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Three Steroidogenic Factor-1 Binding Elements Are Required for Constitutive and cAMP-Regulated Expression of the Human Adrenocorticotropin Receptor Gene

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In the present study, we characterized two new SF-1 binding sites, SF-209 and SF-98, in the promoter of the human ACTH receptor (hACTH-R) gene. Both sites, together with the previously described SF-35 site, are required for full constitutive activity of this gene. This was demonstrated by the use of constructs containing part of the promoter upstream of the luciferase gene and carrying mutation in one of these sites, to transiently transfect H295R cells. Mutations of either SF-35, SF-98, or SF-209 induced a decrease of luciferase activity. This effect was amplified when two or three elements were mutated together in the same construct. Only SF-35 and SF-98 seem to play a major role in the cAMP-induced regulation of the hACTH-R gene, since mutation of either one of these sites reduced the forskolin induction of luciferase activity by 50%. When both elements were mutated, no stimulation was obtained over the control. This indicates that SF-1 protein must bind to both sites for the cAMP response.

The human adrenocorticotropin receptor (hACTH-R) is a seven-transmembrane receptor which belongs to the melanocortin receptor family (1–3) and is expressed almost exclusively in the adrenocortical cells. Its ligand, the pituitary ACTH, regulates the synthesis of adrenal glucocorticoids and mineralocorticoids through the binding to specific receptors, leading to the stimulation of adenylate cyclase and the increase of cAMP production (4). A characteristic of the ACTH-R gene is that its expression is positively regulated by its own ligand through the cAMP pathway (5). Indeed, both ACTH-R mRNA levels and receptor number are increased by a treatment in the presence of ACTH (5, 6). In human, this ACTH up-regulation occurs, at least partly, at the transcriptional level (5).

Part of the hACTH-R gene was cloned in 1992 (1) and we have recently isolated and studied 1.1 kb of its promoter (7). The induction of promoter activity is sensitive to cAMP but there is no evidence of the involvement of the CRE-like sequences, present in some fragments, in this regulation. We have also shown the presence of one Steroidogenic Factor-1 (SF-1) binding element at −35 bp from the transcription start site. This element is involved in the basal promoter activity but is not sufficient for the cAMP regulated expression of the hACTH-R gene (8).

The mouse ACTH-R gene has been recently cloned (9) and an SF-1 element, at −25 bp, appears also important for basal promoter but there is no evidence of transcriptional cAMP-regulated expression of the mACTH-R. As the SF-1 protein is expressed mostly in steroidogenic tissues, in ventromedial nucleus of the hypothalamus and in gonadotropes of the pituitary, it could also play a role in cell-specific expression of the hACTH-R possibly by cooperation with other factors (9).

An SF-1 binding sequence (PyCAAGGPyCPu) (10) is found in various genes encoding steroidogenic enzymes and the SF-1 protein has already been shown to be involved in the basal expression of numerous P450 steroidogenic genes (11), along with its involvement in the development and differentiation of the adrenal and gonads (12, 13). Its role is not limited to the regulation of steroidogenic enzymes, since SF-1 plays also a key role in controlling the expression of the Müllerian inhibiting substance (MIS) in primary cultured Sertoli cells (14) and the glycoprotein hormone α subunit in pituitary gonadotropes (15). It has also been described as involved in the cAMP-regulated expression of several genes including CYP17, CYP11A and StAR (16–18).

In this report, we characterized two SF-1 binding sites not previously described, in the hACTH-R gene

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promoter, located upstream of the first identified one. We have also studied their role in both basal and cAMP-regulated expression, with respect to the SF-1 site located at −35.

