Endocrine Journal 2000, **47** (5), 543–548

Alternate Exon in the 5′-Untranslated Region of the Human ACTH Receptor Gene

MITSUMASA KUBO, CHIKARA SHIMIZU*, HIROMICHI KIJIMA*, TATSUYA ISHIZUKA*, KAZUHIKO TAKANO*, AKIKO TAKANO* AND TAKAO KOIKE*

Health Administration Center, Hokkaido University of Education, Sapporo 002–8501, Japan
** Department of Medicine II, Hokkaido University School of Medicine, Sapporo 060–8638, Japan*

Abstract. The human ACTH receptor (ACTH-R) gene consists of two exons; the first encodes the 5′-untranslated region (UTR) and the second encodes part of the 5′-UTR, the entire coding region, and the whole of the 3′-UTR. While investigating the transcription initiation site of the human ACTH-R gene by 5′-rapid amplification of cDNA ends, we found a 157-bp alternate exon in the 5′-UTR. This newly identified exon was irrelevant to the alternate exon in the mouse ACTH-R gene which we reported previously.

Key words: Gene structure, Alternative splicing, ACTH receptor, Adrenal gland, Human
(*Endocrine Journal* **47**: 543–548, 2000)

THE ACTH receptor (ACTH-R) is a 7-transmembrane spanning protein through which the ACTH signal is transmitted into adrenocortical cells to mount steroidogenesis. In recent years, the structure and regulation of the ACTH-R gene have been elucidated using molecular biological techniques [1–8].

We recently reported that the mouse ACTH-R gene consists of four exons [6]: the first three exons encode the 5′-untranslated region (UTR) and the fourth exon encodes part of the 5′-UTR, the entire coding region, and the whole of the 3′-UTR. Northern blot analysis revealed a 1.8 kb mRNA in mouse adrenals which consists of two mRNA species, one with and one without 57-bp exon 2 by alternative splicing.

The structure of the human ACTH-R gene is somewhat different from the mouse counterpart. It consists of two exons [7], the first of which encodes the 5′-UTR, while the other encodes part of the 5′-UTR, the entire coding region, and the whole of the 3′-UTR. Northern blot analysis revealed multiple mRNA species in human adrenals; 1.8 and 3.8 kb are the predominant species with 4, 7 and 11 kb mRNAs being minor species. All of these mRNAs are produced by alternative usage of different polyadenylation signals located in exon 2 [8].

To examine the transcriptional regulation of the ACTH-R gene, we initially investigated transcription initiation sites in the human ACTH-R gene by 5′-RACE (rapid amplification of cDNA ends), and found an alternate exon in the 5′-UTR of the gene. We here report features of the newly identified alternate exon.

Received: March 5, 1999
Accepted: June 26, 2000
Correspondence to: Mitsumasa KUBO, M.D., 5–3–1 Ainosato, Kita-ku, Sapporo 002–8501, Japan

Materials and Methods

Primers

Sequences of oligonucleotide primers (Fig. 1) were: 1X5S, 5′-CTCCGAGCTCATTCCTTCTC; 1XL, 5′-TTGCCCAGAAAGTTCCTGCTTC; 1XS, 5′-AGTTCCTGCTTCAGAGCTG; IN5AS, 5′-CCCACGTCTTTGTGGCGCT; IN3S2, 5′-CAAAAGGTCACCTGTCACTAATC; IN3S, 5′-GCAGAGGTGAGA-

CACAGAGATT; IN3AS2, 5′-GAATAGATTTGAGGTTGTTCACC; AltS, 5′-TCCAGCTTCCCTCTAGCCAG; AltMAS, 5′-CCCAGCTGTCGCCTGTGCC; AltAS, 5′-CCTCCACATGAAAAAAAGTC; 2INAS, 5′-GGTCTTGAAGGTGCCCAAGC; 5UTAS, 5′-GTAGCACTTGCTGGAGATCTAAG; R2, 5′-TGCGGCGCATGGTCACGAT.

