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Genomic Structure and Promoter Characterization of the Human ACTH Receptor Gene

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One kb of the 5′-flanking region of the human ACTH receptor gene was isolated and partially characterized. Transient transfections with hGH reporter confirmed the promoter activity in Y1 cells. Putative elements for transcription factors involved in regulation were noted in the sequence. The promoter activity of some of the constructs is responsive to a treatment by forskolin, due to the presence of several CRE-like sequences. © 1997 Academic Press

The primary role of adrenocorticotropic hormone (ACTH) is to up-regulate the synthesis of adrenal glucocorticoids and mineralocorticoids through activation of adenylate cyclase and elevation of intracellular cAMP (1, 2). This hormone also stimulates in vivo proliferation of the adrenal cortex and is required for its normal development (3). Moreover, ACTH acts outside the adrenal cortex in the central and peripheral nervous system and the immune system through other melanocortin receptors which are distinct from the ACTH receptor (4, 5).

The human ACTH receptor gene was partly cloned in 1992 (6) and does not contain introns within the coding sequence. This receptor belongs to the superfamily of G protein-coupled receptors but its primary amino acid sequence sets it apart in the unique subfamily of the melanocortin receptors (6, 7). The ACTH receptor transcripts have only been detected, by Northern blot and *in situ* hybridization, in the adrenal cortex across the zona fasciculata-reticularis and the zona glomerulosa (6). Recently, it has been shown, using RT-PCR, that ACTH receptor mRNAs are expressed in the human skin (8). Previous data have shown that cultured human adrenocortical cells contain high affinity ACTH receptors and several transcripts encoding the ACTH receptor have been reported (9, 10). Moreover, ACTH highly increased the expression of the ACTH receptor mRNAs and binding sites in a time- and dose-dependent manner (9). In human, the stimulatory effect of the hormone occurs at the transcriptional level and is mediated by cAMP pathway. The up-regulation of a receptor by its ligand is a novel regulatory phenomenon and implies regulation at the molecular level of the encoding gene. Recently, we determined the structure of the 5′-untranslated region of the ACTH receptor mRNA using 5′-RACE methodology. We demonstrated the utilization of a major transcription start site located in an upstream exon separated from the coding exon by more than 1.8 kb of an intronic sequence (11).

In this study, we have cloned and analyzed about one kb of the 5′ flanking region of this gene and we report that this region conferred basal promoter activity onto chimeric constructs. Moreover, induction of this promoter activity is sensitive to cAMP due to the presence of several putative CRE-like sequences.

MATERIALS AND METHODS

Materials. Nystatin, penicillin/streptomycin, fetal calf serum, trypsin-EDTA and Dulbecco's Modified Eagle's Medium / Ham's nutrient mixture F-12 (DMEM/F12 1:1) were purchased from Gibco-BRL (Cergy-Pontoise, France); forskolin from Sigma-Aldrich (Saint-Quentin-Fallavier, France); restriction enzymes from Boehringer (Meylan, France).

Isolation and characterization of the human ACTH receptor promoter. We used a novel method called PromoterFinder DNA walking kit (Clontech, Ozyme, Montigny-le-Bretonneux, France), to isolate genomic DNA upstream of exon 1 of the human ACTH receptor. This kit contains five "libraries'' that are a pool of genomic DNA fragments obtained after digestion with five different restriction enzymes. Both ends of each DNA fragment are ligated to a PromoterFinder Adaptor. Two PCR were performed using Advantage *Tth* Polymerase Mix (Clontech). The first PCR used a sense oligonucleotide specific of the adaptor and an antisense oligonucleotide (5′-TCAGCTCTGAAGCAGGAACTTTCTGG-3′) specific of the 3′-end of exon 1 of the human ACTH receptor gene as