MATERIALS AND METHODS

Cell culture. H295R (human adrenocortical tumor cell line) (19) were cultured in DMEM/F-12 (1:1) containing 15 mM Hepes, 14 mM $NaHCO_3$, supplemented with 3% fetal calf serum, 2% Ultroser G (BioSepra, Villeneuve-la-Garenne, France) and 1% ITS (insulin 10 mg/L, transferrin 5.5 mg/L, selenium 5 μg/L). All complete media contained gentamycin (20 mg/L), penicillin (100 U/ml) and streptomycin (0.1 mg/ml)

Reporter vector constructions. pGL3 basic (Promega, Charbonnières, France) was used as expression vector. Different fragments of hACTH-R gene promoter were amplified by PCR using specific oligonucleotides containing, at the 5′ end, sequences for *Nhe*I or *Bgl*II restriction endonuclease sites, as 5′ or 3′ primers, respectively, and human genomic DNA as template. After digestion by the appropriate restriction enzyme, the fragments were ligated to the *Nhe*I/*Bgl*II-digested pGL3 basic, upstream of the coding region of the luciferase reporter gene. Four wild type constructs were generated in this way: p(−1017/+22)luc, p(−503/+22)luc, p(−263/+22)luc and p(−63/+22)luc. Sequences were checked to ensure that there were no PCR generated mutations.

Constructions of vectors containing mutated fragments were obtained using the Quik Change Site-directed Mutagenesis kit from Stratagene (Ozyme, Montigny-le-Bretonneux, France) and oligonucleotides containing mutations of the SF-1 site.

Transient transfections. Transfections of H295R cells were achieved in serum-free medium without antibiotics using the lipofectAMINE PLUS reagent (Gibco-BRL, Cergy-Pontoise, France), in the presence of 2 μg of DNA. According to the method suggested by the manufacturer, cells were incubated with the DNA-liposome complexes for 3 h and medium was, then, replaced by 1 ml of fresh complete medium.

To monitor transfection efficiency, the pGL3 Control vector was used. It contains SV40 promoter and enhancer sequences, resulting in strong expression of the luciferase gene.

Luciferase assays. Twenty-four hours after transfection, cells were treated 48 h in the presence or not of 10^{-5} M forskolin. Luciferase activity was measured in cell extracts using the Luciferase Assay system (Promega) and a luminometer (Turner Designs-Bioblock, Illkirch, France) to measure the light produced during 15 s at room temperature. Statistical analysis was performed with Student's *t* test for comparison of two groups.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from H295R cells as described by Dignam *et al.* (20) and the protein concentrations were determined by the BCA protein assay system (Pierce Chemical, Montluçon, France). The double-stranded oligonucleotides, containing a putative SF-1 binding site, were labeled by T4 polynucleotide Kinase (Promega) using [γ-^{32}P]ATP (4000 Ci/mmol, ICN Biomedicals France, Orsay) and incubated with nuclear extracts as previously described (8). For the competition assays, 200-fold molar excess of unlabeled double-stranded oligonucleotide used as competitor was added to the reaction mixture. In additional competition experiments, double-stranded oligonucleotides containing mutations were used. In some experiments nuclear extract proteins were preincubated with an anti-SF-1 polyclonal antibody (21), kindly provided by Dr. K. Morohashi (Okazaki, Japan). In other experiments, SF-1 protein was used instead of total nuclear extracts. It was obtained by *in vitro* transcription/translation using the TNT Coupled Reticulocyte Lysate System (Promega), pBS-SF-1 as template and T3 RNA polymerase (22). pBS-SF-1 was a generous gift of Dr. E. McCabe (Los Angeles, CA). The resulting DNA-protein complexes were analyzed by electrophoresis using a 5% polyacrylamide gel with 0.5X Tris-borate-EDTA as running buffer, during 2 h at 250 V. Gels were dried under vacuum at 80°C for 1 h and exposed to Kodak BioMax MR film (Sigma-Aldrich, Saint-Quentin Fallavier France).

RESULTS

Regulation of luciferase reporter gene by the hACTH-R promoter. We examined the promoter activity of various lengths of the hACTH-R gene 5′-flanking region. When transiently transfected into H295R cells, all DNA fragments regulated the basal expression of the luciferase gene (Fig. 1). The p(−63/+22)luc construct had a basal activity with a 4.4 ± 0.5-fold stimulation over results obtained with the pGL3 basic. The use of longer constructs for the transfection gave higher stimulations: 17.3 ± 1.9, 24.8 ± 2.5, and 40.7 ± 5.8 with p(−263/+22)luc, p(−503/+22)luc and p(−1017/+22)luc, respectively. These variations in the activities of the constructs were statistically significant between −503 and −1017 bp but not between −263 and −503.

