The untranslated first exon 'exon 0S' of the rat estrogen receptor (ER) gene

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Abstract Recently, we have isolated the untranslated first exon 'exon 0N' of the rat estrogen receptor (ER) gene from the liver by the use of the 5'-rapid amplification of cDNA ends (5'-RACE) method. To investigate the existence of other untranslated first exon(s), we further analyzed the 5'-untranslated region (UTR) of ER mRNA in the rat liver in this study. Total RNA from the livers of 8-week-old male Wistar rats was subjected to 5'-RACE with the antisense primers located in exon 1 of the rat ER gene. The inserts of four clones (clones 3, 4, 7 and 8) were sequenced. The nucleotide sequences of the clones revealed the existence of a previously unidentified untranslated first exon (we termed it 'exon 0S') which was spliced onto exon 1 of the rat ER mRNA. The distribution of ER mRNA containing 'exon 0S' (ER mRNA (0S-1)) in several brain regions and various peripheral tissues of 8-week-old male and female Wistar rats was further analyzed by the use of the reverse transcription-polymerase chain reaction. ER mRNA (0S-1) was found to be widely distributed in the rat brain and peripheral tissues. The distribution of the message was different from that of ER mRNA containing exon 0 (the first reported 5'-UTR form of rat ER mRNA) or of ER mRNA with exon 0N which was reported in our recent report. These results indicate that (1) 'exon 0S' is a novel untranslated first exon of the rat ER gene, (2) rat ER mRNAs possess at least three forms of 5'-UTRs which are exon 0, exon 0N, and exon 0S, (3) the tissue specific expression of ER is regulated, at least in part, by the usage of differential promoters in the rat.

Key words: Estrogen receptor mRNA; Untranslated first exon; Multiple promoter; Alternative splicing; Rat

1. Introduction

We have recently identified the untranslated first exon 'exon 0N' of the rat estrogen receptor (ER) gene from the liver [1] by the use of the 5'-rapid amplification of cDNA ends (5'-RACE) method, indicating that at least two forms of 5'-untranslated region (UTR) are present in the rat ER mRNA: the ER mRNA containing the untranslated first exon 'exon 0' (the first reported form of rat ER mRNA [2], exon 0 is also named 'exon 1*' by Keaveney et al. [3]), and the message having the untranslated first exon 'exon 0N' [1]. On the other hand, the recent report by Grandien revealed the existence of a novel untranslated first exon (he termed it 'exon C') of the human ER gene in the liver [4] using the 5'-RACE method. Thus, the human ER mRNAs possess at least three forms of 5'-UTR: the ER mRNA transcribed from the promoter upstream exon 1, the messages containing exon 0 [3,5–9] and exon C [4]. Interestingly, no nucleotide sequence homology was found between the rat exon 0N and the human exon C, suggesting the possibility of the presence of other unidentified untranslated first exon(s) of the rat ER gene. In this context, we further analyzed the 5'-UTR of ER mRNA in the rat liver by 5'-RACE with the antisense primers located in exon 1 of the rat ER gene in the present study.

2. Materials and methods

2.1. Tissues and RNA extraction

Eight-week-old Wistar male and female rats were used. Several regions of the brain (the anterior hypophysis (AP), hypothalamus and preoptic area (HPOA), amygdala (AMY), cerebral cortex (CC) and cerebellum (Ce)) were dissected from three male rats as described previously [10], and various peripheral tissues (the liver (Li), kidney (Ki), spleen (Sp), adrenal gland (Ad), small intestine (Is), large intestine (Il), heart (He), adipose tissue (Fa), and testis (Te)) from three male rats, and the uterus (Ut) and ovary (Ov) from three female rats were also dissected. Total RNA was extracted from tissues according to the procedure of Chirgwin et al. [11]. Briefly, tissues were homogenized in 4 M guanidine isothiocyanate solution and the total RNA was pelleted through a 5.7 M cesium chloride cushion by ultracentrifugation at 35000 rpm (Beckman, SW50.1 rotor) for 12 h at 20°C. The RNA concentration was determined by UV absorption at 260 nm.

