NOTE

Novel Splicing Events of Untranslated First Exons in Human Estrogen Receptor Alpha (ER α) Gene

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Abstract. In order to analyze the structures of the 5'-untranslated region of estrogen receptor alpha (ER α) mRNA in human uterine endometrium (Em), total RNA from Em was analyzed by 5'-rapid amplification of the cDNA ends method with antisense primer located on exon 1 of human ER α gene. Three isoforms of 5'-RACE clones were obtained: ER α mRNAs containing exon (A) (the upstream region of exon 1), exon C, and exons F-E2 (we adopted the nomenclature of 5'-untranslated exons of the Gannon group). The results imply that the major isoforms of ER α mRNA expressed in Em are these three isoforms. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) analysis was carried out on Em, ovary (Ov) and liver (Li) mRNAs to detect the novel isoforms of ER α mRNA in these tissues, using sense primers located on exons (A), B, C, F, and E1, and antisense primer located on exon 1. As a result, in addition to the previously reported ER α mRNA isoforms containing exons (A), B, C, F-E2 and E1-E2 on exon 1, we identified two novel isoform mRNAs in which exons F and E1 were directly spliced onto exon 1. Differential distributions of these isoforms of ER α mRNAs in Em, Ov and Li were demonstrated by RT-PCR-Southern blot analysis. These results, together with the previous reports by others, indicate that there are at least ten isoforms of ER α mRNA containing different 5'-untranslated regions, exons (A), B, C, D, T1-T2, T1, F-E2, F, E1-E2 and E1, expressed in human, and that these are involved in tissue specific expression of the gene.

Key words: Estrogen receptor alpha gene, Messenger ribonucleic acid, Untranslated exons, Alternative splicing, Human

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PREVIOUS reports have revealed that multiple 5'-untranslated first and second exons exist in human estrogen receptor alpha (ER α) gene, and that tissue specific expression of the gene is regulated, at least in part, by the multiple promoters located on 5'-untranslated first exons. The existence of six 5'-untranslated first exons, exons B, C, D, T1, F and E1, as well as two 5'-untranslated second exons; exons T2 and E2, has so far been reported (Fig. 1) [1–9].

Although various names had been applied to these 5'-untranslated exons, we adopted the most recent nomenclature by the Gannon group [5, 7, 9] in the present paper, while the other nomenclatures in the previous reports are indicated in the inset of Fig. 1. From these exons, it is reported that eight isoforms of ER α mRNA with different 5'-untranslated regions (UTRs) are expressed in various tissues to regulate tissue specific expression of the gene; these are ER α mRNA transcribed from exon (A) that is termed ER α mRNA ((A)-1) (the transcript from the upstream region of the exon 1; since this region is not an independent exon, the "A" is put in parentheses), ER α mRNAs with exon B on exon 1 (ER α mRNA (B-1)), exon C (ER α mRNA (C-1)), exon D (ER α mRNA (D-1)), exons T1 and T2 (ER α mRNA (T1-

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T2-1)), exon T1 (ER α mRNA (T1-1)), exons F and E2 (ER α mRNA (F-E2-1)), and exons E1 and E2 (ER α mRNA (E1-E2-1)) (Fig. 1) [1-9].

In addition, our previous studies on 5'-UTR of rat ER α mRNA have demonstrated the presence of two 5'-untranslated first exons, exons 0N [10] and 0S [11], in rat ER α gene. These studies indicated that at least three isoforms of ER α mRNAs containing exons 0 (the first reported 5'-UTR form of rat ER α mRNA [12]), 0N and 0S on exon 1 are expressed in rat. Rat exons 0 and 0S are the homologues of human exons

C and F, respectively, but the homologue of rat exon 0N has not been identified in human gene so far. Therefore, we considered that the previously unidentified 5'-untranslated exons, including the homologue of rat exon 0N, might exist in human gene. Moreover, exon F was spliced onto untranslated second exon E2 that was spliced onto exon 1 in human. On the other hand, exon 0S [11], the homologue of human exon F, is directly spliced onto exon 1 in rat. Thus, we have also hypothesized that a novel isoform of ER α mRNA in which exon F is directly spliced