Figure 2 shows the results obtained for the responsiveness to forskolin. There was no stimulation by a 48-h treatment with 10^{-5} M forskolin, for cells transfected with the p(−63/+22)luc construct. A 2- to 2.5-fold stimulation over the results obtained with pGL3 basic was elicited by the other constructs. The variation in the activities of the constructs between −263 and −1017 were not statistically significant.

These data led us to search for cis elements responsible for basal and cAMP responsiveness between −263 and −63 bp from the transcription start site.

SF-1 binds to elements SF-98 and SF-209. Between −263 and −63 two sequences corresponding to SF-1-like binding sites were present. At −98 to −90, the sequence of the putative SF-1 binding site (SF-98): TCAAGGTAA is very similar to the sequence of the previously described SF-35 (8), with only one base which diverges from the consensus sequence (10). The putative SF-1 binding sequence (TAACCTTGA) located at −201 to −209 (SF-209) is in reverse orientation compared to the other sites SF-35 and SF-98, and is also divergent from the consensus site by one base.

When EMSA was performed, using H295R cell nuclear extracts and a double-stranded labeled probe containing the SF-98 site, one major specific protein–DNA complex was observed (Fig. 3). Formation of this complex was abolished by the addition of a 200-fold molar excess of non-radiolabeled homologous competitor, but not by a mutated oligonucleotide in which the two G present in the core sequence were replaced by two T (M-98). Hence, these two G are required for SF-1 binding. The specific complex migrated in the same manner as a complex formed after incubation of the SF-98 probe with SF-1 protein (Fig. 3). In addition, formation of the specific complex between the probe and the nu-

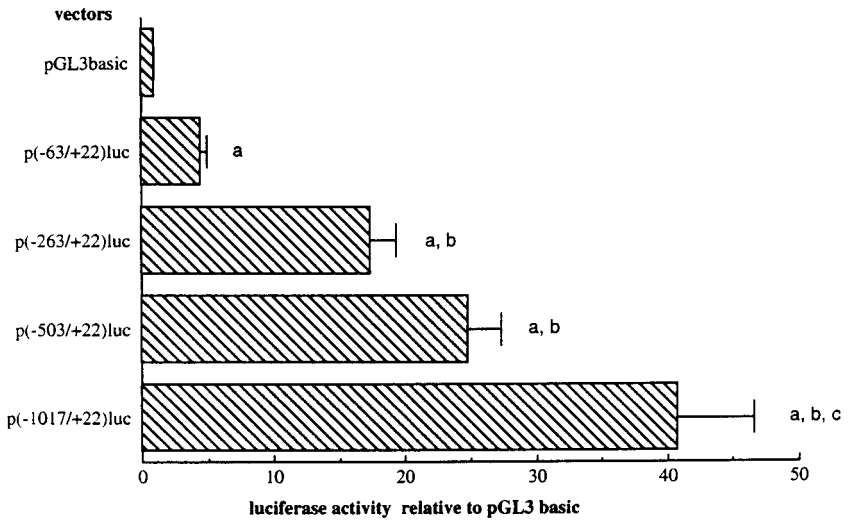

FIG. 1. Analysis of basal promoter activity in H295R cells. Cells were transfected, as described under Materials and Methods, with various hACTH-R constructs. Values are mean ± SEM of four different experiments performed in triplicate and are expressed as luciferase activity relative to the value obtained with pGL3 basic. Values obtained for this plasmid are defined as 1.0. Relative luciferase activity obtained for the pGL3 Control used to evaluate the transfection efficiency was 70.8 ± 6.4. (a) indicates values significantly different from the value obtained with pGL3 basic ($p < 0.05$); (b) indicates values significantly different from the value obtained with p(−63/+22)luc ($p < 0.05$); (c) indicates values significantly different from the value obtained with p(−263/+22)luc ($p < 0.05$).

clear extracts was blocked by an antibody directed against SF-1 protein. These findings indicate that the SF-98 element in the hACTH-R promoter binds SF-1 protein.