5′-rapid amplification of cDNA ends

Marathon cDNA Amplification Kit (Clontech Inc., USA) was employed. Total RNA was extracted from the adrenal of a patient with ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) reported previously [9] by RNAzol (CINNA/BIOTEX Lab. Inc., USA) and poly(A)$^+$RNA was purified with Oligotex-dT30<super> (Japan Roche). Using 1 μg of poly(A)$^+$RNA and 10 μM of Marathon cDNA synthesis primer, single strand cDNA (sscDNA) synthesis was done. Following double strand cDNA (dscDNA) synthesis, 5′-RACE was carried out. Using Marathon cDNA Adaptor-ligated dscDNA as a template, PCR was performed between AP1 primer (supplied in the kit) and 5UTAS corresponding to a complementary sequence of the 5′-UTR of exon 2 by AmpliTaq Gold (Perkin Elmer, USA) under the conditions of activation step at 95°C for 9 min, followed by 45 cycles of denaturation step at 94°C for 50 sec, annealing step at 60°C for 50 sec, and extension step at 72°C for 90 sec. Then aliquots of the PCR product were amplified between AP2 primer (supplied in the kit) and 5UTAS by AmpliTaq Gold under the conditions of activating step at 95°C for 9 min, followed by 45 cycles of denaturation step at 94°C for 1 min, annealing step at 65°C for 1 min, and extension step at 72°C for 1 min. The resultant DNAs were analyzed on a 2.5% agarose gel and approximately 350- and 140-bp bands were purified by DE81 papers as reported previously [6]. These were cloned on TA cloning vector and sequenced on both strands by the dideoxy chain termination method.

Reverse Transcription (RT)-PCR analysis

Human normal adrenal total RNA was purchased from Clontech Inc. (USA) and poly(A)$^+$RNA was extracted with Oligotex-dT30<super>. Five μg of human normal adrenal poly(A)$^+$RNA and AIMAH-derived adrenal poly(A)$^+$RNA described above were reverse transcribed by Superscript II reverse transcriptase (GIBCO-BRL, USA) using oligo-dT$_{15}$ as a primer at 42°C for 1 h in a 50-μl solution. PCR was performed between a set of primers, 1XS and AltAS, AltS and R2, or 1XS and R2 using 1 μl of sscDNA as a template by AmpliTaq Gold under the conditions of activation step at 95°C for 9 min, followed by 35 cycles of denaturation step at 94°C for 1 min, annealing step at 60°C (1XS-AltAS, 1XS-R2) or 65°C (AltS-R2) for 1 min, and extension step at 72°C for 1 min. The PCR products were analyzed on a 2.5% agarose gel.

Long distance PCR

A XL PCR kit (Perkin Elmer, USA) was used for long distance PCR analysis. Briefly, 40 pmoles of each primer, AltS and R2 were mixed in 40 μl of the solution including magnesium acetate and dNTPs (final concentrations were 1.1 mM and 0.8 mM, respectively) to examine the distance between 157-bp alternate exon (described later) and exon 2. One AmpliWax PCR gem (Perkin Elmer, USA) was added in the tube and heated at 80°C for 10 min, followed by standing at room temperature for 5 min. Then a 60-μl mixture of 1 μg of genomic DNA from HeLa cells or peripheral mononuclear cells of two healthy Japanese men, and DNA polymerase (supplied in the kit) was loaded on the wax layer. PCR was performed using GeneAmp PCR System 2400 (Perkin Elmer, USA) under the conditions of initial hold at 94°C for 1 min, followed by 16 cycles of denaturing step at 94°C for 15 sec and annealing/extension step at 68°C for 10 min, then 12 cycles of denaturing step at 94°C for 15 sec and annealing/extension step at 68°C for 10 min plus 15 sec increments per cycle, and finally additional hold at 72°C for 10 min. The PCR product was analyzed on a 0.75% agarose gel, and also purified with Wizard PCR Preps (Promega, USA) followed by sequencing from 5′ and 3′ ends using primers AltS and R2, respectively. Primers 1X5S and 2INAS (this primer was synthesized according to the sequence of the long distance PCR product described above) were used to determine the distance between exon 1 and alternate exon, and PCR was performed under the same conditions except for the annealing/extension temperature at 66°C. Aliquots of the PCR products were

