a]

The detailed sequences of the forward and reverse primers are shown in Supplemental Material 1. Exons X3, X4, X6, and B have alternative splice sites. The subscript letter "S" on each exon indicates a short form. Absence of the subscripts on the exons indicates that both variants, long and short forms, were identified. The amplicons listed in this table were physically cloned and identified. Please note that the identification of amplicons is not comprehensive.

[a] 40 PCR cycles were required for detection.

cloned multiple mRNA variants with unique 5′-UTRs using rapid amplification of 5′-cDNA ends (5′-RACE) and RT-PCR, and mapped the newly cloned and the previously reported cDNA sequences onto the mouse genome. Furthermore, the expression profiles of the 5′-UTR variants were determined using RT-PCR and RT-PCR/Southern blotting.

2. Materials and methods

2.1. Animals

All experiments were performed with the approval of the Nippon Medical School Animal Care Committee. Ten-week-old male

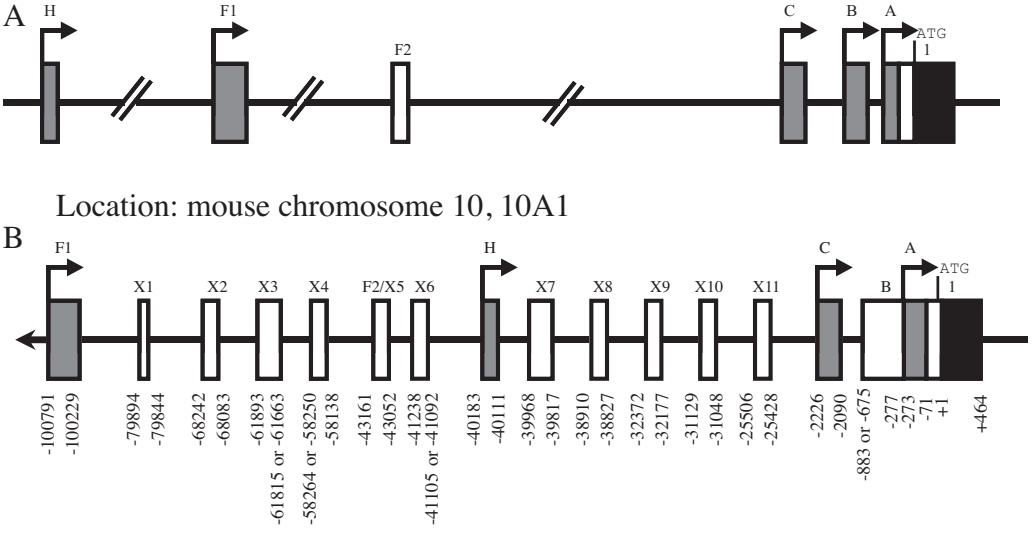

Fig. 1. Genomic organization of the mouse ERα gene. Schematic representations of the previously assumed mouse ERα gene structure (A) and the structure determined in the present study (B). The image and the nomenclature in Fig. 1A are based on the report of Kos et al. [3]. cDNA sequences of multiple mouse ERα variants are mapped on the Build 37 mouse genome assembly. The mouse ERα gene is located at 10A1 on mouse chromosome 10. The numbers below the ends of the exons correspond to the distance from the adenine of the canonical start codon, which is located at the nucleotide position 5634157 on mouse chromosome 10, and is defined as nucleotide position +1 in Fig. 1B. Exons X3, X4, X6, and B have alternative splice sites. The numbers separated by "or" indicate the positions of the alternative splice sites. The black, gray and white boxes indicate coding regions, untranslated leader exons, and untranslated internal regions, respectively. The arrow on the central line indicates the orientation of the chromosome. The bent arrows on the exons symbolize the putative transcription start sites. Nucleotide sequences are shown in detail in Supplemental Material 2. The images are not to scale.

and female C57BL/6J mice (three males and six females for 5′-RACE, and four males and twelve females for RT-PCR) were used in the present study. The mice had free access to water and chow, and were kept under a 14-h light, 10-h dark cycle.