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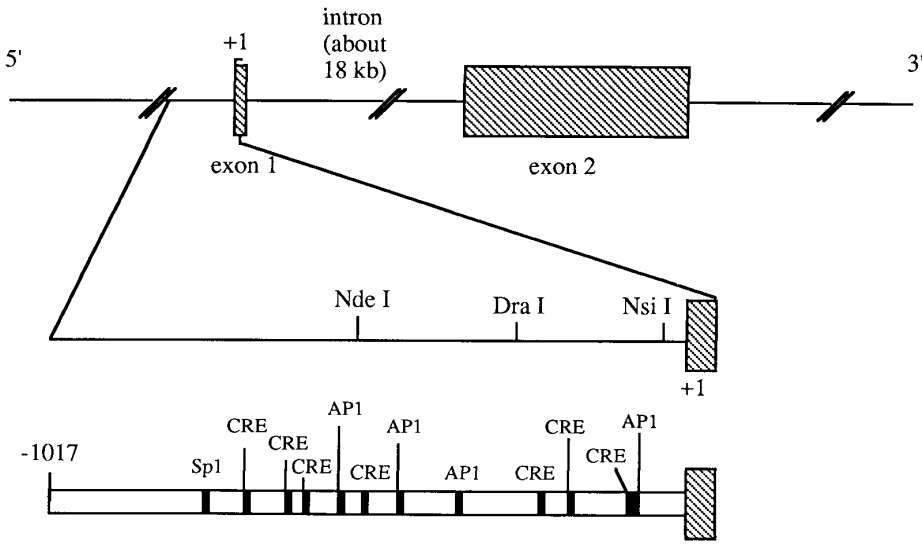

FIG. 1. Genomic structure of the human ACTH receptor. An enlargement of the promoter region shows the restriction sites used to obtain the different constructs. Relative positions of putative regulatory elements are also shown at the bottom.

primers, and one of the library as template. This primary PCR reaction mixture was used as template for the second PCR that was performed using nested primers either specific of the adaptor (sense) or specific of the 5′-end of exon 1 (antisense, 5′-TCTGGGCAAAATGAATGAGAAGGAA-3′). PCR were carried out according to the instructions provided with the kit. The PCR products, corresponding to sequences upstream of exon 1, were then subcloned into *Sma*I-cleaved pTZ18 (Pharmacia Biotech, Orsay, France) and sequenced in both directions using the dideoxy chain termination method (12) and [α-^{35}S] dATP with the T7 sequencing kit (Pharmacia) according to the instructions provided.

PCR amplification of the intronic sequence. Human genomic DNA, prepared from blood lymphocytes, was used as template for PCR with the Expand Long Template PCR system (Boehringer), which is a mixture of thermostable Taq and Pwo DNA polymerases. For the first PCR, the forward primer (5′-CTAGCTGTATCTCCGGTGATGCATGTGTTCCG-3′) was specific of a sequence upstream of exon 1 of the human ACTH receptor gene, and the reverse primer (5′-AGCCAGAAGTTCTGCATCTC-3′) was located 1.4 kb upstream of the ATG codon, inside the intron. PCR conditions were: 30 cycles of denaturation (10 s at 92°C), annealing (30s at 62°C) and elongation (13 min to 17 min for the last 5 cycles at 68°C). This primary PCR reaction mixture was used as template for a second PCR using nested primers. The forward primer (5′-ATTCCTTCTCATTCATTTTGCCCAGAAAG-3′) was specific of exon 1 and the reverse primer (5′-ACAGGGATGAGTCACCTTCGTGCCTCCT-3′) was located 1.6 kb upstream of the ATG codon inside the intron. PCR conditions were the same as those used in the primary PCR.

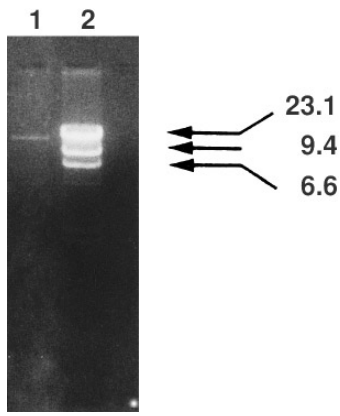

FIG. 2. PCR amplification of the intronic sequence. The sequence located between exon 1 and the coding exon was amplified by PCR as described in Materials and Methods (lane 1). Lane 2 corresponds to MW marker Lamda/ *Hind*III (Oncor-Appligene, Illkirch, France).

Vector constructions. The p(−1017/+22)GH vector was generated by ligating the *Bam*HI/*Eco*RI-digested ptZ18 containing products of PCR using PromoterFinder DNA walking technology (Clontech) to the *Hind*III-digested p0GH (13). Sites of digestion were filled in with the Klenow fragment of DNA polymerase to obtain blunted ends before ligation. The p0GH vector contains a promoterless human Growth Hormone (hGH) structural gene subcloned into pUC12. Fragments of different sizes in p0GH were obtained by digestion of p(−1017/+22)-GH with various enzymes, to generate: p(−503/+22)GH, p(−268/+22)GH and p(−56/+22)GH.

As a positive control, the pTK-GH vector was used (13). This vector contains the hGH structural gene placed under the control of the thymidine-kinase promoter.

