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Short Sequence-Paper

cDNA cloning and sequence analysis of the bovine adrenocorticotrophic hormone (ACTH) receptor

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We isolated five independent cDNAs of nearly 3000 bp for the bovine ACTH receptor by screening adrenal cortex cDNA libraries with a PCR cloned cDNA fragment. The deduced receptor sequence includes 297 residues ($M_r = 33\,258$) with 81% identity with the human ACTH receptor, and shows seven hydrophobic transmembrane domains. The calculated M_r of the receptor is smaller than the 40–45 kDa observed in crosslinking studies with labeled ACTH. Since the bovine and human receptors have two glycosylation motifs in the N-terminus, the difference may result from glycosylation of the receptor. Analysis of the sequences of both bovine and human receptors revealed a single protein kinase A phosphorylation motif located in the third intracellular loop (Ser-209) juxtaposed to a protein kinase C phosphorylation motif (Thr-204). Thus, the involvement of protein kinase A and C pathways in ACTH action may be mediated in part by phosphorylation of the ACTH receptor at these motifs. The 3'-untranslated region of the bovine cDNA is > 2000 bp and includes two inverse repeats giving an extensive and strong secondary structure to the ACTH receptor RNA.

The process of steroidogenesis in the adrenal cortex is mainly regulated by the pituitary adrenocorticotrophic hormone (ACTH). ACTH stimulates the synthesis and secretion of steroids (short-term effects), and enhances transcription of genes encoding steroidogenic enzymes and mitochondrial genes (trophic effects) [1–3]. Like other peptide hormones, ACTH effects these processes by binding to specific G-protein-coupled receptors on the cell surface, and activating intracellular signal transduction systems, e.g., cAMP, calcium, and phosphoinositides [1,4–9].

To clone cDNAs for the bovine ACTH receptor we amplified bovine adrenal cortex mRNA fragments using oligonucleotide primers. Poly(A)⁺ RNA was isolated from powdered bovine adrenal cortex tissue with a FastTrack kit (Invitrogen), and reverse transcribed to cDNA with oligo(dT) primers. To amplify receptor cDNAs, polymerase chain reactions (PCR) were carried out using ACTH receptor specific primers (forward: 5'-CCTCGTGTGGTTTTGCCGGAGGAGA; reverse: 5'-TGCGTCCCTGAGCTCTGGGGTCCG-GAA), and degenerate primers corresponding to the

conserved 2nd, 3rd, and 6th membrane spanning domains of G-protein-coupled receptors based on comparison of the sequences of human ACTH [9] and other G-protein-coupled receptors. Reactions with the ACTH receptor specific primers yielded one major fragment of about 800 bp with strong homology to the human ACTH receptor, whereas degenerate primers led to the cloning of fragments homologous to previously cloned cDNAs encoding neuropeptide Y and interleukin-8 receptors.

Our screening of two bovine adrenal cortex cDNA libraries in λ gt11 (one generously provided by Drs. Morohashi and Omura [10], and ours [11]), with the radiolabeled 800 bp fragment yielded five positive clones out of about 105 000 plaques. Phage DNA from the clones was isolated [12], and the cDNA inserts of about 3000 bp were subcloned into the *EcoRI* site of pBluescript II KS-Phagemid. We determined the complete sequences of two cDNAs (Fig. 1) using both Sequenase II (USB) and an automatic DNA Sequencer (Applied Biosystems 373A). Both strands of the cDNAs were sequenced using synthetic oligonucleotide primers.

Features of the ACTH receptor

The largest open reading frame (ORF) of the cDNA sequence represents a protein of 297 residues ($M_r = 33\,258$) with 81% identity with the human ACTH re-

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The nucleotide sequence data reported in this paper is listed in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X74501 as 'B. taurus mRNA for ACTH receptor'.

