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A Steroidogenic Factor-1 Binding Element Is Essential for Basal Human ACTH Receptor Gene Transcription

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We have previously shown that the promoter of the human ACTH receptor (ACTH-R) contains, at −35 bp, a binding site for the steroidogenic factor 1 (SF-1), an orphan nuclear receptor which could be responsible for the transcriptional activity of this promoter. In the present study, electrophoretic mobility shift assays demonstrated that the sequence −43/−19 bound the SF-1 protein present in the nuclear extracts of adrenocortical cells. Mutation of the SF-1 binding site markedly reduced (40%) the basal transcription of the reporter gene in Y-1 cells transfected with the mutated p(−56/+22)GH construct compared to the wild-type construct. These results demonstrate that the SF-1 binding element present in this fragment is required for the basal promoter activity of the human ACTH-R gene. In addition, other binding elements located upstream from this characterized SF-1 binding site are involved in the full basal promoter activity of the human ACTH-R since transfection studies with a longer p(−1017/+22)GH construct resulted in a higher GH release than with the p(−56/+22)GH construct. © 1998 Academic Press

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ACTH is the main hormone that regulates the synthesis of glucocorticoids through activation of adenylate cyclase (1). ACTH plays also a trophic role in the expression and maintenance of the specific differentiated functions of the adrenal cortex and positively regulates its own receptors (2, 3). The cloning of the human ACTH-R cDNA has shown that the coding sequence is contained in a single exon (4). This receptor belongs to the superfamily of G-protein coupled receptors and more precisely to the sub-family of the melanocortin receptors (4-6).

The human ACTH-R is expressed primarily in the adrenal cortex although there is some evidence for expression of its mRNA in the skin by using RT-PCR (7). Northern blot analysis of human ACTH-R mRNAs revealed the presence of several transcripts (8, 9) which could be explained by the use of alternative polyadenylation signals (10). In cultured human adrenocortical cells, ACTH and Angiotensin-II increased the expression of ACTH-R mRNA and the number of binding sites in a time- and dose-dependent manner (8). Other activators of the cAMP pathway are also able to up-regulate the expression of the ACTH-R mRNA (9).

The cloning and the characterization of the 5′-flanking region of the human ACTH-R gene have shown that the human gene is composed of two exons separated by an intronic sequence of about 18 kb (11, 12). One major transcription start site is located in the first, non-coding, exon (13). One kb of the promoter region of the human ACTH-R gene has been cloned (11, 12). It does not show any typical characteristics of promoter regions such as TATA- or CAAT-boxes but a sequence resembling an Initiator (Inr) sequence was found spanning part of exon 1 (12). The presence of several putative binding sites for transcriptional factors has been reported in the promoter of the ACTH-R. In particular, an SF-1 binding site was described for the first time (12). Presence of such an element was further confirmed in the murine promoter (14).

The basal activity of the 1-kb region of the human ACTH-R promoter was tested *in vitro* after transient transfection of Y-1 cells with chimeric constructs containing a part of the promoter region and a reporter gene encoding the hGH. The results have shown that the minimal promoter contained the putative SF-1 element at −35 bp from the transcription initiation site (12). SF-1 is an orphan nuclear receptor which regulates many genes directly involved in steroidogenesis or other target genes from the hypothalamo–pituitary–gonadal axis (15-22). SF-1 is also implicated in the development of the adrenal gland and gonads (23-25). The aim of this study was to characterize this SF-1-like sequence at position −35 bp in the human ACTH-R promoter and to determine its role in the transcription of the corresponding gene.

MATERIALS AND METHODS

Materials. Ham's nutrient mixture F-12/ Dulbecco's Modified Eagle's Medium (F12/DMEM 1:1), fetal calf serum, gentamycin, penicil-

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lin/streptomycin, nystatin and LipofectAMINE reagent were purchased from Gibco-BRL (Cergy-Pontoise, France). Restriction and modification enzymes were from Boehringer Mannheim (Meylan, France). All chemicals were obtained from Sigma Chemical Co. (St Quentin-Fallavier, France).