Similar results have been obtained using a labeled oligonucleotide containing the sequence located at −209 to −201 bp (Fig. 4). After EMSA, a major specific complex was obtained, running at the same position in the presence of the SF-1 protein as with H295R cell nuclear extracts. It was displaced by the unlabeled homologous oligonucleotide and by an antibody directed against the SF-1 protein but not by an unlabeled oligonucleotide carrying a mutation of the two C (replaced by two T) in the core sequence (M-209).

Some light faster non specific bands could also be observed using either one of the labeled probes.

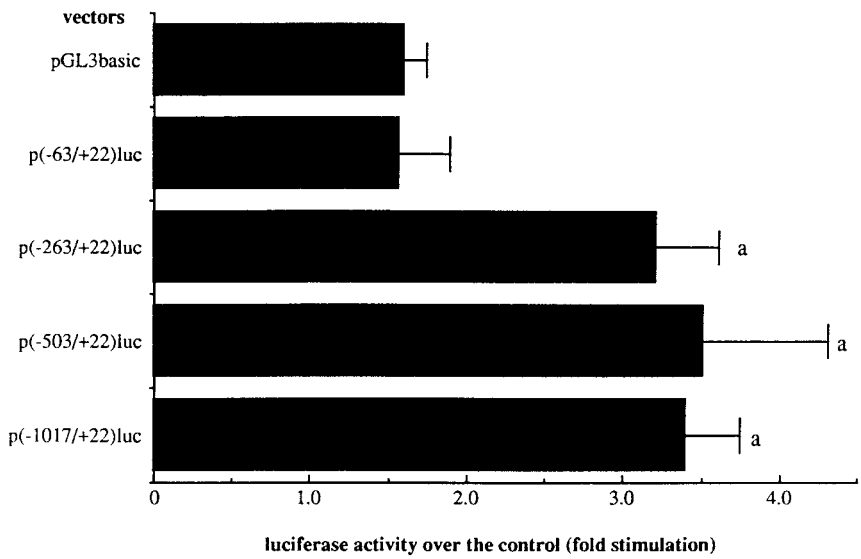

FIG. 2. Analysis of cAMP-stimulated promoter activity in H295R cells. Cells were transfected with various constructs and, then, treated for 48 h with 10^{-5} M forskolin. Values are mean ± SEM of four to five experiments performed in triplicate and are expressed, for each construct, as luciferase activity relative to the respective unstimulated control. a indicates values significantly different from values obtained with pGL3 basic ($p < 0.03$).

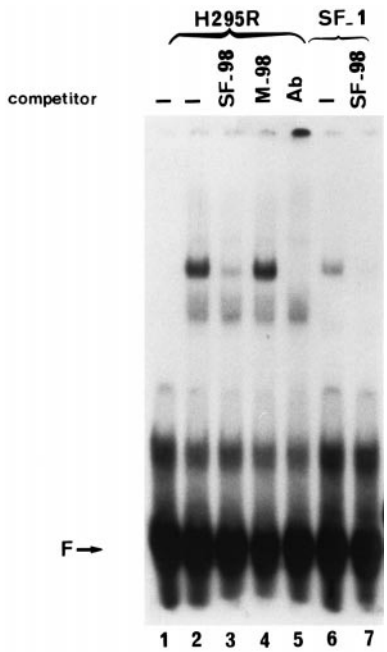

FIG. 3. Binding of H295R nuclear proteins and SF-1 to the −98/−90 element. H295R nuclear extracts (lanes 2 to 5) and SF-1 protein (lanes 6 and 7) were incubated with 10,000 dpm of the radiolabeled probe containing the −98/−90 element, in the presence (lanes 3 and 7) or not (lanes 2 and 6) of a 200-fold molar excess of unlabeled probe. In lane 4, incubation was performed in the presence of a 200-fold molar excess of unlabeled mutated oligonucleotide (M-98) and lane 5 in the presence of antibodies directed against SF-1 protein. Lane 1 corresponds to the labeled probe alone, without nuclear extract. DNA–protein complexes were separated from free probe (F) by gel electrophoresis.

