A novel mutation in the steroidogenic acute regulatory protein gene promoter leading to reduced promoter activity

Andres J Casal, Victoria J P Sinclair¹, Alessandro M Capponi, Jérôme Nicod¹, Uyen Huynh-Do¹ and Paolo Ferrari^{1,2}

Division of Endocrinology, Diabetology & Nutrition, University Hospital, Geneva 1211, Switzerland

¹Division of Nephrology, Inselspital, Berne 3010, Switzerland

²Department of Nephrology, Fremantle Hospital, University of Western Australia, Perth 6160, Australia

(Requests for offprints should be addressed to P Ferrari; Email: paolo.ferrari@health.wa.gov.au)

Abstract

We have identified a novel cytosine/thymidine polymorphism of the human steroidogenic acute regulatory (*StAR*) gene promoter located 3 bp downstream of the steroidogenic factor-1 (SF-1)-binding site and 9 bp upstream of the TATA box (ATTTAAG). Carriers of this mutation have a high prevalence of primary aldosteronism. In transfection experiments, basal *StAR* promoter activity was unaltered by the mutation in murine Y-1 cells and human H295R cells. In Y-1 cells, forskolin (25 μ M, 6 h) significantly increased wild-type promoter activity to $230 \pm 33\%$ ($P < 0.05$, $n = 4$). In contrast, forskolin increased mutated promoter activity only to $150 \pm 27\%$, with a significant 35% reduction compared to wild type ($P < 0.05$, $n = 3$). In H295R cells, angiotensin II (AngII; 10 nM) increased wild-type *StAR* promoter activity to $265 \pm 22\%$ ($P < 0.01$, $n = 3$), while mutated *StAR* promoter activity in response to AngII only reached $180 \pm 29\%$ of controls ($P < 0.01$, $n = 3$). Gel mobility shift assays show the formation of two additional complexes with the mutated promoter: one with the transcription repressor DAX-1 and another with a yet unidentified factor, which strongly binds the SF-1 response element. Thus, this novel mutation in the human *StAR* promoter is critically involved in the regulation of *StAR* gene expression and is associated with reduced promoter activity, a finding relevant for adrenal steroid response to physiological stimulators.

Journal of Molecular Endocrinology (2006) **37**, 71–80

Introduction

Recent reports suggest that variations at the gene *CYP11B2* that encodes the aldosterone synthase enzyme are associated with essential hypertension, and may influence aldosterone secretion (Davies *et al.* 1999, Paillard *et al.* 1999, Takeda *et al.* 1999, Lim *et al.* 2002, Nicod *et al.* 2003). Two variants, a cytosine/thymidine (C/T) substitution in the 5'-promoter region, which disrupts a putative binding site for steroidogenic factor-1 (SF-1), and a gene conversion in intron 2 (Int2 C) of *CYP11B2* are in tight linkage disequilibrium (White & Slutsker 1995, Lim *et al.* 2002, Nicod *et al.* 2003). This SF-1 T/Int2 C haplotype is increased in frequency in hypertensive patients (Davies *et al.* 1999) and has subsequently been associated with a high aldosterone-to-renin ratio (ARR) in selected hypertensive patients (Lim *et al.* 2002, Nicod *et al.* 2003). These findings indicate an increased sensitivity to angiotensin II (AngII) in the presence of the TT genotype of SF-1 in the promoter of *CYP11B2*.

The exact mechanisms for increased aldosterone synthase activity, associated with the T allele in the *CYP11B2* promoter, are still objects of debate. Alternative explanations are increased transcription factor

availability at other functional sites of the gene, linkage of the SF-1 and Int2 C sites with a quantitative trait locus in the regulatory elements or the possibility of transcriptional activation of the steroidogenic acute regulatory (*StAR*) gene by an SF-1-dependent mechanism (Garbers & Dubois 1999). The latter is supported by the observation that only some, but not all, carriers of the T allele show increased aldosterone synthase activity.