GAAAGTTCCTGCTTCAGAGCAGAAGATCTTCAGCA 35

AGAACTACAAGAAGAAAGATTCTGGAGAATCAATCAAGTTTCTGCAAGTTCAGTAACGTTTCTGTCTTAACGACACAGGAAAG 125

ATGAAACACATTCTCAATCTGTATGAAAACATCAACAGTACAGCAAGAAATAACTCAGACTGTCTGCTGTGATTTTGGCCAGAGAGATA 215

M K H I L N L Y E N I N S T A R N N S D C P A V I L P E E I 30

TTTTTACAGTATCCATTGTGGGGTTTGGAGAACCCTGATGGTCTCTGGCTGTGGCCAGAATAAGAGTCTTCAGTCGCCCATGTAC 305

F F T V S I V G V L E N L M V L L A V A K N K S L Q S P M Y 60

TTTTTCATCTGCAGCTTGGCTATTCCGATATGCTGGGAGCCTGTACAAGATTTGGAAAAAGCTTGTGATCATGTTCAAAAACATGGGT 395

F F I C S L A I S D M L G S L Y K I L E N V L I M F K N M G 90

TACCTCGAGCCTCGAGGCAGTTTAAAAGCACAGCAGATGATGGTGGACTCCCTGTTTCATCCTCTCCCTTCTCGGCTCCATCTGCAGC 485

Y L E P R G S F E S T A D D V V D S L F I L S L L G S I C S 120

CTGTCTGTGATGCGCCTGACCGCTACATCACAATCTTCCAGCTCTGCAGTACCACCGCATCATGACCCCGCAGCTGCCCTCGTCATC 575

L S V I A A D R Y I T I F H A L Q Y H R I M T P H R A L V I 150

CTGACGGTCTCTGGCAGGCTGCACAGGCAGTGGCATTACCATCGTACCTTCTCCATCAGCTCCCACAGTATGCCCTTACACAGG 665

L T V L W A G C T G S G I T I V T F S H H V P T V I A F T A 180

CTGTTCCCGCTGATGCTGGCCCTCATCCTGTGCTCTACGTGCACATGTTCTGCTGGCCCGCTCCACACCAGGAGGACCCCTCCCTT 755

L F P L M L A F I L C L Y V H M F L L A R S H T R R T P S L 210

CCCAAAGCCAACATGAGAGGGGCGCTCACACTGACTGTCTGCTGGGGTCTTCAATTTCTGTGGGCACCTTGTCTTTCATGTCTC 845

P K A N M R G A V T L T V L L G V F I F C W A P F V L H V L 240

TTGATGACATTCTGCCAGCTGACCCCTACTGTGCTGTACATGTCCCTTCCAGGTGAATGGTGTGTTGATCATGTGTAATGCCATC 935

L M T F C P A D P Y C A C Y M S L F Q V N G V L I M C N A I 270

ATCGACCCCTTCAATATGCTTACGGAGCCAGAGCTCAGGGTGCATTCAAAAAGATGGTTATCTGCAACTGTTACCAGTAGAATGAT 1025

I D P F I Y A L R S P E L R V A F K K M V I C N C Y Q 297

TGGTCCCTGATTTAGGAGCCACAGGGATATACTGTCCAGGACAGAGTAGCGTGACAGCAACAACACTAGGACTCCAACCTGTCTCTCC 1115

CTTCCCTAGAGCATGAGGATAATCCACTAATCTTGTGTAATAGTCAATTCACGTAACAGCCTTGGCTATAGGGTACAACCTCTAT 1205

AGCTTAGAGAGGCAAAATAATACCAAAAGGCCAATAACAAGCACATACAGCAATACACATGTGAGAATCTGAGTAAAGAAAAG 1295

TGCTCTCTATAGATAACTTGGCAAGGAAATGCCAACCCACTCCAGTGTCTTGGCTGGAGAATCCAGGGATGGGGAGCCTGGTGG 1385

GCTGCCATCTATGGGGTCGGACAGAGTCGGACATGACTGAAGCGACTTAGCAGCAGCAGCATCACAGGGTGGCCTCTCTAAGTGGTAGG 1475

ACAAGCACTCAGGGTATGGACTACAGGTTTTTTGTAAGCCAAAGGTGCCAATTAGTGTGCTACCGCCTGCTCTCTTCTGTGCCACC 1565

TGCTTTGTGTGTACCATCTCCAGTCTTCTACAAGCAGGCAGGATGCATCTATAGTCAATAACAGCTTTGAGTTCCAATAATAAAA 1655

GGAAAGCTTCTCATTAGTCTTCTGGCTAAGTCAAGTCCACTGACTTTAAAGTATTTCTGGTAAAACCTTATGCTTTAGAAGCA 1745

TAAATTTCAAAGGAGAAATAAATATATAATCCATTTGGTCTTATGTAGGAATAAATAAGGCCAGAGGAAGACTGCGGCAAAATGAAG 1835

AAGGTATACCAACCTTGTGAATCTGTCAGTGTGCTGCTCAGTCCGCTCAGTGGTTTCCAACCTTTTGAGACCCCATGGACTGCAG 1925

ACCGCCAGGCTCTCTGTCTGTAGGATTTCCAGGCAAGAATACTGGAGGGGGTTGCCATTTCTCTCCAGGGACTCTTTCCAACCCAG 2015

CGATAGAACCTGCATCTCTGTGTCTCTGTATCAGCAAGCAGATCTCTACCCTGCACCACATAGGAAGCCCATACCTGTCCAACAC 2105