Cell culture and transfection. The mouse adrenocortical tumor cell line Y1 (14) was used to study the promoter activity of the different constructs. The day before transfection, cells were plated on 6-well dishes at 200,000 cells per well, in Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F-12 (DMEM-F-12 1/1) supplemented with NaHCO3 (14 mM) and Hepes (10mM) and containing gentamycin (20μg/ml), penicillin (100U/ml), streptomycin (0.1 mg/ml) and fetal calf serum (10%). Transient transfections were performed as described before (15) using LipofectAMINE reagent (Gibco-BRL, Cergy-Pontoise, France) and 5μg of chimeric constructs. Twenty-four hours after transfection, cells were treated or not with 10^{-5}M forskolin. After 24h treatment, medium was collected and the concentration of hGH was quantitated by Radio Immunological Assay (hGH COATRIA, bioMérieux, Marcy-L'Etoile, France).

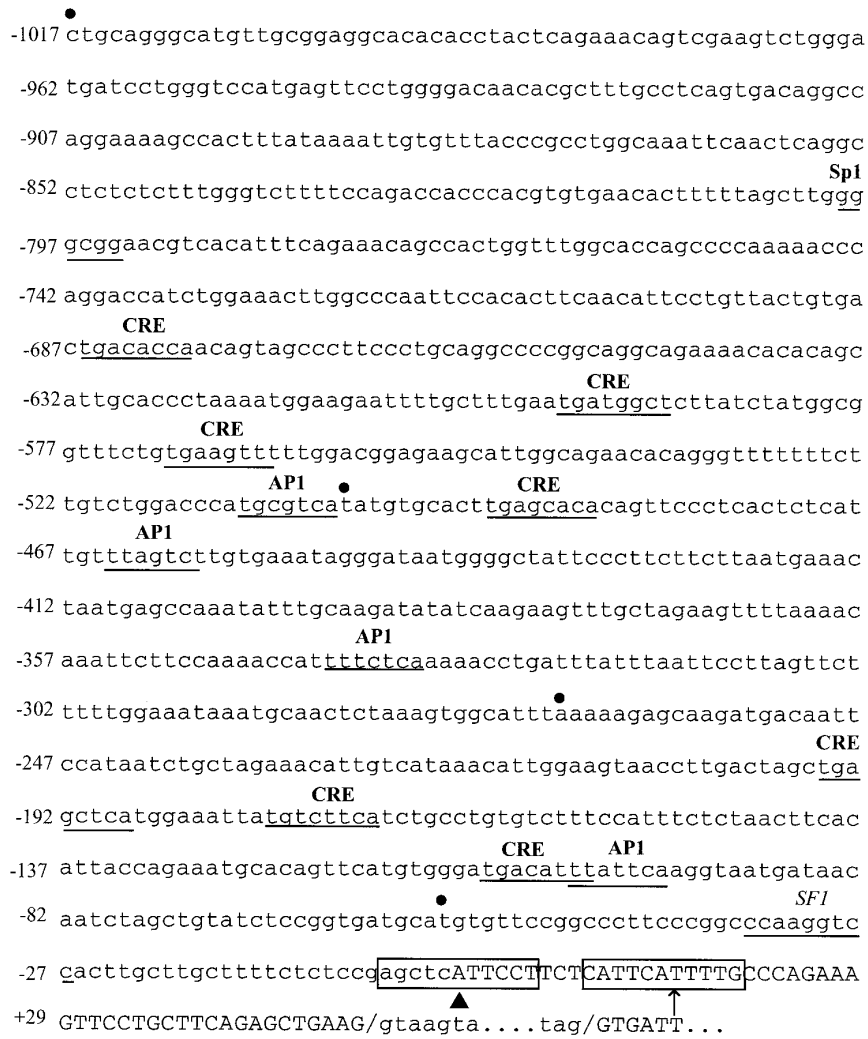

FIG. 3. Nucleotide sequence of the 5′-flanking region of the human ACTH receptor gene. The sequences of one kilobase of the promoter region, the exon 1 and exon/intron junction are shown. Upper cases indicate exonic sequences. Nucleotide numbering starts with +1 corresponding to the major transcription start site (▲) defined by 5′-RACE. The minor transcription start site is also indicated (arrow). Potential transcription regulatory sites are underlined and the putative Inr are boxed. The first nucleotide of each fragment used to obtain the different constructs is shown (●).