Fig. 1. Schema of 5'-untranslated exons of human ER α gene and locations and directions of PCR and 5'-RACE primers. Human ER α gene contains eight 5'-untranslated exons, termed exons B, C, D, T2, T1, E2, F and E1 by the Gannon group [5, 7, 9]. Various names have been defined on these exons by the previous reports listed in the inset [1-5]. Note that the same alphabet was defined on the different exons, and one exon name was defined on the region consists of two exons (indicated by # and ##) in the previous reports. Transcription is started from 5'-untranslated first exons; exons B, C, D, T1, F and exon E1, as well as the upstream region of exon 1 (the exon (A), because this region is not a independent exon, "A" is put in the parentheses). The previously reported splicing manners of these exons are indicated by solid lines [1-5]. Eight isoforms of ER α mRNAs containing the different 5'-UTRs were previously identified, in which exons (A), B, C, D, exons T1-T2, exon T, exons F-E2 and exons E1-E2 are spliced on exon 1 [1-5]. The present study revealed the previously unidentified splicing manner indicated by dashed lines which generate two novel isoforms of the ER α mRNAs, in which exon F and exon E1 are directly spliced on the exon 1. Location and direction of 5'-RACE and PCR primers in this region are indicated by arrows.

onto exon 1 might exist in human.

To analyze the structure of 5'-UTRs of ER α mRNA in human uterine endometrium (Em), we carried out the 5'-rapid amplification of the cDNA ends (RACE) method [13] on RNA from the tissue using an anti-sense primer located on exon 1 of ER α gene. Moreover, in order to identify the novel splicing events of previously reported 5'-untranslated exons, reverse transcription-polymerase chain reaction (RT-PCR) was carried out on RNAs from Em, ovary (Ov) and liver (Li) using sense primers located on exons (A), B, C, F and E1, and antisense primer located on exon 1.

Materials and Methods

Tissues and RNA extraction

Normal uterine endometrium, ovary and liver were collected after obtaining the patient's informed consent according to guidelines of our institute. Uterine endometrium in proliferative phase (Em) and normal ovarian tissue (Ov) were collected from a resected uterus during an operation for uterine leiomyoma. Liver tissue (Li) was collected from the surrounding part of a resected liver tumor during an operation for liver hemangioma. Histological examination of adjacent regions of these samples did not show any abnormal findings. Total RNA was extracted from the tissues by a guanidine-cesium chloride method. The RNA concentration was determined by UV absorption.

Primers for 5'-RACE and PCR

The locations and directions of primers for 5'-RACE and PCR in human ER α gene are shown in Fig. 1. The nucleotide sequences of 5'-RACE and PCR primers were as follows: RACE I: 5'-CTCGAG GTCGACTCTAGATTTTTTTTTTTTTTTTTT-3' [13], RACE II: 5'-CTCGAGGTCGACTCTAGA-3' [13], hEAs (forward): 5'-GAGCTCGCGTGTCTGGCGGG ACAT-3', hEBs (forward): 5'-CTGGATCGTCTTTC GCGTT-3', hECs (forward): 5'-GACTTCTACAAG CCCATGGA-3', hEFs (forward): 5'-TCTCCTGCGa ATTCTATAG-3', hEE1s (forward): 5'-TCTagAAAA TCTGATACCAATCCT-3', hE2s (forward): 5'-TTA TGGAGTCTGGTCCTGTG-3', hE1Ras (reverse): 5'-gaaTTCAGGGGCTCCAGCTCGTT-3', hE1as (re-