2.2. Total RNA isolation

Mice were decapitated under ether anesthesia, and mouse tissues were quickly removed and stored in liquid nitrogen until use. The non-reproductive organs (the adrenal, brain, intestine, kidney, liver, lung, spleen and stomach), epididymis and testis were collected from male mice, and the ovary and uterus were from female ones. Total RNA was extracted using Isogen (Nippongene, Tokyo, Japan) following the manufacturer's instructions. Total RNA was then treated with *Turbo* DNase (RNase-free DNase I; Ambion, Austin, TX), and purified. The concentration was quantified by absorption at 260 nm.

2.3. 5′-RACE cloning

CapFishing 5′-RACE was performed in the following manner. Total RNA isolated from the kidney, liver, ovary, testis and uterus was reverse-transcribed in the RT reaction mixture with a gene-specific primer designed on mouse ERα exon 1, 0.1 mg/ml BSA and $MnCl_2$. The reaction was carried out at 42 °C for 60 min, then added with the CapFishing adapter (Seegene, Seoul, Korea) and 100 U of fresh reverse transcriptase (RTase), and incubated at 42 °C for 30 min. The reaction was stopped by heating at 75 °C for 15 min and treated with RNase H (TaKaRa Bio, Shiga, Japan) at 37 °C for 30 min. 5′-RACE products were amplified in two-round of PCR with a common 5′-RACE universal primer (Seegene) and gene-specific primers.

The conditions for the first- and second-round PCRs consisted of 28 cycles 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 a 50-μl of reaction mixture comprising the 5′-RACE cDNAs 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 for 5′-RACEs are listed in Supplemental Material 1. 5′-RACE products were excised after agarose electrophoresis, purified, and cloned into pGEM-T-Easy vectors (Promega, Madison, WI).

2.4. RT-PCR

Total RNA was reverse-transcribed into first-strand cDNA using an oligo(dT) primer. The RT reaction mixtures (final volume, 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* (RNase inhibitor; Promega), and 200 U ReverTra *Ace* (M-MLV reverse transcriptase, RNase H(−); Toyobo, Osaka, Japan). The reaction was carried out at 42 °C for 60 min, and stopped by heating at 75 °C for 15 min. cDNA was treated with RNase H at 37 °C for 30 min and stored at −20 °C until use.

PCR was performed in 25 μl of reaction 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 in three steps, with a PCR cycle reaction 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 oligonucleotide primers used for RT-PCR are listed in Supplemental Material 1, and the information on the primer pairs is shown in Table 1.

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 cloned into pGEM-T-Easy vectors (Promega). Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Fluorescent signals were detected using ABI PRISM 310 or 3100 Genetic Analyzers (Applied Biosystems).

2.7. Southern blotting

Electrophoresed PCR products were transferred to nylon membranes (Hybond N+; GE Healthcare, Fairfield, CT), and then subjected to Southern blot analysis. Hybridization was carried out for 16 h at 45 °C in 6× standard saline citrate (SSC: 1× SSC is 150 mM NaCl and 15 mM sodium citrate), 0.5% SDS, 10 μg/ml salmon sperm DNA, 0.2% BSA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 50 mM Tris–HCl (pH 8.0) with digoxigenin (DIG)-labeled probes. Filters were washed at 45 °C for 20 min, twice in 1× SSC and 0.1% SDS, and twice in 0.2× SSC and 0.1% SDS. The hybridized probes were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche Diagnostics, Mannheim, Germany). The results were visualized using a chemiluminescent substrate (CSPD; Roche Diagnostics) and a Light-Capture Cooled CCD Camera System (ATTO, Tokyo, Japan).

3. Results

3.1. Identification of mouse ERα variants with novel 5′-UTRs

Fig. 1A shows the previously assumed structure of the 5′-UTR of the mouse ERα gene. The nomenclature of promoters and exons are based on the system proposed in Kos et al. [3]. At least six upstream exons (A, B, C, F1, F2, and H) were reported in the ERα gene of the mouse [9,12].