Oligonucleotides. Complementary single-stranded oligonucleotides were synthesized (Genosys, Cambridge, England) and annealed to generate the double-stranded oligonucleotides representing either wild-type (ONSF1) or mutant (ONSF1m) human ACTH-R promoter elements. SF-1 like element is bolded and the mutation is underlined. The sequence containing consensus CRE is also shown (ONCRE). (a) ONSF1 (−43/−19), 5′TTCCCGGC**CCAAGGTCC**ACTTGCTT 3′, 3′AAGGGCCG**GGTTCCAGG**TGAACGAA 5′; (b) ONSF1m (−45/−17), 5′CCTTCCCGGC**CCAA*TT*TCC**ACTTGCTTGC 3′, 3′GGAAGGGCCG**GGTT*AA*AGG**TGAACGAACG 5′; (c) ONCRE consensus, 5′TGACTAGCTGACGTCATGGAAATTA 3′, 3′ACTGATCGACTGCAGTACCTTTAAT 5′.

Vector constructions. The p0GH reporter gene plasmid (Nichols Institute Diagnostics, San Juan Capistrano, CA) was used as expression system. The plasmids p(−1017/+22)GH and p(−56/+22)GH were obtained as previously described (12). Stratagene Quik Change Site directed Mutagenesis kit (Ozyme, Montigny-le-Bretonneux, France) was used to generate plasmids p(−1017/+22)mGH and p(−56/+22)mGH where the SF-1-like element is mutated as described above. Sequences of all mutated constructions were checked using the T7 sequencing kit (Pharmacia Biotech, Orsay, France). pTKGH, where hGH gene is under the control of the thymidine kinase promoter, was used as a positive control for transfection.

Cell culture and transfections. The mouse adrenocortical tumor cell line Y-1 was used to study the promoter activity of the different constructs. The day before transfection, cells were plated on 6-well dishes at 150 000 to 200 000 cells/well in F12/DMEM medium supplemented with $NaHCO_3$ (14 mM) and Hepes (10 mM) and containing gentamycin (20 μg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (20 U/ml) and fetal calf serum (10%). Transient transfections were performed in serum-free medium without antibiotics using the LipofectAMINE reagent as described before (12, 26) and 5 μg of chimeric constructs. Twenty-four hours after transfection, the medium was changed. After twenty-four hours, the medium was harvested and the hGH secreted in the cell medium was quantitated by RIA (hGH COATRIA, bioMerieux, Marcy-l'Etoile, France). The cells were dissolved in 0.5M NaOH, 0.4% sodium deoxycholate and the protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Montluçon, France).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from Y-1, COS-7, M3 and bovine adrenocortical cells and from human adrenal tissue as described by Dignam *et al.* (27). The double-stranded oligonucleotides were end-labeled using T4 polynucleotide kinase and γ (^{32}P) ATP (4000 Ci/mmole, ICN Biomedicals, Orsay, France). Ten micrograms of nuclear extracts were incubated with 2 μg poly(dI-dC).poly(dI-dC) as non-specific competitor in a final volume of 20 μl of EMSA buffer (10 mM Hepes pH 8, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 4 mM spermidine, 4% Ficoll). The incubation was performed for 10 min at 4°C after addition of 10,000 dpm of labeled probe. For the competition assays, a molar excess of unlabeled oligonucleotide was added simultaneously with the labeled probe. The resulting DNA-protein complexes were analyzed by electrophoresis using a 5% polyacrylamide gel and 0.5X Tris-Borate-EDTA as running buffer. The gels were dried and exposed at −80°C to an autoradiographic film. In some experiments, EMSA was performed using *in vitro* transcribed/translated SF-1, which was prepared using the TNT Coupled Reticulocyte Lysate System (Promega, Charbonnieres, France) with pBS-SF-1 as a template and T3 RNA polymerase (20). pBS-SF-1 was a generous gift of Dr. E. McCabe (Los Angeles, CA). In other experiments, nuclear extracts were preincubated with an anti-SF-1 (Ad4BP) polyclonal antibody, provided by Dr. K. Morohashi (Okazaki, Japan), before addition of the labeled probe (15).