verse): 5'-aaGCTTGCTGTTGTCCACGTAC-3', hE5as (reverse): 5'-GCCAGACGAGACCAATCATC-3', hBAAs (forward): 5'-ACCGCGAGAAGATGACCCAG-3', hBAAs (reverse): 5'-GTCGCCCTGGACTTTCGAGC A-3'. The sequences of primers hEAs, hEBs, hECs, hEFs, hEE1s, hE2s, hE1Ras, hE1as and hE5as were derived from the sequences of the respective exons (indicated by the number or alphabet) of human ER α gene [1-9, 14-16]. The sequence of primers hBAAs (forward) and hBAAs (reverse) were derived from exons 2 and 3 of human beta actin gene [17], respectively. Some nucleotide substitutions indicated by the lower case letters were introduced into the primers to facilitate subcloning of the products. In order to distinguish the product of the RNA, from the product of the genomic DNA, the primer pairs used in reverse transcription (RT)-PCR flanked the region including one or more introns on genomic DNA (Fig. 1) except for the primer pair hEAs and hE1as (see the section of "RT"-PCR without RTase).

5'-RACE

5'-RACE libraries were constructed as described by Frohman *et al.* [13]; in brief, about 10 μ g of total RNA from Em was reverse transcribed with the primer hE1as (Fig. 1). The poly A tailed cDNA was subjected to PCR with primers hE1Ras, RACE I, and RACE II. PCR was carried out for 60 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min as described below. The products were subcloned into the pBS M13+ vector (Stratagene, La Jolla, CA, USA). The RACE libraries were screened by ³²P-labeled human ER α (hECs/hE1as) cDNA probe, which was synthesized by the random priming method using the RT-PCR product from Em with primers hECs and hE1as, as a template. The nucleotide sequences of the positive clones were determined by a dideoxy method.

Screening and sequencing of human ER α genomic DNA

The human genomic DNA library constructed from peripheral leukocytes was purchased from Clontech (Cat. #CLHL1111j, Palo Alto, CA, USA). The library was screened by the Sal I-Bgl II fragment of the 5'-RACE clone Em 74 isolated in the present study. This clone Em 74 included exon F (Fig. 2).

Fig. 2. Nucleotide sequence of 5'-RACE clone Em 74.

The nucleotide sequence of the 5'-RACE clone Em 74 that contains the longest 5'-UTR including exons F and E2 is indicated. The nucleotide of A of translation initiation codon in exon 1 (indicated by #) is assigned as the position number +1 [10–12]. The sequences of primers for 5'-RACE are indicated by lower case letters. Exon/exon junctions are indicated by solid triangles [3, 4]. The underline indicates the position of PCR primer hEFs. The nucleotide of the position number -316 of exon F (indicated by *) is "A" in the present study. The nucleotide at this position is reported as "A" by Flouriot *et al.* [4], but as "T" by Schuur *et al.* [15].

The positive clone termed G351 was isolated, subcloned and sequenced.

RT-PCR

The cDNA was synthesized from 200 ng of total RNA from each tissue (Em, Ov and Li) with 2.0 units of RAV-2 reverse transcriptase (RTase) (Takara, Kyoto, Japan) in a 10 µl reaction volume at 42°C for 60 min. PCR was performed as described by the manufacturer (Perkin Elmer, Branchburg, NJ, USA) with minor modifications. Briefly, 1 µl of the synthesized cDNA was amplified in a 10 µl PCR reaction volume containing 0.25 units of *Taq* DNA polymerase (Perkin Elmer), and 1.5 mM MgCl₂ (for primers hEAs/hE1as, hEBs/hE1as, hECs/hE1as, hEFs/hE1as and hBAs/hBAas) or 1.0 mM MgCl₂ (for primers hEE1s/hE1as and hE2s/hE5as) and 0.5 µM each of the respective primers. The samples were

amplified for 16 cycles (for primers hBAs/hBAas), 32 cycles (for primers hEBs/hE1as, hEFs/hE1as, hEE1s/hE1as and hE2s/hE5as) or 40 cycles (for primers hEAs/hE1as and hECs/hE1as) by incubation at 94°C for 1 min, 55°C (for primers hEBs/hE1as, hECs/hE1as, hEFs/hE1as, hEE1s/hE1as, hE2s/5as and hBAs/hBAas) or 60°C (for primers hEAs/hE1as) for 1 min, and 72°C for 1 min. The RT-PCR products were subcloned into PBS vector and sequenced.