AGAGGTAAACGAAAAAGTGCAGATGTCCTCAAAATGCAATTTAAGTAGAGAAATGTGTGTTTACCAGGTTTTTGGATTATGAGGGAAAC 2195

CAAGACCAAAACAACATTTCAAGACAGAGCTATTATGGAGACTCAACCAGAGACTGGTAAAAACATTTAACTCATTCCCTAGATGAA 2285

TCTGAGGCAGACAGCATCACTGAAGAATTTCTCCTACTAGAGCCACAGGAAAGATGCCAGATCTAGCAAGTGGCATCCCCAGCAGGAT 2375

AAAAGAAACATAAATGTAGGTTTCTGTTTTTAAAGCAAAGCTTAAAGAAGAAGCAGATTCTGATCCTAAGTCTTTGTGAGTACACA 2465

GATTAATTTACAGGGATGAGCGATGGAAGGGACAGAGGCCAGATCTGCAGGTACACAGCACTGAGCGGCTCAGGAGCCAGCAAGAGGGAT 2555

GGTATCTTGGGAAGAGGCACCTTCTTCCCGCCAGAGTTTTCTCCTGAGTGTCTGAGAGGAGAAGCTAGCCATAAACAGGACTGCTGGTCAGG 2645

TGATGGGTAGAGTATTCGGTGTGGGGAGGCGAGGCTTAAAGAAGCAGAGGGAAACACAAGAATTAATGGGGAGGCTGCTGGGGTAGTT 2735

ATCCAAAAGGTCAATAATTCACCTGAAGCCGAAACCCAGTGTCTCTGCACCTCCATCTCCAGTAATCCTCTTTGATGCTCCCCCTC 2825

ACCAAAATGAATGACAAATACCCCTTTCTCCTATTCTCTTGTAGAGAACATCCAGGACAAAGGTGAAAAGAAGCAAAGACTGA 2909

Fig. 1. Sequences of the bovine ACTH receptor cDNA and the encoded protein. The sequence shown is that of the cDNA insert from clone R3. The sequence of the second clone (R1) starts at nucleotide 61 and shows two bp differences: a C at position 1776 and a C at 2734. The two repeats homologous to the artiodactyl A-type short interspersed nuclear elements (SINE) are underlined.

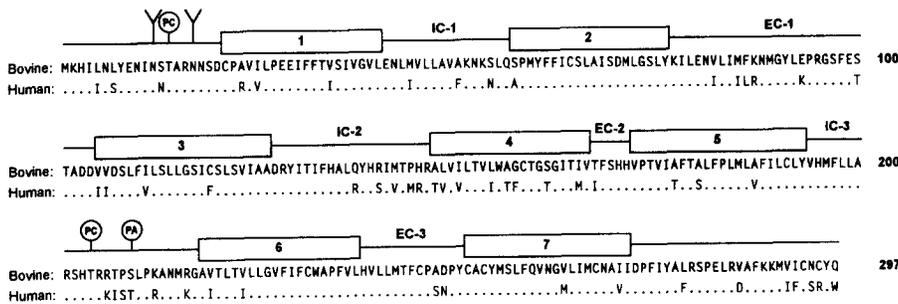


Fig. 2. Locations of the transmembrane (TM) domains and the putative post-translational modification sites of the ACTH receptor. Dots mark residues of the human [9] ACTH receptor that are identical to the bovine. The boxes demarcate the seven hydrophobic TM domains identified using the method of Eisenberg et al. [13]. IC and EC mark intracellular and extracellular loops respectively. The Asn residues predicted to be glycosylated are marked by a Y-like sign. Putative protein kinase A and C phosphorylation sites are marked by encircled PA and PC.

ceptor (Fig. 2). The sequence includes seven highly hydrophobic segments predicted as transmembrane domains (Fig. 2). In contrast to most G-protein-coupled receptors, both the bovine and human sequences have short extracellular N- and intracellular C-terminal domains and a short second extracellular loop, and lack cysteines in the first and second extracellular loops. The methods we used [13,14] do not indicate a short fifth domain as noted for the human receptor [9]. The conserved Asp (D) in the second transmembrane domain, and the triplet 'DRY' at the end of the third domain [15] also appear in both ACTH receptor sequences (Fig. 2). Since bovine and human ACTH are identical, the ACTH binding site of the receptors would be expected to be highly conserved. The homology between the bovine and human receptors is highest in the putative ligand binding sites observed in other receptors [15], and clearly distinguishes the ACTH receptors from the most closely related MSH receptors.