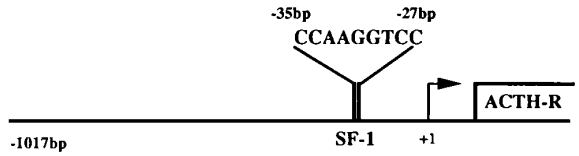

FIG. 1. Schematic diagram of the 5′ flanking region of the human ACTH-R gene. The arrow indicates the transcription start site.

RESULTS

The potential SF-1 binding site interacts specifically with SF-1. Examination of the proximal promoter region of the human ACTH-R gene (Fig. 1) showed a putative binding site for the steroidogenic factor 1 located from −35 to −27 bp (CCAAGGTCC) that differed by a single nucleotide (underlined) from the reported SF-1 binding elements (PyCAAGGPyCPu) (16, 28).

We used electrophoretic mobility shift assays (EMSA) to study the proteins that bind to the labeled probe containing the binding site for SF-1 (ONSF1). As shown in Fig. 2, in the presence of Y-1 nuclear extracts, two complexes were obtained, one major protein-DNA complex (CI) and one minor (CII). Formation of both complexes was competed in a dose-dependent manner by the homologous unlabeled ONSF1 probe but not by adding a 200-fold molar excess of the unlabeled ONSF1m probe, containing a mutated binding site (CCAA**TT**TCC), showing that the two mutated bases are crucial for the protein binding (Fig. 2). Also, no competition was observed when using a heterologous probe (ONCRE) (Fig. 2). Both complexes, CI and CII, were then specific. To determine which protein is contained in the CI complex, different approaches were then taken. First, EMSA was performed using *in vitro* transcribed/translated SF-1 instead of nuclear extracts. When SF-1 or Y-1 nuclear extracts were incubated with ONSF1 probe, the same specific binding pattern was observed (Fig. 2). Second, the specific complex CI was displaced when the Y-1 nuclear extracts were preincubated with a specific antibody against bovine SF-1 (Fig. 2). Third, the complex CI, formed with ONSF1 probe by using nuclear extracts from adrenocortical cells of different origins (murine, bovine or human) (Fig. 3A) was not observed by using nuclear extracts from non-steroidogenic cells (monkey kidney cell line COS-7, murine melanoma cell line M3) (Fig. 3B). All of these results demonstrate that a SF-1 related protein, present in the nuclear extracts of adrenocortical cells, interacts specifically with the SF-1 binding element localized in the human ACTH-R proximal promoter, to form the complex CI in EMSA. The complex CII was not observed in all the experiments and could

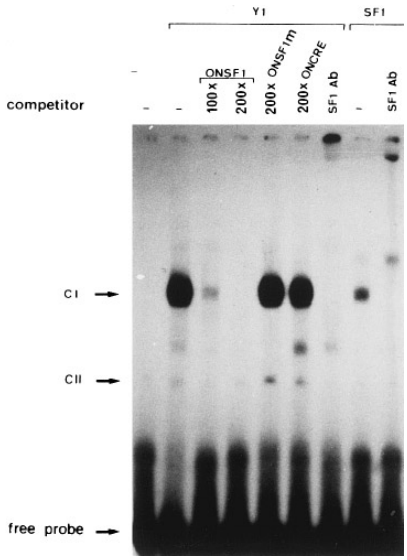

FIG. 2. Specificity of binding to the human ACTH-R SF-1 binding site. 10 μg of Y1 cell nuclear extracts were incubated with the ^{32}P-end labeled ONSF1 probe with or without increasing molar excess of unlabeled ONSF1 competitor or with a 200x molar excess of the ONSF1m competitor or the heterologous ONCRE competitor, or in the presence of 1 μl of SF-1 polyclonal antiserum (SF-1 Ab) as indicated. ^{32}P-end labeled ONSF1 probe was also incubated with 1 μl of in vitro synthesized SF-1 in the presence or absence of 1 μl of SF-1 polyclonal antiserum. Position of free probe, complexes I and II is indicated.