Southern blotting

Two microliters of the RT-PCR products from each tissue were electrophoresed in 2.0% agarose gel and analyzed by Southern blotting as described previously [10, 11]. The hybridization signal was analyzed by BAS-2000 image analyzer (Fuji Film, Tokyo, Japan).

"RT"-PCR-Southern blotting without RTase and RT-PCR blank

Since the region flanked by the primer pair hEAs/hE1as does not containing any introns on the genomic DNA, we carried out "RT"-PCR-Southern blotting without the RTase on the RNA samples from the same tissues under the same conditions. However, no signals were generated, indicating that the signals with primers hEAs and hE1as under the present RT-PCR conditions originated from the mRNA (data not shown). Moreover, in order to examine whether contamination of reagents occurred, distilled water was subjected to RT-PCR (Bl), but no specific signal was obtained from Bl (Fig. 4A-F, lane Bl), indicating no contamination in these experiments.

Results

5'-RACE clones from Em

The structures of 5'-UTR of ER α mRNA in Em were analyzed by 5'-RACE with antisense primer located on exon 1. Twenty-one positive 5'-RACE clones were obtained: 2 clones derived from ER α mRNA ((A)-1), 5 clones originated ER α mRNA (C-1) and the remaining 14 clones corresponded to

ER α mRNA (F-E2-1)). No clones derived from the other previously identified mRNA isoforms in human or the homologue of rat exon 0N [10] were found.

The nucleotide sequence of the 5'-RACE clone, clone Em 74, that contained the longest 5'-UTR including exon F and exon E2 is shown in Fig. 2. The nucleotide at the position -316 of exon F (indicated by * in Fig. 2) is "A" in the clone Em 74. In the previous reports, the nucleotide at the same position is reported as "A" by Flouriot *et al.* [4], but as "T" by Schuur *et al.* [6].

Genomic analysis of exon F

In order to confirm the nucleotide at the position -316 of exon F, genomic cloning of the exon was carried out. The nucleotide at the position -316 is "A" in the clone G351 (Fig. 3 "j"). In addition, although the nucleotides at the positions -336 and -333 have been reported as "A and C" by Flouriot *et al.* [4] and "G and G" by Schuur *et al.* [6], the nucleotides at the same positions are "A and G" in the clone G351 (Fig. 3, "e" and "f").

Novel splicing event of exon F and exon E1

RT-PCR with primers hEFs/hE1as on Em RNA generated two different molecular sizes of the products (Fig. 4D). Sequence analysis revealed that the

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-375          ---atttcatgggcataacagcgtcctgtcta
                                a      b

-346  ccgactcagaaacggattttaccAAACTGAAATGCAGGCTCCATGCTCAGAAGCTCTTT
      c      d e f      gh i      j      k      l m

-286  AACAGGCTCGAAAGGTCCATGCTCCTTTCTCCTGCCCATTCTATAGCATAAGAAGACAGT

-226  CTCTGAGTGATAATCTTCTCTTCAAgtaggtaggtagtactcc---

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Fig. 3. Nucleotide sequence of insert of genomic clone G351 (exon F)

The genomic clone G351 was obtained by screening of the human genomic library with the Sal I-Bgl II fragment of 5'-RACE clone Em 74 which includes exon F. The nucleotide number is defined in the same manner as in Fig. 2. Exon F cloned by 5'-RACE (Fig. 2) is shown by the uppercase letters. Different transcription start sites have been reported; Flouriot *et al.* have reported the start sites at the position "d" and "h" [4]. Moreover, Schuur *et al.* [6] have reported ten transcription start sites of the exon F at "a", "b", "c", "e", "g", "i", "j", "k", "l" and "m". Some nucleotides at the positions -336 ("e"), -333 ("f") and -316 ("j") are different from those reported by previous reports. The nucleotides at the positions of -336, -333 and -316, are "A, G and A" in the present study, whereas they are reported as "A, C and A" by Flouriot *et al.* [4], as "G, G and T" by Schuur *et al.* [6], and as "A, G and A" in human genomic sequence of chromosome 6q.24 (GenBank AL035695), implying that the "A, G and A" in these positions might be a wild type.