amplified with primers 1XL and AltMAS under the same conditions except for the annealing/extension temperature at 68°C. The PCR products were analyzed on a 0.75% agarose gel, and also purified with Wizard PCR Preps followed by sequencing from 5′ and 3′ ends using primers 1XL and AltMAS, respectively.

Results and Discussion

By 5′-RACE, two broad bands of approximately 350 and 140 bp were generated and the density was slightly more intense in the latter (data not shown). After cloning and sequencing, 7 clones contained cDNA fragments of both exons 1 and 2. The length of exon 1 was variable; the longest was 59 bp (Fig. 1), and other cDNA clones contained exon 1 sequences of 39 (2 clones), 27, 25 (2 clones) and 19 bp. Naville *et al.* reported that the length of exon 1 of the human ACTH-R gene was 49 bp by 5′-RACE [7], and this was confirmed by primer extension and S1 nuclease protection studies [10]. We obtained no cDNA clones containing exactly 49 bp of exon 1. This might be due to adrenal RNA from the patient with AIMAH we utilized in the 5′-RACE study. Alternatively, the quality of adrenal poly(A)$^+$RNA and/or extension reaction of reverse transcription might be inadequate, because most of the cDNA clones we obtained contained shorter sequences of exon 1.

One clone contained an additional 157-bp sequence

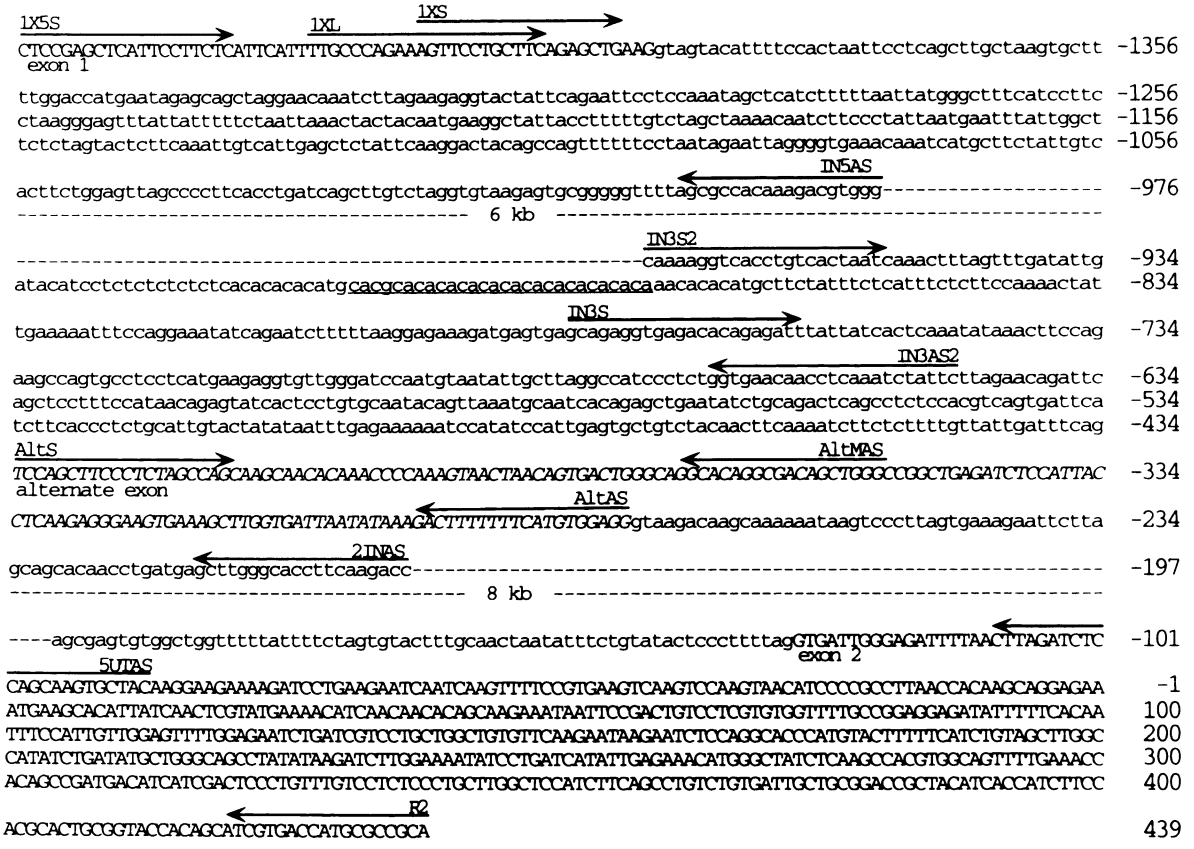

Fig. 1. Nucleotide sequence of the human ACTH-R gene. Genomic sequences of exon 1 and its 3′-flanking region are at the top, the alternate exon and its surrounding regions are in the center, and exon 2 and its 5′-flanking region are at the bottom. Numbers are relative to the translational start. Exons 1 and 2 are shown in uppercase, alternate exon in uppercase italics, and intronic sequences in lowercase. The positions and orientation of oligonucleotide primers are indicated above the sequence. The CA dinucleotide repeat is underlined. The nucleotide sequence of the alternate exon and its flanking regions shown in this figure appears in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB013459.