RESULTS AND DISCUSSION

Genomic Structure of the Human ACTH Receptor Gene

We have isolated about 1 kilobase of the 5′-flanking region of exon 1 of the human ACTH receptor gene. The presence of this exon 1 upstream of the coding exon has been previously described by using 5′ Rapid Amplification of cDNA end methodology (5′ RACE) (11). The existence of this exon upstream of the coding exon (Figure 1) was confirmed by Clark and Cammas (16) by comparison of results obtained by primer extension and S1 nuclease protection analysis. On the contrary, in the mouse ACTH receptor gene, two 5′ exons have been reported (17, 18). A third exon of 57 bp located between exon 1 (109 bp) and exon 3 (113 bp) is present in only some transcripts. In this gene, the coding exon is exon 4.

The intron separating exon 1 from exon 2 in the human ACTH receptor gene had a size of about 18 kb, as demonstrated by using a long range PCR (Figure 2). Consensus acceptor and donor splice sites (tag/G and AAG/g respectively) are present and shown in Figure 3.

Analysis of the 5′-Flanking Region of the Human ACTH Receptor

Examination of the human ACTH receptor gene sequence, at 5′ position of the major transcription

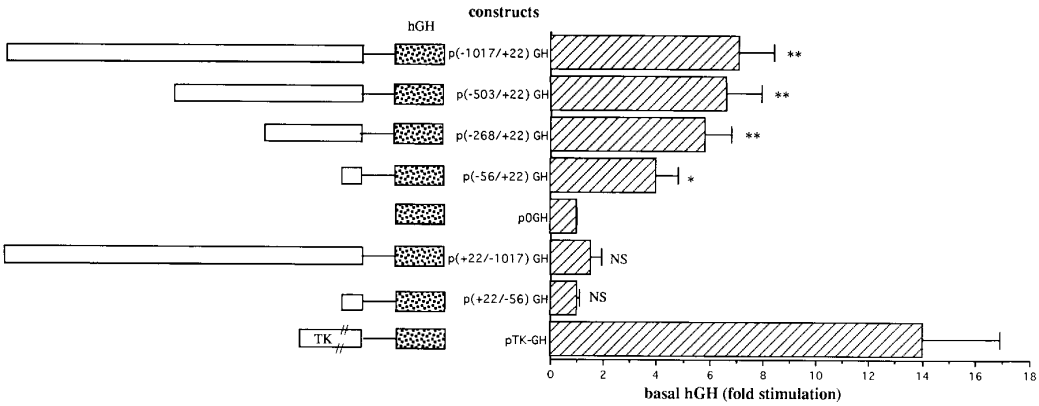

FIG. 4. Basal promoter activity of the different constructs. Y1 cells were transiently transfected with different constructs containing fragments of the 5′-flanking region of the human ACTH receptor gene. Activity was calculated by measuring the hGH secreted for 24 hours by transfected but not treated cells and is expressed as fold stimulation over the value obtained with cells transfected with p0GH. pTK-GH served as positive controls for transfection. Values are mean±SEM of 4 to 9 experiments. Significant differences between constructs and p0GH are indicated: **, $p<0.005$ and *, $p<0.025$. NS = not significant.

initiation site designated as +1 in Figure 3, did not reveal the presence of consensus CAAT- or TATA-boxes. One sequence resembling an Initiator (Inr) element (19), extending from nucleotide −5 to +6 directly overlapped the major transcription start previously described (11). A second putative Inr was present, extending from nucleotide +10 to 20, and overlapped the minor transcription start site (11). Such elements (PyCCTCAPyTCTG) have been described in several genes without TATA box and are involved in determining the transcription initiation site (19, 20). A GGGCGG sequence was found at position −799, corresponding to the consensus sequence that binds the transcription factor Sp1 (21). A near consensus sequence of the TPA responsive element (TRE) (22) was identified at four positions: −510 (TGcGTCA), −464 (TtAGTCt), −339 (TttCTCA) and −102 (TtAtTCA). The small characters correspond to differences between TRE-like sequences and the consensus TRE (TGAGTCA) that interacts with phosphoproteins Jun and Fos in the form of heterodimers (AP1). There are seven putative cAMP responsive el-