Fig. 4. Distributions of ER α mRNA isoforms containing different 5'-exons in uterine endometrium (Em), ovary (Ov) and liver (Li).

Two hundred nanograms of total RNA from uterine endometrium (Em), ovary (Ov) and liver (Li) were subjected to RT-PCR with primers hEAs/hE1as (A. ER α mRNA ((A)-1)), primers hEBs/hE1as (B. ER α mRNA (B-1)), primers hECs/hE1as (C. ER α mRNA (C-1)), primers hEFs/hE1as (D. ER α mRNA (F-E2-1) and ER α mRNA (F-1)), primers hEE1s/hE1as (E. ER α mRNA (E1-E2-1) and ER α mRNA (E1-1)), primers hE2s/hE5as (F. ER α mRNA (2-5)), and primers hBAs/hBAas (G. beta actin mRNA). Two molecular sizes of the product were generated with primers hEFs/hE1as (D), and primers hEE1s/hE1as (E). Analysis of the nucleotide sequences has revealed that the shorter products are generated from the novel isoforms of the ER α mRNAs, ER α mRNA (F-1) and ER α mRNA (E1-1), in which exon F and exon E1 are directly spliced onto exon 1, respectively. ER α mRNA (2-5) is considered to be the sum of ER α mRNA isoforms with distinct 5'-untranslated exons. Differential distributions of these isoforms of ER α mRNAs in Em, Ov and Li clearly indicate that tissue specific expression of human ER α gene is regulated, at least in part, by the multiple promoters that exist on the respective 5'-untranslated first exons as well as alternative splicing by which exons F and E1 are spliced directly onto exon 1.

longer product corresponded to ER α mRNA (F-E2-1), while the shorter one originated from the novel mRNA isoform in which exon F was directly spliced

onto exon 1. We termed this isoform ER α mRNA (F-1). Moreover, two distinct sizes of the products were obtained by RT-PCR with hEE1s/hE1as on Li RNA (Fig. 4E). Nucleotide sequencing indicated that the longer product originated from ER α mRNA (E1-E2-1), while the shorter one corresponded to the novel isoform mRNA in which exon E1 was directly spliced onto exon 1. This isoform was termed ER α mRNA (E1-1).

Distributions of ER α mRNA isoforms in Em, Ov and Li

In order to investigate tissue-specific expression of ER α mRNA isoforms containing different 5'-UTRs, RT-PCR was performed on the total RNA of Em, Ov and Li with primers hEAs/hE1as, hEBs/hE1as, hECs/hE1as, hEFs/hE1as and hEE1s/hE1as (Fig. 4A-E). In the present study, the total RNA sample was extracted from a single tissue. The results demonstrated that ER α mRNA ((A)-1) and ER α mRNA (C-1) were expressed in Em and Ov, but were undetectable in Li (Fig. 4A, C). Moreover, ER α mRNA (B-1) was detected only in Em (Fig. 4B). ER α mRNA (F-E2-1) was detected not only in Em, but also in Ov and Li, in contrast to the novel ER α mRNA (F-1) that was observed only in Em (Fig. 4D). On the other hand, ER α mRNA (E1-E2-1) and novel ER α mRNA (E1-1) were detected only in Li (Fig. 4E). RT-PCR with primers hE2s/hE5as was also carried out on the same tissues to analyze the sum of isoforms of ER α mRNAs containing the different 5'-UTRs (Fig. 4F). Among the three tissues, Em contained the highest amount of total ER α mRNAs.