2.2. RACE and PCR primers

The locations and sequences of the RACE and PCR primers are shown in Fig. 1. The sequences of the primers RACE I and RACE II were identical to those reported by Frohman et al. [12]. The sequence of the primers rE0s (forward), rE1as (reverse) and rE1RACEas (reverse) were derived from the rat ER cDNA sequence [2]. The sequence of the primer rE0Ss (forward) was derived from the nucleotide sequence of RACE clone 3. The expected lengths of the RT-PCR products with primers rE0Ss/rE1as, primers rE0s/rE1as and primers rE0s/rE1RACEas were 338 bp, 271 bp and 207 bp, respectively. Since these primer pairs were considered to flank the regions including the intron on genomic DNA (Fig. 1), the expected lengths of the RT-PCR products could be generated only from the respective mRNAs, and not from genomic DNA. In order to confirm the validity of the RNA concentration which was determined by UV absorption, the distribution of β actin mRNA in the tissues was analyzed with primers βAs/βAas which flanked the rat β actin cDNA sequence from base 100 to 343, as numbered by Nudel et al. [13]. The RT-PCR product of 244 bp with the primers βAs/βAas was also generated only from β actin mRNA because the amplified region consisted of the first and second exons.

2.3. 5'-RACE

5'-RACE was carried out essentially as described by Frohman et al. [12]; briefly, 1 μg of total RNA of the liver was subjected to reverse transcription (RT) with primer rE1as (Fig. 1) under conditions described in Section 2.4, followed by poly(A) tailing with 15 units of terminal deoxynucleotidyltransferase (Bethesda Research Laboratories, Gaithersburg, MD, USA) at 37°C for 15 min. The poly(A)-tailed cDNA was subjected to the polymerase chain reaction (PCR) with primers rE1RACEas, RACE I, and RACE II (Fig. 1). The reaction was carried out for 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The amplified product, digested with *Xba*I and *Eco*RI, was subcloned into pBS M13$^+$ vector (Stratagene, La Jolla, CA, USA). The RACE library was screened by Southern blotting with the ^{32}P-labeled rat ER (exons 0–1) cDNA probe. The rat ER

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(exons 0–1) cDNA probe was synthesized by the random priming method using the RT-PCR product from the uterus with primers rE0s/rE1as as a template. The nucleotide sequences of the inserts of clones 3, 4, 7 and 8 were determined on both strands by the dideoxy method [14] with a Sequenase DNA sequencing kit (USB, Cleveland, OH, USA).

2.4. RT-PCR

Total RNA from each tissue was reverse transcribed to synthesize single stranded cDNA. 200 ng of total RNA from the tissues was incubated at 42°C for 60 min with 2 units of RAV-2 reverse transcriptase (Takara, Kyoto, Japan) in a 10 μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM each of dNTP, 10 μM random hexadeoxynucleotide primer (Takara). PCR was performed as recommended by the manufacturer (Perkin Elmer, Branchburg, NJ, USA) [15] with minor modifications. Briefly, 1 μl of cDNA was amplified in a 10 μl reaction volume containing 0.25 unit of *Taq* DNA polymerase (Perkin Elmer), 1.5 mM $MgCl_2$ and 0.5 μM of primers rE0Ss/rE1as or primers βAs/βAas. To analyze the distribution of ER mRNA containing exon 0S, the reaction was performed with primers rE0Ss/rE1as for 32 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. To analyze the distribution of β actin mRNA, the reaction was performed with primers βAs/βAas for 17 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min.

2.5. Southern blotting

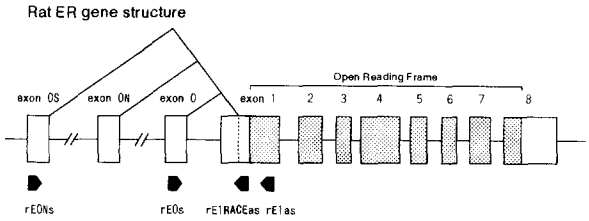
Two microliters of the RT-PCR products from each tissue RNA were electrophoresed in 2.0% agarose gel and transferred onto a nylon membrane (Hybond N^+, Amersham, Buckinghamshire, UK) with 400 mM NaOH as a transfer solution for 3 h. The membrane was incubated in a prehybridization buffer containing 6×SSC (1×SSC: 15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0), 150 μg/ml yeast total RNA, and 1.0% sodium dodecyl sulfate (SDS) at 42°C for 1 h. Then the membrane was hybridized with ^{32}P-labeled rat ER (exons 0–1race) cDNA probe or rat β actin cDNA probe in the same buffer at 65°C for 12 h. The rat ER (exons 0–1race) cDNA probe and rat β actin cDNA probe were synthesized by the random priming method using the RT-PCR products from the uterus with primers rE0s/rE1RACEas and primers βAs/βAas as templates, respectively. The templates were confirmed to be part of rat ER cDNA or rat β actin cDNA by nucleotide sequencing. The specific activity of the probes was approximately $1.0–2.0\times10^9$ cpm/μg DNA. After hybridization, the membrane was washed under stringent conditions. The hybridization signal was analyzed with a Bioimage Analyzing System, BAS2000 (Fuji Film, Tokyo, Japan).