Through an extensive homology search in DDBJ/EMBL/Genbank using the Basic Local Alignment Search Tool (BLAST) [22] and the sequences of exons F1, F2, and 1 as queries, we identified three EST clones (BB634555, BY021551, and BY236474) containing novel inserted sequences between exons F1 and F2, or exons F2 and 1. To confirm their authenticity and to determine their positions in the mouse genome, we mapped the EST clones onto the Build 37 mouse genome assembly using the BLAST-like alignment tool (BLAT) [23]. Each inserted sequence was composed of one or two untranslated internal exon(s). We named the exons inserted between exons F1 and 1 as exons "X" and numbered them according to 5′–3′ order in the gene. The EST clones correspond to F1-$X3_L$-$X4_L$-1 for BB634555, F1-F2/X5-X11-1 for BY021551, and F1-$X4_L$-F2/X5 for BY236474 (Exons X3 and X4 have alternative splice sites, and alternative usage of the splice sites generates long and short forms of the exons. The subscript letter, "L", indicates a long form.).

To further identify mouse ERα splice variants with novel 5′-UTRs and to characterize novel untranslated exons, we performed 5′-RACE and RT-PCR cloning. CapFishing 5′-RACE cDNAs were prepared from kidney, liver, ovary, testis, and uterus total RNAs, and amplified using a CapFishing universal sense primer and antisense

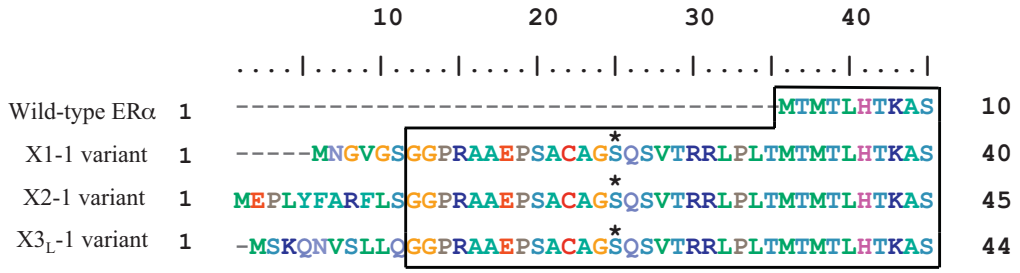

Fig. 2. Amino acid sequences of mouse wild-type ERα, X1-1, X2-1, and $X3_L$-1 variant proteins. The amino acid sequences of N-terminal extremities of mouse wild-type ERα, X1-1, X2-1, and $X3_L$-1 variant proteins predicted from their mRNA transcripts are shown. The asterisks indicate putative phosphorylation sites.

primers located on exon 1. We identified 11 independent 5′-RACE clones corresponding to C-1, H-1, F1-1, F1-X2-1, F1-X4-1, F1-F2/X5-1, F1-X11-1, F1-X2-F2/X5-1, F1-X4-F2/X5-1, F1-F2/X5-X11-1, and F1-F2/X5-X7-X11-1 (The absence of the subscripts on exon X4 indicates that both variants, long and short forms, were identified.). H-1 and F1-F2/X5-X7-X11-1 variants were amplified only in the liver and the other clones were identified in all five organs tested. For RT-PCR cloning, oligo-dT-primed cDNAs were prepared from kidney, liver, ovary, testis, and uterus total RNAs, and amplified using PCR primers designed on the respective leader and internal exons, and exon 1. Variants identified using RT-PCR are listed in Table 1. In total, we newly determined ten untranslated internal exons (exons X1, X2, X3, X4, X6, X7, X8, X9, X10, and X11). The overall structure of the mouse ERα 5′-flanking region is schematically represented in Fig. 1B. Nucleotide sequences are shown in detail in Supplemental Material 2.