between exons 1 and 2. To examine the presence of the extra sequence in mRNA populations from normal adrenals, RT-PCR analysis, using primers AltS corresponding to the 5′-sequence of the 157-bp fragment and R2 corresponding to a complementary sequence of exon 2 was carried out. A 732-bp fragment was detected (Fig. 2A, lane 2). RT-PCR with primers 1XS corresponding to a sequence in exon 1 and AltAS corresponding to the complementary sequence of the 3′ end of the 157-bp fragment revealed a 179-bp fragment (Fig. 2A, lane 3). Direct sequencing of the 732- and 179-bp RT-PCR products defined the identical sequence of part of each exon and the 157-bp fragment. Using primers 1XS and R2, RT-PCR analysis showed only a 597-bp fragment (Fig. 2A, lane 1) which was devoid of the 157-bp sequence. The densities of the 732- and 179-bp RT-PCR product bands were very weak as compared to the 597-bp RT-PCR product. The data indicated that the transcript containing this alternate exon is an extremely minor species in normal adrenals. The similar finding was demonstrated in adrenals derived from our patient with AIMAH. A 732-bp DNA fragment amplified between primers AltS and R2 was faint (Fig. 2B, lane 6), and only a 597-bp fragment was amplified between 1XS and R2 (Fig. 2B, lane 5) in AIMAH-derived adrenals, whereas a 179-bp fragment was relatively evident (Fig. 2B, lane 7).

We determined the location of the 157-bp alternate exon by long distance PCR. An approximately 8.6-kb DNA fragment was identified with primers AltS and R2 in HeLa cells and two healthy Japanese men (Fig. 3A, lanes 2–4) and had sequences identical to the 157-bp fragment and part of exon 2, as determined by direct sequencing of the PCR product from both ends. Since multiple bands were generated by long distance PCR between primers 1X5S corresponding to a sequence of exon 1 and 2INAS corresponding to a complementary sequence of the 3′-flanking region of the 157-bp alternate exon, aliquots of the PCR products were amplified with primers 1XL and AltMAS corresponding to a complementary sequence of the alternate exon. A 6.8-kb DNA fragment was identified (Fig. 3B, lanes 6–8), and contained identical sequences of part of exon 1 and the 157-bp fragment. Thus, the alternate exon is located approximately 6.7 kb downstream of exon 1, and the distance between exons 1 and 2 is approximately 15 kb.