Role of the SF-1 elements on the basal and cAMP-regulated expression. To evaluate the functional role of the three SF-1 binding elements and their relative importance, H295-R cells were transfected with the p(−263/+22)luc either wild-type or carrying mutation(s) of the SF-1 sites. These sites were mutated as described above in EMSA for SF-98 and SF-209 or as described by Marchal *et al.* (8) for SF-35.

Mutation of either one of the SF-1 binding sites decreased the basal luciferase activity of the p(−263/+22)luc after transfection of H295R by 47, 63, and 58%, respectively, for M-35, M-98, and M-209 (Fig. 5). These variations were statistically significant ($p < 0.006$). Decreases obtained with M-98 and M-209 were not significantly different from each other, indicating that both elements have similar effectiveness on the basal expression of the hACTH-R gene. But the site at −35 is significantly less effective. When double mutations of the SF-1 sites were generated, the constructs obtained gave an equivalent reduced luciferase activity (decrease of about 80%), compared to constructs carrying only one mutation. Effects seem rather additive than synergistic. The basal promoter activity of a construct displaying three mutated SF-1 elements, was even more reduced compared to the double mutated constructs (statistically significant differences: $p < 0.05$). This triple-mutated construct retained a basal promoter activity which corresponded to a 4.9-fold increase compared to the promoterless pGL3 vector. This residual activity is fully explained by the presence of the Initiator (Inr) element overlapping the transcription start site (7). Indeed, the triple mutated construct carrying a mutated Inr has no significant basal activity over the pGL3 basic (data not shown).

When cells transfected with the wild-type p(−263/+22)luc construct were treated by forskolin during 48 h, luciferase activity was increased by 2.5-fold over the control value of the same transfected cells. Mutations M-35 and M-98 substantially diminished this value by about 50% ($p < 0.001$) (Fig. 6). The M-209 mutation elicited only a 20% decrease (Fig. 6), which is statistically significant ($p < 0.02$). The double mutations M-35/M-98 and M-98/M-209, and the triple mutation completely abolished the forskolin-induced increase of promoter activity.

DISCUSSION

We have used for this study the human adrenal carcinoma NCI-H295 cells and, more precisely, the

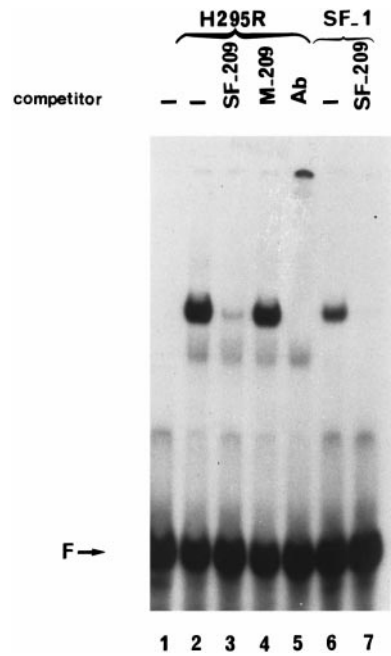

FIG. 4. Binding of H295R nuclear proteins and SF-1 to the −209/−201 element. H295R nuclear extracts (lanes 2 to 5) and SF-1 protein (lanes 6 and 7) were incubated with labeled SF-209, in the presence (lanes 3 and 7) or not (lanes 2 and 6) of a 200-fold molar excess of unlabeled SF-209. In lane 4, incubation was performed in the presence of a 200-fold molar excess of unlabeled mutated SF-209 (M-209) and in lane 5 in the presence of antibodies directed against SF-1 protein. Lane 1 corresponds to the labeled probe alone, without nuclear extract. F, free probe.