Mouse exon H is highly homologous to rat exon C/I6 [9,24]. However, rat exon C/I6 is not a leader exon but an internal exon utilized by the 0S isoforms that are counterparts of the mouse F1 isoforms [5]. To determine whether exon H is a leader exon or not, we performed 5′-RACE with antisense primers located on exon H and RT-PCR with primers designed on exons F1 and H. No splicing events between exons F1 and H were observed in either experiment (data not shown). Mouse exon B was assumed to be a leader exon. However, we demonstrated that mouse exon B is an internal exon utilized by the C isoforms (Supplemental Material 3).

Exons X3, X4, X6, and B have alternative splice sites, which contribute to further diversity of mouse ERα transcripts. The utilization of untranslated internal exons is somewhat exclusive: only F1 isoforms utilize exons X1, X2, X3, X4, F2/X5, X6, and X8, and the C isoforms use exon B. Exons X7, X9, X10, and X11 are used by both F1 and H isoforms. The splice acceptor site of exon 1 is common to the F1, H, and C isoforms. Although the gene has the potential to generate thousands of 5′-UTR mRNA variants, almost all transcripts identified in the present study are predicted to encode identical ERα proteins because there are in-frame termination codons upstream of the canonical start codon. The exceptions are the variants generated by direct splicing events from exons X1, X2, and $X3_L$ to exon 1. The X1-1 (F-X1-1), X2-1 (F-X2-1 and F-X1-X2-1), and $X3_L$-1 (F-$X3_L$-1, F-X1-$X3_L$-1, F-X2-$X3_L$-1, and F-X1-X2-$X3_L$-1) variants potentially encode ERα proteins with 30, 35, and 34 additional N-terminal amino acids, respectively (Fig. 2). These additional amino acid sequences contain putative phosphorylation motifs, suggesting possible regulation(s) and/or putative function(s) of the variants. The putative start codon in exon X2 has a typical Kozak consensus sequence (ACCATGG).

3.2. Expression patterns of mouse ERα mRNA isoforms

The expression pattern of each mouse ERα mRNA isoforms was analyzed using RT-PCR with a specific forward primer designed from a respective leader exon (Fig. 3). To assess the overall expression of the mouse ERα gene, the coding region between exons 5 and 7 was amplified (Fig. 3A). The expression of mouse ERα mRNAs was detected in all tissues tested. The expression level was high in the reproductive organs, adrenal, kidney, and liver, moderate in the brain, lung, and spleen, and low in the intestine and stomach. The F1 isoforms were widely expressed (Fig. 3B). At low PCR cycle numbers (27 and 30 cycles), two bands corresponding to F1-1 and F1-F2-1 were amplified. Using a greater number of PCR amplification cycles (33 cycles), we observed multiple amplicons of the F1 isoforms, which were generated by various insertions of alternatively spliced internal exons (exons X1, X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, and X11). The expression of the H isoforms was restricted in the epididymis, kidney, liver, and testis. Internal exons X7, X9, X10, and X11 were alternatively utilized by the H isoforms (Fig. 3C). The C isoforms were expressed highly in the reproductive organs, adrenal, and liver, and moderately in the brain (Fig. 3D). The expression level of the A isoforms was extremely low, and could not be detected using 30–36 cycles of PCR (Fig. 3E).

3.3. Expression patterns of mouse ERα mRNA variants containing untranslated internal exons

To determine the expression patterns of mRNA variants containing untranslated internal exons, RT-PCR/Southern blot analysis was performed with primers designed from respective internal exons and exon 1, and with a DIG-labeled oligonucleotide probe against exon 1 (Fig. 4). Consistent with the tendency of 5′-RACE cloning, the variants containing exons X2, X4, F2/X5, or X11, were detectable at a relatively low number of PCR cycles (30 cycles for F2/X5-containing variants and 33 cycles for X2-, X4-, or X11-containing variants). Each internal exon-containing variant was preferentially expressed in certain organs, suggesting that the alternative splicing may be regulated in a tissue-specific manner.