2.6. RT-PCR blank

In order to examine whether contamination of reagents occurred in the present experiments, distilled water was simultaneously subjected to RT-PCR (RT-PCR blank). No specific signal was obtained from the RT-PCR blank (Fig. 3A,B, lane Bl), indicating no contamination occurred in these experiments.

3. Results and discussion

Four positive clones (clones 3, 4, 7 and 8) were isolated by screening with the rat ER (exons 0–1) cDNA probe from the 5′-RACE library constructed from rat liver total RNA with the antisense primers located in exon 1 of the rat ER cDNA. The nucleotide sequence of the clones revealed that clones 4 and 7 were part of clone 3 which was identical with clone 8 (Fig. 2). The nucleotide sequence of 458 bp of the insert of the clone 3 included 24 bp of the primer RACE I in the 5′ end and 23 bp of the primer rE1RACEas in the 3′ end (Fig. 2). The sequence of 411 bp of the region flanked by the primers included the translation initiation site of the rat ER gene [2]. The nucleotide sequence from nucleotide (nt) −71 to +60 corresponded to part of exon 1 of the rat ER cDNA except for one nucleotide substitution at nt −28 (G instead of A in the first reported rat ER cDNA) which was mentioned in our recent report on exon 0N [1]. On the other hand, the nucleotide sequence of the region from nt −351 to −72 of clone 3 (Fig. 2, indicated by italics) did not show any homology with the sequences of exon 0 [2] and the recently identified exon 0N [1] of the rat ER gene. Moreover, no homology was found between the nucleotide sequence of the region and the recently reported exon C of the human ER gene [4]. In addition, the sequence of the region was different from the upstream sequence of nt −72 of exon 1 of the rat ER gene analyzed by genomic cloning (data not shown). From these results, we concluded that clones 3, 4, 7 and 8 originated from the ER mRNA containing the previously unidentified untranslated first exon (which we termed 'exon 0S') which was spliced onto the 5′-UTR of exon 1 at nt −61. Although we have not cloned the genomic DNA encoding exon 0S and not investigated the transcription start site in the present study, the presence of RACE clones 4 and 7 which encode the sequences downstream of nt −231 and −201, respectively, implies that multiple cap sites may exist in the rat 0S gene.



Nucleotide sequences of the RACE and PCR primers;

RACE I : 5′-CTCGAGGTCGACTCTAGATTTTTTTTTTTTTTTTT-3′
RACE II : 5′-CTCGAGGTCGACTCTAGA-3′
rE1RACEas: 5′-GAATTCAGGGGCTCCAGCTCGTT-3′
rE1as : 5′-AAGCTTGCTGTTGTCCACGTAC-3′
rE0s : 5′-GAATTCTACAAACCCATGGA-3′
rE0Ss : 5′-TCTAGAAAACACAAGGCTCCATGCT-3′
βAs : 5′-ATCTGGGCCGCCCTAGCC-3′
βAas : 5′-TGGCCTTAGGGTTCAGAGG-3′

Fig. 1. Location and sequences of the PCR and RACE primers. The sequences of primers rE0s (forward), rE1as (reverse) and rE1-RACEas (reverse) were derived from the rat ER cDNA sequence [2]. The sequence of primer rE0Ss (forward) was derived from the nucleotide sequence of RACE clone 3. The sequences of primers RACE I and RACE II were identical to those reported by Frohman et al. [12]. In order to facilitate subcloning, substitution or addition of a few nucleotides was introduced in primers rE1RACEas, rE1as, rE0s and rE0Ss at the positions indicated by the underline. The dashed line indicates a splicing acceptor site on exon 1. Although the locations of exons 0S and 0N on the rat genomic DNA have not been determined yet, the positions of these exons are indicated in the figure for convenience of explanation. Primers βAs/βAas flank the rat β actin cDNA sequence from base 100 to 343, as numbered by Nudel et al. [13].