Discussion

In the present study, we analyzed the structure of ER α mRNA in Em in order to investigate whether any novel untranslated 5'-exons existed. The 21 positive 5'-RACE clones contained 2 clones from ER α mRNA ((A)-1), 5 clones from ER α mRNA (C-1), 14 clones from ER α mRNA (F-E2-1), and no clones originated from the other ER α mRNA isoforms. Although 5'-RACE method was not a quantitative analysis, it was inferred that these three isoforms were major transcripts of ER α gene in Em. Since RT-PCR in the present study revealed that Em also

contained a low but definite level of the other isoforms of ER α mRNAs, ER α mRNA (B-1) and ER α mRNA (F-1) (Fig. 4B, D), it remains possible that the other isoforms of ER α mRNAs with the presently unidentified 5'-exon (s) such as the homologue of rat exon 0N [10] might be present in Em.

In the previous reports, exon F was demonstrated to be spliced onto exon E2 that was spliced onto exon 1 [3-8] (Fig. 1). On the other hand, the rat exon 0S [11] that was the homologue of the human exon F was reported to be spliced directly onto the exon 1. Thus, we carried out the RT-PCR analysis on RNA of Em and Li in order to identify the novel splicing events of the previously reported 5'-exons. The results indicated that a novel isoform of ER α mRNA, ER α mRNA (F-1) in which exon F was directly spliced onto exon 1 (Fig. 1, dashed lines), was present in Em. Moreover, the presence of another novel isoform of ER α mRNA, ER α mRNA (E1-1) in which exon E1 was directly spliced onto exon 1 (Fig. 1, dashed lines), was also demonstrated in Li.

Distribution of ER α mRNA isoforms containing the different 5'-UTRs including the novel isoform of ER α mRNA (F-1) and ER α mRNA (E1-1) identified in the present study in the Em, Ov and Li were analyzed by RT-PCR-Southern blotting with primers hEAs/hE1as, hEBs/hE1as, hECs/hE1as, hEFs/hE1as and hEE1s/hE1as (Fig. 4A-E). Distributions of the ER α mRNA ((A)-1), ER α mRNA (B-1) and ER α mRNA (C-1), ER α mRNA (F-E2-1) and ER α mRNA (E1-E2-1), which are essentially similar to the results by RNase protection assay reported by Flouriot *et al.* [4], were clearly different among these tissues, indicating that the multiple 5'-untranslated exons and promoters system was involved in tissue specific expression of the gene as demonstrated by the previous reports [1-9]. We have not analyzed the distributions of ER α mRNA (D-1), ER α mRNA (T1-T2-1) and ER α mRNA (T1-1), because the levels of the isoform mRNAs in Em, Ov and Li were considered to be very low or absent based on the data in the previous reports [4-9]. Moreover, distributions of novel isoforms of ER α mRNA (F-1) and ER α mRNA (E1-1) were demonstrated in the present study (Fig. 4D, E); ER α mRNA (F-1) was observed

only in Em, and ER α mRNA (E1-1) was detected only in Li. It is interesting to note that the level of ER α mRNA (F-1) is very low or absent in Ov and Li where definite levels of ER α mRNA (F-E2-1) are observed (Fig. 4D). It should be noted that the difference in distribution might be greater different among tissues since we analyzed the distribution of mRNAs in a single sample of Em, Ov and Li in the present study.

Our present study, together with previous reports by others [1-9], clearly demonstrated that in addition to the previously reported eight different isoforms, two novel isoforms of ER α mRNAs are generated by the multiple 5'-untranslated exon system which consists of exon (A) (the upstream region of exon 1), the six 5'-untranslated first exons, exons B, C, D, T1, F and E1, and the 5'-untranslated second exons, exons T2 and E2, in the human tissues (Fig. 1). Differential distributions of these isoform transcripts (Fig. 4) indicated tissue specific expression of human ER α gene is regulated, at least in part, by the multiple promoters that exist on the respective 5'-untranslated first exons as well as mechanisms that control alternative splicing by which exons E1 and F are spliced directly onto exon 1. Interestingly, a recent report [18] has shown two novel ER α mRNA isoforms in which exons F-E2 and exons E1-E2 are directly spliced onto exon 2 to generate the exon 1-skipping ER α mRNA isoforms. Further studies are necessary to clarify the entire structure and the splicing manners of the gene to comprehend the regulatory mechanism of the tissue specific expression of ER α mRNA.

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