4. Discussion

Recently, we identified the complexity of the rat ERα gene [5]. The present study shows that, similarly, the mouse ERα gene is also a complicated transcription unit, with alternative promoter usage and alternative splicing generating multiple mRNA transcripts. The previous image of the mouse ERα gene structure was relatively simple: transcription was initiated from one of five promoters (promoters A, B, C, F1, and H), and one internal exon, exon F2, was alternatively inserted between exons F1 and 1. Consequently, six 5′-UTR variants that is, A-1, B-1, C-1, F1-1, F1-F2-1, and H-1, could be generated [9,12]. However, our analysis of mouse ERα mRNAs has revealed the presence of numerous splice variants with unique 5′-UTRs. These variants are based on four leader exons (exons A, C, F1, and H) and twelve internal exons (exons X1,

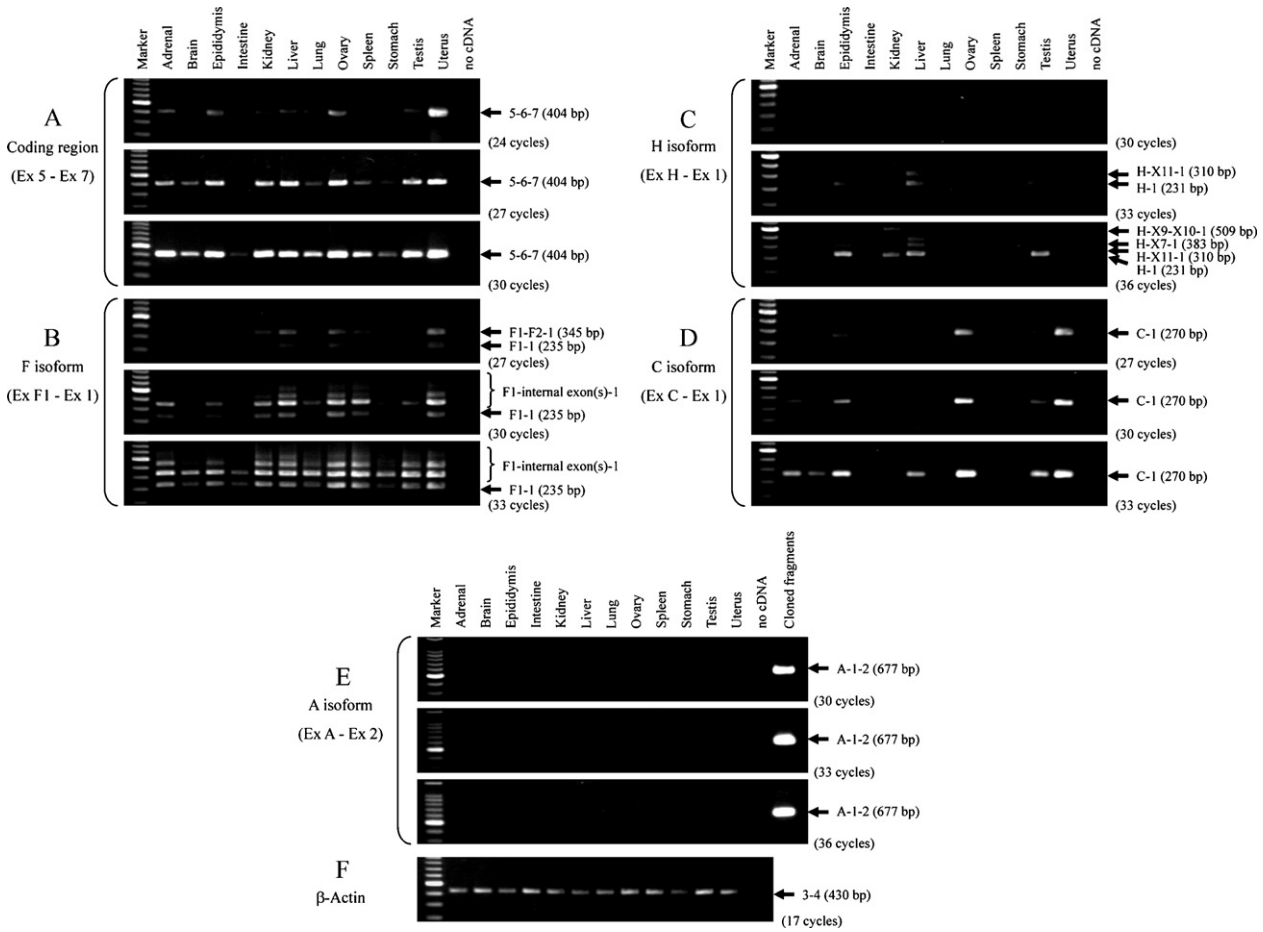

Fig. 3. Expression patterns of mouse ERα mRNA isoforms. The tissue distribution of mouse ERα mRNA isoforms (F1, H, C, and A isofroms) was analyzed using RT-PCR. Total RNAs were subjected to RT-PCR using specific primers designed on the respective leader exons (exons F1, H, C, and A) and exon 1. To assess the overall ERα expression, the coding region between exons 5 and 7 was amplified. Expression of total ERα isoforms (A), F1 isoforms (B), H isoforms (C), C isoforms (D), A isoforms (E), and β-Actin (F). β-Actin was used as an internal control. The number of PCR cycles is indicated on the right bottom of each panel. From the upper to the lower panels, the number of PCR cycles increases in increments of three cycles. The similar results were observed for four separately prepared samples.

X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, X11, and B). Exon B, which was regarded as a leader exon, was demonstrated to be an internal exon.

Kos et al. [9] cloned A-1, C-1, and F1-1 mouse ERα variants using 5′-RACE and an F1-F2-1 variant using RT-PCR, and further identified a B-1 variant from the analogy with the human ERα gene. Although they used RT-PCR analysis to determine the expression of F1 isoforms, the main analysis was performed using S1 nuclease protection assays. The assays can be used to estimate the relative abundance of particular mRNAs with specific probes, but are not suited to determine the splicing patterns. Although our RT-PCR and RT-PCR/Southern blotting analyses did not