be due to a proteolytic product of the full length SF-1 protein, formed during the preparation of nuclear extracts (29). As the −56/+22 bp region of the promoter of the human ACTH-R gene is transcriptionally active (12), the finding that SF-1 interacts specifically with

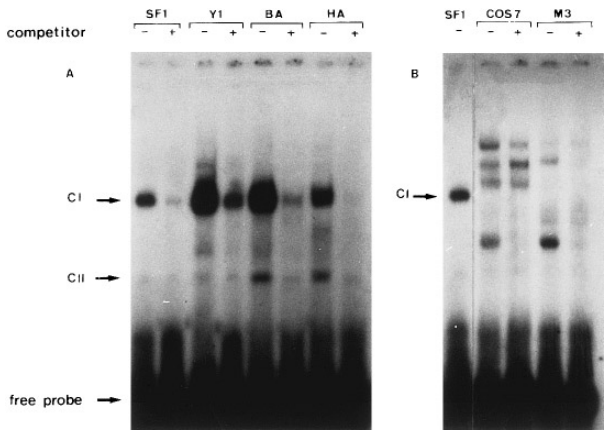

FIG. 3. EMSA using nuclear extracts from adrenocortical cells (A) and non-steroidogenic cells (B). ^{32}P-end labeled ONSF1 probe was incubated with 1 μl of in vitro synthesized SF-1 or with 10 μg of nuclear extracts, in the absence (−) or presence (+) of a 200x molar excess of unlabeled ONSF1 competitor. Nuclear extracts are from Y1 cells, bovine adrenal cells (BA), human adrenal tissue (HA), monkey kidney cells (COS-7) or murine melanoma cells (M3).

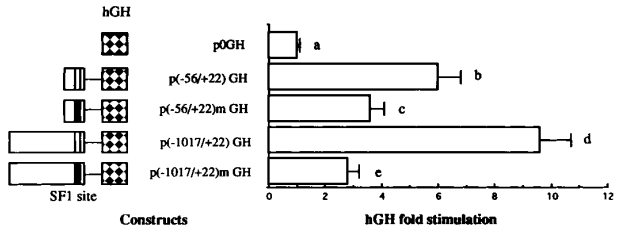

FIG. 4. Basal promoter activity of the p0GH constructs in Y-1 transfected cells. Y1 cells were transiently transfected with 5 μg of each plasmid schematically represented on the left side of the figure. Transcriptional activity was calculated by measuring the hGH secreted during 24 hours and is expressed as fold stimulation over the value obtained with cells transfected with p0GH. Transfections were performed in triplicate, and values are mean ± SEM of 5 different experiments. Statistical analysis was performed with Student's t-test for comparison of two groups. Significant differences between each construct are indicated by different letters ($p < 0.05$). (Levels of hGH secretion for p0GH and pTKGH were 2.3±0.3 and 46.2±8.9 ng/mg protein, respectively).

the −43/−19 bp DNA sequence is consistent with a model where SF-1 could activate the transcription of the human ACTH-R gene.

The SF-1 site is required for basal promoter activity. In order to test the basal promoter activity of the 5′-flanking region of the human ACTH-R, several constructs were prepared by ligating deleted fragments of this region into the p0GH reporter vector (12). The p(−56/+22)GH was the smallest of our constructs able to stimulate the secretion of hGH by transiently transfected Y-1 cells and this construct contains the SF-1 binding element studied above by EMSA experiments. Transfection of Y-1 cells with this construct increased basal GH release by 6-fold as compared to p0GH (Fig. 4). Mutation of the SF-1 binding site by site directed mutagenesis reduced by 40 per cent the basal secretion of hGH in Y-1 cells transfected with this construct p(−56/+22)mGH compared to the wild type construct but did not abolish it (Fig. 4). These results suggest that the SF-1 binding site is essential but is not the only sequence involved in the basal promoter activity of ACTH-R gene in Y-1 cells.