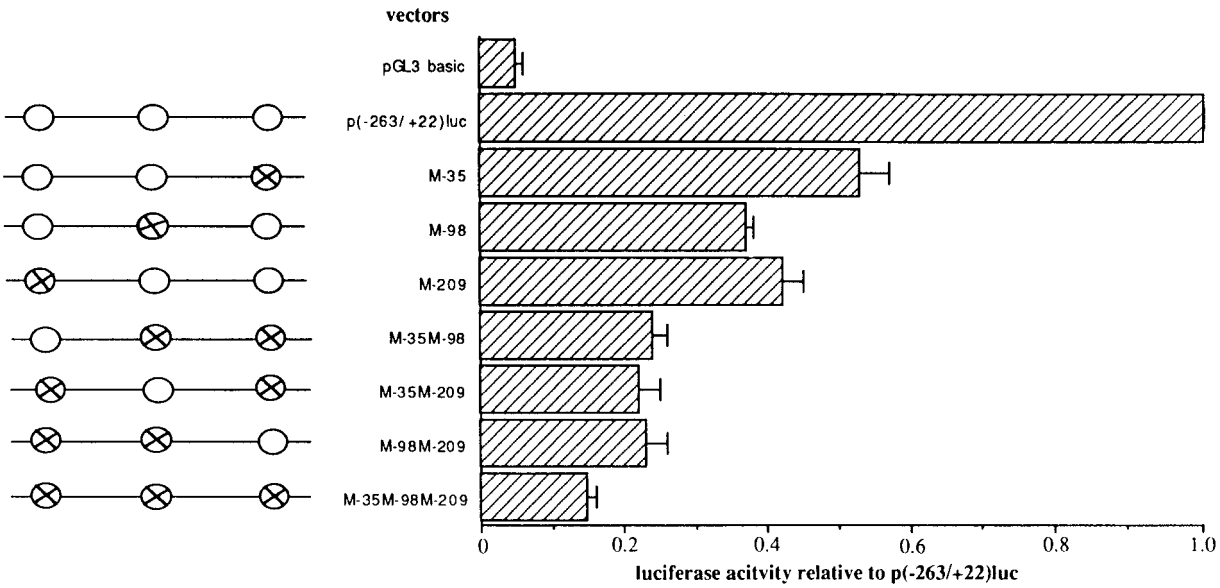

FIG. 5. Basal promoter activity after transfection of H295R cells with mutated p(−263/+22)luc constructs. H295R cells were transiently transfected with various constructs carrying at least one mutated SF-1 element (scheme on the left). Values are mean ± SEM of seven different experiments performed in triplicate and are expressed as luciferase activity relative to the value obtained with wild type p(−263/+22)luc.

subline of adherent H295R (19), which has been shown to secrete corticosteroids, mineralocorticoids and androgens. Moreover, H295R cells retained, to some extent, the capacity to produce steroid hormones in response to ACTH (19). These cells represent therefore a good model to study the regulation of human genes expressed in adrenals.

The *cis* acting elements, which seem important for the constitutive expression of the hACTH-R gene, are present between −63 and +22 bp, between −63 and −263 bp and between −503 and −1017 bp. The first −263 bp upstream from the transcriptional start site, seems to be responsible for the cAMP-induced transcriptional activity.