Biochemical studies using photoaffinity labeling and crosslinking techniques reported molecular masses ranging from 40 000 to 100 000 for ACTH binding proteins assumed to be the ACTH receptor [5]. Recent binding studies indicate only one class of ACTH receptor [6]. Even the value of 40 000 is still higher than the ~ 33 000 calculated from the amino acid sequence. Since both the bovine and human receptors have two glycosylation motifs in the N-terminus (Fig. 2), the difference between the weights of the predicted sequence and gel electrophoretic data may result from glycosylation of the receptor.

Analysis of the sequences revealed additional important consensus motifs in both bovine and human receptors (Fig. 2). A single motif for protein kinase A phosphorylation (R/K-R/K-X-X-S/T) [16] is located in the third intracellular loop (Ser-209) juxtaposed to a protein kinase C phosphorylation motif (T-X-R) [17] (Thr-204). A second kinase C motif is located at the extracellular N-terminus (Thr-14), and a casein kinase II (a Ser/Thr kinase) phosphorylation motif (S/T-X-X-D) in the second extracellular loop. Both the bovine and human receptors have no tyrosine phosphorylation motif characteristic of growth factor receptors.

The motifs located in the intracellular loops could be substrates for intracellular phosphorylation. The role of the cAMP-protein kinase A pathway in mediating the effects of ACTH is well established [7]. Activation of the protein kinase C pathway by a phorbol ester and angiotensin II was shown to modify the steroidogenic responses of the adrenal cortex to ACTH [18–20]. The present findings suggest that these effects may be mediated in part by phosphorylation of the ACTH receptor.

The bovine ACTH receptor cDNA shows a second smaller ORF of 219 residues located on the comple-

mentary strand within the limits of the first. Similar ORFs are also found in the human ACTH and MSH receptor genes. We do not know whether the cryptic ORF on the complementary strand is transcribed and translated. Among the sequences in the EMBL database, this ORF shows highest similarity (61% identity in 174 bp of overlap) to the 5'-terminus of a human α_{1A} -adrenergic receptor [21]. Yet, this region of the α_{1A} -adrenergic receptor cDNA represents an exact duplicate copy of an internal coding segment in reverse orientation, shows no similarity to the highly homologous rat cDNA [21], and therefore it may be a cloning artifact.

Do the sequences of ACTH and its receptor have complementary relationship?

The finding of an ORF on the complementary strand of the ACTH receptor gene led us to examine the 'Molecular Recognition Theory' which implicates an evolutionary relationship between peptide ligands and their receptors. The theory has been expounded mainly with studies suggesting that peptides encoded by the complementary strand of ACTH mRNA (dubbed 'HTCA') are similar to the ACTH receptor [22–26]. Our comparison of the complementary strands of the sequences presented in this paper with the sequence of the ACTH precursor POMC gene did not reveal a specific similarity. Moreover, the amino acid sequences of all the ORFs encoded by the ACTH receptor cDNA also did not show any specific similarity with either ACTH or HTCA. Whereas the maximum identity between the HTCA and the ACTH receptor is 21%, randomly selected proteins show stretches with 25% identity. Thus, our sequence comparisons, as well as other previous analyses [27], do not provide evidence for the Molecular Recognition Theory.

Reverse repeats in the 3'-untranslated region (UTR)

The bovine cDNAs reported here include a much longer 3'-UTR than the human cDNA (~ 2000 vs. 260 bp). The overlapping 3'-UTRs of the bovine and human sequences show only 64% identity in 247 bp with 12 gaps. Further downstream, the bovine 3'-UTR includes two repeats (Fig. 1) homologous to the artiodactyl A-type short interspersed nuclear elements (SINE). These elements, first observed in the bovine POMC gene encoding the ACTH precursor [28], are commonly found in intergenic regions, introns and UTRs of many genes [29,30]. The sequence of the bovine ACTH receptor cDNA is unique in the EMBL database in having two copies of this repeat in the 3'-UTR. These repeats apparently represent two sub-families of A-type SINE: only the distal one has a common deletion in position 110 of the 117 bp bovine consensus repeat [29], and also a unique microsatellite tail as compared to (AGC)₄ in the proximal (Fig. 2).

Therefore, the two repeats probably originated by independent insertions rather than local duplication of one repeat. The opposite orientation of the two repeats favors the formation of a hairpin loop yielding an extensive and strong secondary structure in this region. It remains to be determined whether the structure they afford to the RNA affects the stability or expression of the ACTH receptor mRNA.

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