provide a quantitative determination of variant mRNAs, they allowed us to find larger number of splice variants. Kos et al. [9] deduced the existence of further upstream exons or alternatively spliced mRNA variants from the discrepancy between the total amount of ERα and the sum of the relative amounts of A-1, B-1, C-1, F1-F2-1, and H-1 variants. Therefore, the presence of numerous splice variants identified in the present study can resolve, at least, one part of the discrepancy.

Compared with the human and rat ERα genes, the mouse gene has conserved nucleotide sequences in the coding exons and in untranslated exons B, C, and F1. Between mouse and human, mouse exon A is equivalent to human exon A, however, the mouse A isoforms were scarcely expressed in all tissues tested: this agrees with a previous report [9]. Between mouse and rat, mouse exon H is homologous to rat exon C/I6, though exon C/I6 is not a leader exon but an internal exon [5]. Mouse internal exons X1-11 are not homologous to rat internal exons I1-10 or human internal exons E2 and E3. The splicing patterns of the mouse F1 isoforms are different from those of human and rat counterparts. The main variants of the mouse F1 isoforms are F1-1 and F1-F2-1. In contrast, the main splicing event among the rat 0S isoforms, that are counterparts of the mouse F1 isoforms, is direct splicing to exon 1 [5,6,18]. In humans, an F-E2-1 variant is predominantly expressed [11,17], although the existence of several variants in F isoforms has been reported [20,21]. These differences in the genomic organization and splicing patterns among species suggest that, to a certain extent, the ERα gene might be regulated in a species-specific manner.

The mouse ERα gene is known to be highly expressed in the reproductive organs, and is widely distributed in the non-reproductive organs. Four potential promoters direct these tissue-specific expression patterns of the mouse ERα gene. Among four alternative isoforms in mouse, the C and F1 isoforms are detectable at relatively low numbers of PCR cycles, suggesting that promoters C and F1 are predominantly important in the mouse ERα expression. The distribution of the mouse isoforms indicates that the abundant expression in the reproductive organs is mainly due to the utilization of promoter C, and that promoter F1 contributes to the wider distribution in the non-reproductive organs.