The relative importance of this SF-1 binding site compared to the other putative binding sites for transcription factors was estimated by transfection of Y-1 cells with p(−1017/+22)GH containing or not the mutation in the −35/−27 bp SF-1 sequence element. The results of Fig. 4 show that the expression of the reporter gene was stimulated 9.6-fold when the transfection was performed with the construct p(−1017/+22)GH compared to p0GH whereas this expression was stimulated 6-fold with p(−56/+22)GH. When the SF-1 response element was mutated, the level of expression of the reporter gene was similar in Y-1 cells transfected with either p(−1017/+22)mGH or p(−56/+22)mGH.

DISCUSSION

The promoter of the human ACTH-R contains a sequence corresponding to a SF-1 binding site located at −35 bp relatively to the transcription initiation site (11, 12). A SF-1 binding site has also been described in the mouse ACTH receptor proximal promoter (14).

In the present study, we demonstrated that this SF-1 binding site is critical for the constitutive activity of the human ACTH-R gene promoter. The SF-1 protein present in nuclear extracts from adrenocortical cells formed a specific complex with the probe containing the putative SF-1 binding site located at −35 bp on the ACTH-R promoter as shown by EMSA. This complex appears similar to the complex obtained with a recombinant SF-1 protein. By contrast, when nuclear extracts from non-steroidogenic cells were incubated with this probe, the binding pattern was different and no specific complex corresponding to that observed with the recombinant SF-1 protein was observed. This result was probably due to the presence and absence of SF-1 in steroidogenic and non-steroidogenic tissues, respectively (30-33).

In Y1 cells, the p(−56/+22)GH construct containing the SF-1 binding site is the smallest of our constructs conferring a basal activity. The mutation of the SF-1 binding site in this construct leads to a significant reduction (40%) of promoter activity thereby revealing an important role of SF-1 in the constitutive expression of the human ACTH-R gene. Since the promoter activity is not completely abolished by the mutation, it appears that SF-1 is absolutely necessary but not sufficient. Similar results were reported for the Steroidogenic Acute Regulatory Protein (StAR) mouse promoter (34). Other regulatory elements may be required for a full basal activation of this promoter. An initiator element encompassing the start site of transcription was identified in the promoter of the human ACTH-R gene (12) which may serve as a positive regulator of the transcription of the gene. It has been reported that mutations in Inr elements could affect transcriptional activity of some other genes (35, 36).

Transfection studies in Y1 cells with the p(−1017/+22)GH construct containing other putative regulatory elements in addition to SF-1 led to a higher promoter activity than those obtained with the p(−56/+22)GH construct suggesting that SF-1 is not the unique regulator of this promoter. Other transcriptional factors which could act independently or as coactivators must be involved as demonstrated for other genes (33, 37). However, the mutation of the SF-1 binding site in both the smallest and the longest constructs led to the same reduced promoter activity suggesting that an intact SF-1 binding site is absolutely required for the recruitment of the other regulatory factors which could interact with SF-1.

This finding is very interesting since SF-1 plays a pivotal role in determining the tissue specific expression of its target genes. SF-1 is predominantly expressed in adrenal gland, ovary and testis where it regulates the expression of the steroid hydroxylases and some other genes expressed in steroidogenic cells (38). Nonetheless, SF-1 cannot be the sole determinant of the specific expression of the ACTH-R, limited to the adrenal cortex, as SF-1 is present in all steroidogenic cells. Other regulatory proteins must also participate in cell-selective expression of this receptor.

In both human and murine ACTH-R promoter, there is no evidence for the presence of a consensus cAMP responsive element despite the fact that ACTH is recognized to stimulate ACTH-R expression via cAMP (12, 14).

The role of SF-1 in the hormone or cAMP-induced expression has been reported for several genes (39). We have demonstrated in a previous report that forskolin treatment of the Y-1 cells transfected with the p(−56/+22)GH construct did not significantly alter promoter activity (12). This result showed that the −35/−27 bp SF-1 binding site of the promoter of the human ACTH-R is critical for basal activity but is not necessary or not sufficient to confer cAMP or ACTH stimulation. Similar results have been reported for the SF-1 binding site of the mouse promoters of the StAR and the P450 side chain cleavage genes (34, 40).

Further studies are in progress to determine the other transcriptional factors involved in addition to SF-1 and their potential interactions with the SF-1 protein in the regulation of the human ACTH-R gene transcription.

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