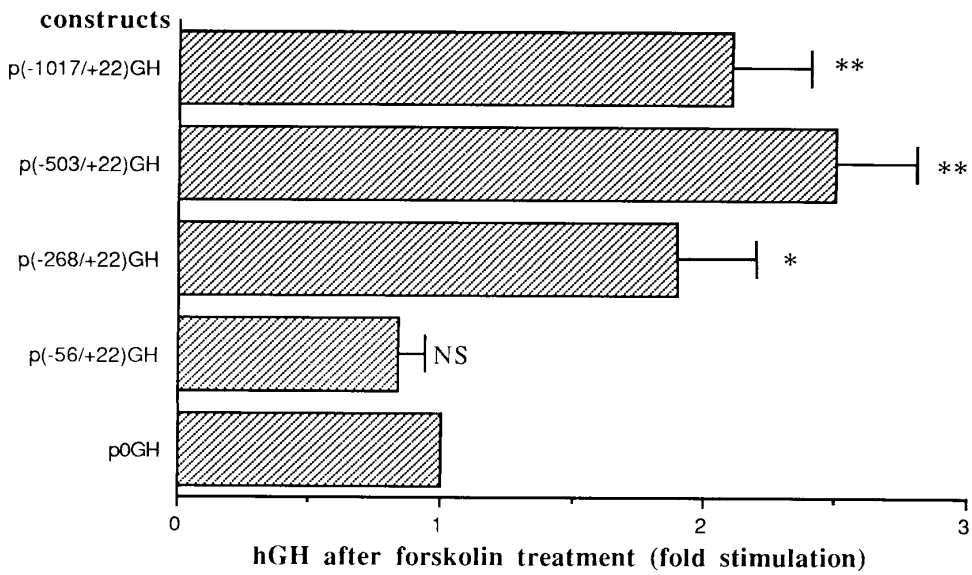

FIG. 5. Effect of forskolin on constructs containing deletions of the ACTH receptor promoter. Y1 cells were transiently transfected with different constructs. Results are expressed as fold effect of forskolin in cells transfected with the different constructs over cells transfected with p0GH. Value obtained for p0GH was fixed at 1. Values are mean±SEM of 5 to 9 experiments. Significant differences with 1 (no stimulation by forskolin) are indicated: **, $p<0.01$ and *, $p<0.05$.

ements (CRE) (consensus CRE=TGACGTCA) (23), at positions −686 (TGACacCA), −598 (TGAtGgCa), −570 (TGAaGTtt), −493 (TGAgCaCA), −196 (TGAgcTCA), −178 (TGtCtTCA) and −107 (TGACaTtt).

At position −35, the sequence CCAAGGTCc was present, which differs by only one base from the consensus Steroidogenic Factor 1 site (SF1) : PyCAAGGPyPyPu (24). This sequence has been described in the gene of steroidogenic enzymes (24) and is important in the expression of these enzymes.

Demonstration of Promoter Activity in the 5′-Flanking Region of the Human ACTH Receptor

To test for the basal promoter activity of the 5′-flanking region, four lengths of genomic fragments were subcloned, in correct orientation, into the p0GH reporter vector (13). These fragments were obtained by digestion with the different restriction enzymes indicated in Figure 1. All these fragments contained also the 22 first nucleotides of exon 1. Fragments in reverse orientation were also cloned in the same vector, for the 1kb construct and the smallest one, as negative controls.

The concentrations of hGH secreted by control cells transfected with the constructs p(1017/+22)GH, p(−503/+22)GH or p(−268/+22)GH were in the same range and not significantly different between them: 7.1±1.3, 6.6±1.3 and 5.8±1.0 fold stimulation as compared to the value obtained with cells transfected with p0GH, respectively (Figure 4). For cells transfected with the p(−56/+22)GH construct, we obtained about 56% of the hGH levels secreted by cells transfected with the 1kb construct. This 56bp fragment still contains the necessary elements to confer basal transcription. An SF1-like element was present in the sequence of this fragment (Figure 3) and could then confer basal promoter activity as described for several steroid hydroxylase genes (24). No activity over the value obtained with cells transfected with p0GH was demonstrated for cells transfected with constructs containing promoter fragments in reverse orientation (Figure 4).

Lebrethon et al (9) have demonstrated that the human ACTH receptor gene expression is positively regulated by ACTH via the cyclic AMP pathway. This hormone upregulated also the number of ACTH binding sites. To look for the presence of functional cAMP responsive elements, Y1 cells transfected with the different constructs were treated with 10^{-5}M forskolin. For cells transfected with the p(−56/+22)GH construct, no significant stimulation by forskolin over the value obtained with cells transfected with p0GH was obtained (Figure 5). This result could be explained by the absence of putative CRE in the sequence located between −56 and +1. On the contrary, for cells transfected with the other constructs, a 2-to 2.5-fold stimulation by forskolin was observed. These values were significantly different from the value obtained with cells transfected with p0GH but differences were not significant between the three constructs. Three putative CREs were present in the sequence located between −268 and +1 and further experiments will be necessary to demonstrate the involvement of one or several of these elements or other ones in the cAMP regulation.

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