In addition to the differential promoter usage, alternative splicing contributes to mRNA 5′-heterogeneity of the mouse ERα gene. Each isoform except A possesses several splice variants. The splicing

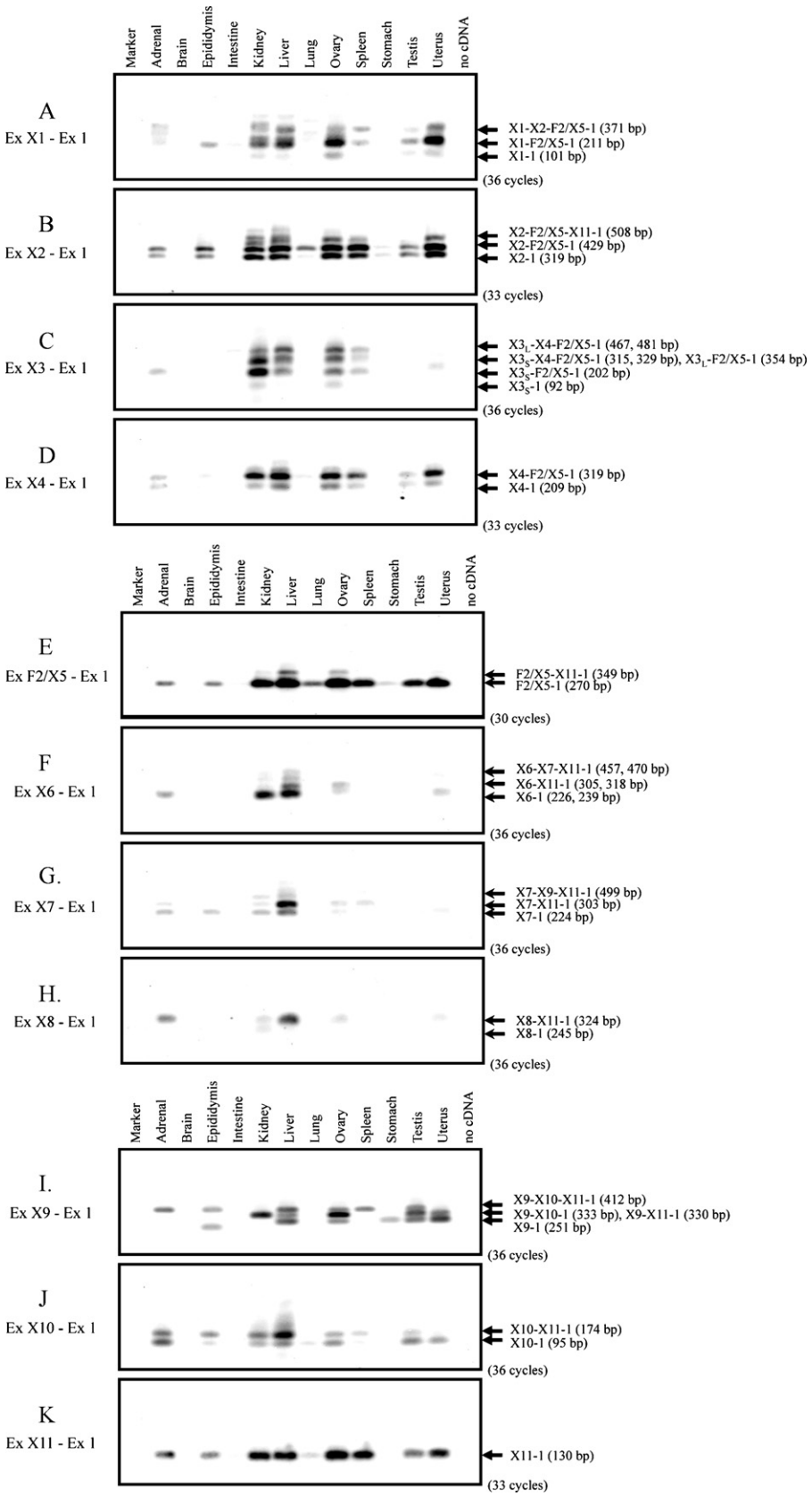

Fig. 4. Expression patterns of mouse ERα mRNA variants containing untranslated internal exons (exons X1-11). The tissue distribution of mouse ERα mRNA variants containing untranslated internal exons (exons X1, X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, and X11) was analyzed using RT-PCR/Southern blotting. Total RNAs isolated were subjected to RT-PCR using specific primers designed on the respective internal exons and exon 1. The number of PCR cycles is indicated on each panel. An oligonucleotide probe located on exon 1 was used for Southern blot analysis. The "Marker" lanes are shown to confirm the specificity of the probe, not to indicate the molecular weight. The similar results were observed for four separately prepared samples.

patterns of the F1 isoforms are particularly complicated. Alternative combinations of exons X1, X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, and X11 are inserted between exons F1 and 1. We also found several splice variants of the H isoforms: H-1, H-X7-1, H-X11-1, H-X7-X11-1, H-X9-X10-1, and H-X9-X19-X11-1. RT-PCR with primers on exons C and 1 amplified only the C-1 variant because the expression level of C-B-1 was extremely low compared with that of C-1. However, using primers on exons C and B, we observed the splicing events between exons C and B. Moreover, the usage of alternative splice sites of exons X3, X4, X6, and B increases the diversity of variants.

All of human ERα 5′-UTR variants containing exon 1 encode identical ERα proteins because there is an in-frame termination codon upstream of the translational start codon in exon 1 [21]. In contrast, mouse 5′-UTR variants have the potential to generate different ERα proteins due to the lack of in-frame stop codons in the UTR of exon 1. In numerous mouse 5′-UTR variants, only seven variants, that is, F-X1-1, F-X2-1, F-X1-X2-1, F-X3$_L$-1, F-X1-X3$_L$-1, F-X2-X3$_L$-1, and F-X1-X2-X3$_L$-1, potentially encode ERα proteins with longer N-termini. Several studies