The nucleotide sequence and structure of the human ACTH-R gene confirmed in this study are illustrated in Fig. 1. The sequences of the 5′- and 3′-flanking regions of the alternate exon in the human ACTH-R gene were compatible with the AG/GT

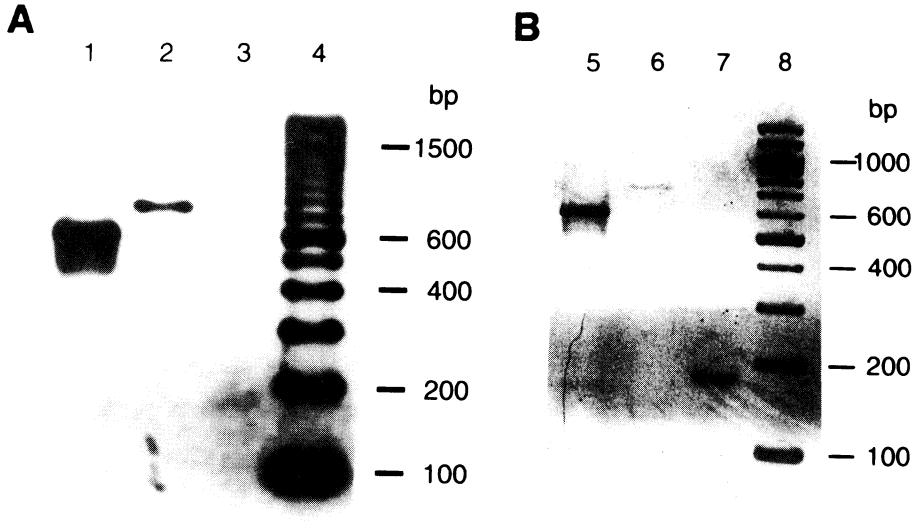

Fig. 2. Electrophoretic analysis of cDNA fragments generated by RT-PCR. Human normal adrenal mRNA (panel A) and AIMAH-derived adrenal mRNA (panel B) were reverse transcribed using oligo-dT_{15} as a primer, followed by PCR. PCR primers shown in Fig. 1 were 1XS (exon 1) and R2 (exon 2) in lanes 1 and 5, AltS (alternate exon) and R2 in lanes 2 and 6, and 1XS and AltAS (alternate exon) in lanes 3 and 7. Lanes 4 and 8: 100-bp DNA ladder.