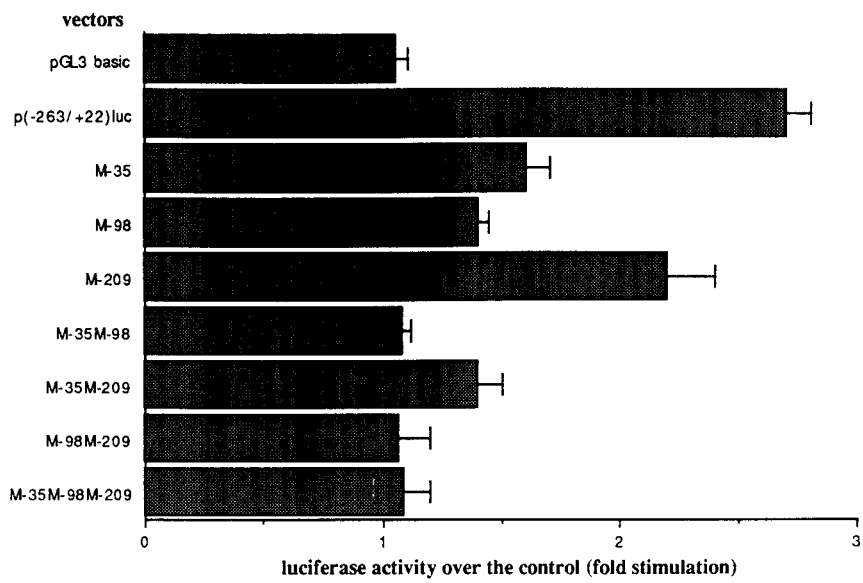

FIG. 6. AMP-stimulated promoter activity of mutated p(−263/+22)luc constructs. H295R cells were transiently transfected with the same mutated constructs as in Fig 5. Twenty-four hours after transfection, cells were treated for 48 h in the presence of 10^{-5} M forskolin. Values are mean ± SEM of seven different experiments performed in triplicate and are expressed as fold stimulation over the respective control value for each construct.

We have previously shown that an SF-1 element located at −35 bp, (then contained in the p(−63/+22)luc construct) is important for the constitutive expression of the hACTH-R gene (8). In this paper, we demonstrate that two other SF-1 binding sites are present, at −98 bp and −209 bp from the transcription start site. Both elements were specifically bound by SF-1, as demonstrated by EMSA experiments. To study their respective involvement in basal and cAMP-mediated expression of the hACTH-R gene, mutations that abolished the binding of SF-1 to these sites, were introduced into the p(−263/+22)luc construct for transfection experiments in H295R cells. Above results demonstrate that these three SF-1 elements are major contributors to basal promoter activity, since mutation of any of these elements significantly decreased promoter activity. Mutation in all three binding sites in the same construct induced the higher decrease (85%). Thus, binding of SF-1 protein to all three SF-1 binding sites is required for a full activity of the hACTH-R gene promoter.

Concerning the forskolin-stimulated promoter activity, only SF-35 and SF-98 appear to be major contributors, with a slightly, but statistically significant ($p < 0.04$), higher effectiveness for SF-98. SF-209 has only a slight effect on this cAMP-induced stimulation. Interestingly, SF-35 alone seems not capable of giving any response to cAMP. Indeed, the construct p(−63/+22)luc, which contains only this SF-1 element was inefficient in stimulating the promoter activity after a forskolin treatment of H295R cells. So, it seems that SF-98 and SF-35 must be both present to elicit the response to cAMP. The importance of the SF-98, acting with the SF-35, is also supported by results obtained after transient transfection of the double-mutated construct M-98M-209, where only SF-35 could bind SF-1 protein. In this case, there was no stimulation of the promoter activity by forskolin, suggesting some cooperation between SF-1 sites.

It is interesting to compare results obtained here with the hACTH-R gene promoter and those reported by Sugawara *et al.* (18) for the human StAR gene promoter. A similar situation was observed, with three SF-1 elements (in reverse orientation) located at −926/−918, −105/−95 and −42/−35 bp from the transcription start site. After transient transfection of human granulosa–lutein cells by a 1.3-kb human StAR promoter-luciferase fusion construct carrying mutation on either one of the SF-1 element, they demonstrated a role for the three sites on basal expression. However, only the two more proximal sites are important for cAMP responsiveness in these cells.

We have clearly demonstrated, in this study, the important role of SF-1 in the control of the expression of the human hACTH-R gene, specifically expressed in the adrenal cells. Further studies will be necessary to evaluate the potential cooperativity between the different SF-1 sites in the cAMP regulation of the hACTH-R gene expression.

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