have demonstrated the presence of C-terminally and N-terminally truncated ERα variants in the mouse [25–27], while the existence of mouse ERα variants with extra-N-termini has not been reported. Therefore, to our knowledge, the present study is the first report that identified mRNA variants encoding mouse ERα proteins with additional N-terminal amino acids.

Although the seven ERα mRNA variants potentially encode different ERα proteins, almost all mRNA variants analyzed in this study are predicted to be translated into identical proteins because most of the untranslated leader and internal exons have in-frame termination codons. The 5′-ends of mRNAs encoding estrogen-related genes such as ERα, ERβ, and P450 aromatase have been reported to contribute to posttranscriptional regulation through the modulation of mRNA stability and/or translational efficiency [21,28–30]. Kos et al. [28] showed that the 5′-UTRs of mouse and human ERα affected their translational efficiency, and that the 5′-UTRs of mouse F1-F2-1 and human counterpart, F-E2-1, variants repressed translation. Our recent study also revealed that several 5′-UTRs of human ERα F isoforms decreased the translation efficiency but had no effect on mRNA stability [21]. Regarding the 5′-UTRs of human ERα, the untranslated upstream ORFs in the 5′-regions are mainly involved in the translation processes [21,28]. It is implied that the diverse structures among the 5′-UTRs of the mouse ERα transcripts support the appropriate expression of the gene.

In conclusion, we have identified twelve internal exons (exons X1, X2, X3, X4, F2/X5, X6, X7, X8, X9, X10, X11, and B) in the 5′-UTR of the mouse ERα gene, and have characterized the numerous mouse ERα mRNA variants generated by alternative promoter usage and alternative splicing. We have thus comprehensively defined the genomic organization and structure of the mouse ERα gene. These findings contribute to a further understanding of the regulatory mechanisms of mouse ERα gene expression.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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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.2011.03.004.

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