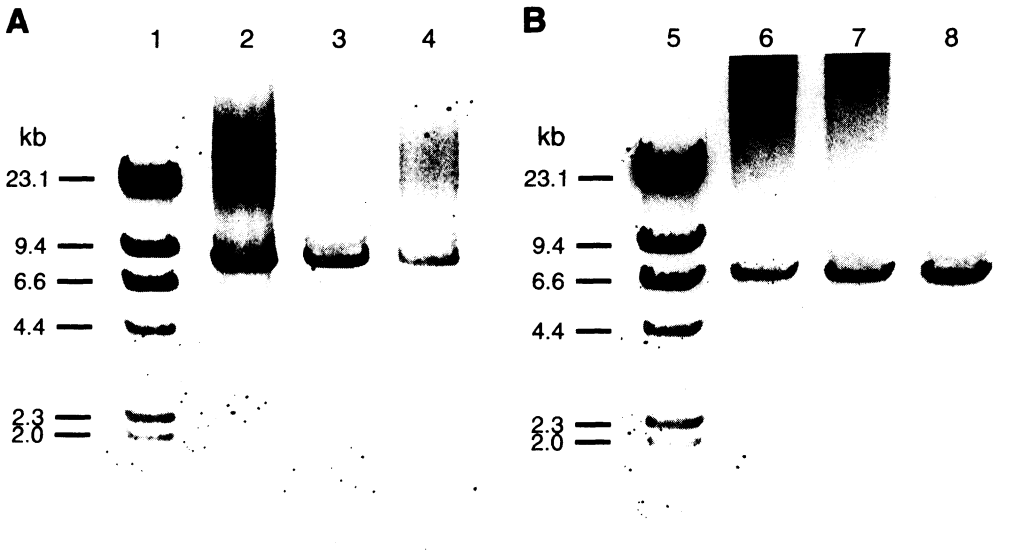

Fig. 3. Electrophoretic analysis of DNA fragments generated by long distance PCR. Long distance PCR was performed using genomic DNA from HeLa cells (lanes 2 and 6) and two healthy Japanese men (lanes 3, 4, 7 and 8) as a template. A; PCR primers shown in Fig. 1 were AltS (alternate exon) and R2 (exon 2) in lanes 2–4. B; PCR primers were 1XL (exon 1) and AltMAS (alternate exon) in lanes 6–8. Lanes 1 and 5: *λHind*III-digested DNA marker.

rule. Although there were high degrees of nucleotide sequence homology between mouse and human ACTH-R gene exon 1, and the mouse and human coding region, the alternate exon in the human ACTH-R gene was irrelevant to the alternate exon in the mouse counterpart which we reported earlier [6], suggesting that the human and/or mouse alternate exon could have been absent in the common ancient ACTH-R gene.

Alternative splicing in the 5′-UTR of the ACTH-R gene could affect translational efficiency as documented in many genes including receptor genes [11–13]. Parola and Kobilka [11] demonstrated that a short open reading frame (sORF) located in the 5′-UTR of β_2 adrenergic receptor mRNA was translated despite a poor translational initiation sequence context of sORF, and the resultant peptide inhibited translation of the major open reading frame, leading to reduction of β_2 adrenergic receptor synthesis. The human ACTH-R transcript containing alternate exon revealed in this study possessed a 27-bp sORF (ATG at −283 and TAA at −113 in Fig. 1), and if it could take place in the ACTH-R gene, alternative splicing might relate to undifferentiation of adrenal tumors. Reincke *et al.* [14] reported that loss of heterozygosity (LOH) on the ACTH-R gene was found in 2 of 4 adrenocortical carcinomas and an oncocytic nonfunctioning adenoma associated with markedly reduced levels of ACTH-R mRNA, but not in 15 hyperfunctioning adenomas. They speculated that loss of ACTH-R function would contribute to cellular dedifferentiation in adrenal tumors. Thus, it is of interest to investigate the species of the 5′-UTR of ACTH-R mRNA in adrenal tumors, whereas ACTH-R transcripts containing alternate exon and exon 2 in AIMAH-derived adrenals were scarce, as seen in normal adrenals described above (Fig. 2).

Searching for a similar sequence in the alternate exon and its flanking regions of the human ACTH-R gene in the GenBank database, the 5′-flanking sequence from position −782 to −657 of this alternate exon proved to be identical to that from position 234 to 359 of the clone AFMa152wg9 which contained a CA dinucleotide repeat as a microsatellite marker (GenBank accession number Z67345). We amplified a DNA fragment containing the CA dinuleotide repeat using HeLa genomic DNA as a template with primers IN3S2 corresponding to the sequence from position 41 to 63 of AFMa152wg9, and IN3AS2 corresponding to a complementary sequence of the 5′-flanking region of the alternate exon (Fig. 1). A CA dinucleotide repeat was confirmed (Fig. 1). As

this microsatellite marker is located in an intron of the human ACTH-R gene, it may serve as an informative marker for investigating the pathophysiology of ACTH-R gene-related disorders together with restriction fragment length polymorphisms by *Pst*I located in an approximately 3-kb region upstream of exon 2 [14] in the gene.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We thank I. Yamakawa for excellent technical assistance and M. Ohara for helpful comments.

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