Using the forward primer rE0Ss whose sequence was derived from RACE clone 3 and the reverse primer rE1as located in exon 1, we investigated the distribution of ER mRNA containing exon 0S (termed ER mRNA (0S-1)) in several regions of the rat brain and various peripheral tissues by RT-PCR. ER mRNA (0S-1) was found to be widely distributed in the tissues examined and the intensity of the signals of the message was high in the AP, Li, Ki, Ad, Ov, Ut and Te, with low but definite signals of the message in the other brain regions and peripheral tissues (Fig. 3A). As shown in Fig. 3B, the intensity of the signals of β actin mRNA in the same tissues was less varied, indicating that the intensity of the signals of ER mRNA (0S-1) in Fig. 3A seemed to reflect

the levels of the message in each tissue. Our recent report on exon 0N [1] revealed the distribution of ER mRNA having exon 0 (ER mRNA (0–1)) which was the first reported form of rat ER mRNA [2], and that of ER mRNA containing exon 0N (ER mRNA (0N-1)). As shown in that report, ER mRNA (0–1) was widely distributed in the rat brain and peripheral tissues but the distribution of the message was clearly different from that of ER mRNA (0S-1); the levels of ER mRNA (0–1) were highest in the AP, HPOA, AMY, Ov and Ut, high in the Li and Sp, with low levels of the message in the other tissues [1]. Furthermore, the distribution of ER mRNA (0N-1) was quite different from that of ER mRNA (0S-1) because the distribution of ER mRNA (0N-1) was almost limited in the peripheral tissues [1].

From these results, it is apparent that rat ER mRNAs are transcribed from at least three different promoters which are located upstream of the untranslated first exons: exons 0, 0N and 0S. The differential distribution of these three ER mRNAs in the rat brain and peripheral tissues suggests that the promoters for the transcription from these three untranslated first exons are independently present and are differentially regulated in a tissue-specific fashion. Interestingly, we have not identified the rat homologue of the untranslated first exon C which is reported in the human ER gene [4]. It is unclear at the present time whether ER mRNA containing the homologue of exon C exists. Further studies on the existence of the other untranslated first exon(s) and the regulatory mechanism of the promoters for the transcription from the different untranslated first exons are important to clarify the role of the multiple untranslated first exons and multiple promoters system on rat ER gene expression.

Fig. 2. Nucleotide sequence of RACE clone 3. The RACE library constructed from rat liver total RNA was screened by the rat ER cDNA (0-1) probe and four clones (clones 3, 4, 7 and 8) were sequenced. Clone 8 was identical with clone 3, and clones 4 and 7 were part of clone 3. The insert of 458 bp of clone 3 includes 24 bp of primer RACE I in the 5′ end and 23 bp of primer rE1RACEas in the 3′ end (indicated by the boxes). The A of the first codon ATG (indicated by the double underline) is assigned nucleotide number +1. The nucleotide sequence from nucleotide (nt) −71 to +60 corresponds to part of exon 1 of the rat ER cDNA except for one nucleotide substitution within the 5′-UTR. As previously reported [1], G(*) was found at nt −28 instead of the A in the reported rat ER cDNA [2]. The region from nt −351 to −72 (indicated by italics) was different from exon 0 [2] and the recently identified exon 0N [1], and did not show any homology with the recently reported exon C of the human ER gene [4]. In addition, the sequence of the region was different from the upstream sequence of nt −72 of exon 1 of the rat ER gene analyzed by genomic cloning (data not shown). We concluded that clones 3, 4, 7 and 8 originated from the ER mRNA containing the previously unidentified untranslated first exon (which we termed 'exon 0S') which is spliced onto the 5′-UTR of exon 1 at nt −71 (▼). The location of primer rE0Ss is underlined.

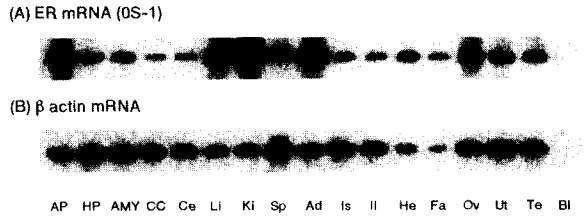


Fig. 3. The distribution of ER mRNA (0S-1) and β actin mRNA in several regions of the rat brain and various peripheral tissues. 200 ng of total RNA from the various tissues from 8-week-old male and female rats indicated in Section 2 was subjected to RT-PCR using primers rE0Ss/rE1as (A) or primers βAs/βAas (B). PCR was performed for 32 cycles (primers rE0Ss/rE1as) or 17 cycles (primers βAs/βAas) at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The signals of the RT-PCR products were analyzed by Southern blotting under stringent conditions. AP, anterior hypophysis; HP, hypothalamus and preoptic area; AMY, amygdala; CC, cerebral cortex; Ce cerebellum; Li, liver; Ki, kidney; Sp, spleen; Ad, adrenal gland; Is, small intestine; Il, large intestine; He, heart; Fa, adipose tissue; Te, testis, were dissected from the male rats; Ut, uterus and Ov, ovary were dissected from the female rats; Bl, RT-PCR blank.

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