Delivery of the substrate cholesterol to the inner mitochondrial membrane and to the cytochrome P450 side-chain cleavage enzyme is the rate-limiting step in steroid hormone synthesis (Crivello & Jefcoate 1980, Privalle *et al.* 1983). The transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where it is cleaved to pregnenolone, is mediated by the *StAR* protein, which was identified and cloned by Clark *et al.* (1994). Mutations in the *StAR* gene are the cause of the potentially lethal condition known as congenital lipoid adrenal hyperplasia (lipoid CAH) (Lin *et al.* 1995). Mutations causing lipoid CAH are all found in the C-terminal region of the *StAR* protein (Miller 1997). *StAR* expression can be regulated both positively and negatively by agents that presumably act on its promoter (Stocco 2001). SF-1 was

the first transcription factor to be studied as a potential regulator of the *StAR* gene. Several SF-1 consensus-binding sites have been identified in the *StAR* promoter (Caron *et al.* 1997, Sugawara *et al.* 1997). Two of these sites, located at positions –97 and –42, are highly conserved in several species whose promoter regions have been sequenced. It is presently unknown whether polymorphisms at these sites are present and whether they might affect *StAR* promoter activity, thereby providing an explanation for the variable aldosterone synthase activity associated with the SF-1 polymorphism in the CYP11B2 promoter. Thus, in search of potential mechanisms underlying primary aldosteronism, we have screened the *StAR* gene for polymorphisms at SF-1-binding sites in hypertensive patients whose aldosterone and renin status was known.

Materials and methods

Patients

We screened for polymorphisms in the SF-1-binding sites of the human *StAR* gene promoter in 20 control subjects and 40 hypertensive patients, who had measurements of plasma renin and immunoreactive plasma aldosterone under standardised conditions as previously described (Nicod *et al.* 2003). Thereafter, the prevalence of the identified polymorphism was assessed in an additional cohort of 172 hypertensive patients who also had measurements of plasma immunoreactive renin and plasma aldosterone under standardised conditions and 76 healthy controls. Among all 212 hypertensive patients, 36 had an ARR above the diagnostic cut-off level for primary aldosteronism (Ferrari *et al.* 2004), but were not homozygous for the T allele of CYP11B2, which is commonly associated with an increased ARR (Nicod *et al.* 2003). Finally, a group of 98 patients with diabetes mellitus type 2 was also tested for the presence of the same identified polymorphism. Thus, genotyping for the *StAR* gene promoter was performed on a total of 406 subjects.

Screening for polymorphisms of the human *StAR* gene promoter

The promoter region of the *StAR* gene harbouring the two conserved SF-1-binding sites and the TATA box was screened for the presence of polymorphisms by single strand conformation polymorphisms (SSCP) in 60 subjects using genomic DNA, isolated from peripheral blood leucocytes. The DNA (100–200 ng) was amplified in a 50- μ l reaction mixture containing 3 mM MgCl₂, 0.4 μ M of each primer, 0.2 mM dNTP and 1 U AmpliTaq Gold polymerase (Perkin Elmer Corp., Foster City, CA, USA) in the presence of the buffer provided with the enzyme. Thirty two cycles of PCR were performed: 30 s at 94 °C, 30 s at 62 °C and 1 min at

72 °C. Primers were 5'-CTGTCCTCCCTACTCTCCCC-3' and 5'-CTCTCAAGGGTGGTTCTTCG-3' and yielded a 258 bp fragment amplifying the –328 to –71 bp region upstream to the start codon. All PCR products were analysed by SSCP on 12% acrylamide gels containing 7.25% glycerol using a two-buffer system. Four microlitres of the PCR sample were loaded and DNA was visualised by silver staining (Lovati *et al.* 2001). Any variations detected by this technique were characterised by directly sequencing the PCR amplified fragment with an ABI PRISM Model 3700 (Applied Biosystems, Foster City, CA, USA). This screening allowed us to identify a C/T single nucleotide polymorphism (SNP) in the promoter region of the *StAR* gene, with loss of a MspA1 I restriction site in the mutant.

Genotyping

In the remaining sample of 366 subjects, genotyping of the identified SNP in the promoter of the *StAR* gene was done by specific restriction enzyme digestion. In all subjects, genomic DNA was amplified using the primers and conditions designed for the SSCP screening analysis. After amplification, the 258-bp long PCR fragments were digested for 2 h at 37 °C with the MspA1 I restriction enzyme (New England Biolabs, Beverly, MA, USA), and the digestion products were separated by gel electrophoresis. The wild-type allele contains a recognition site for the restriction endonuclease so that digestion leads to two fragments of 103 and 155 bp. In the mutant allele, the recognition site is disrupted and therefore the amplified fragment is not digested.

Plasmids and construction of *StAR* promoter mutants

The wild-type pGL2-h*StAR* promoter construct was kindly provided by D Stocco (Texas Tech University, Lubbock, TX, USA). The mutated pGL2-h*StAR* bearing the C/T substitution in the position –33 bp from the transcription start site was generated by using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek TX, USA) according to the instructions of the manufacturer. Mutagenic primers (*StAR*-mutprom-fwd 5'-GATGCACAGCCTTCAGTGGGGACATTTAAGAC-3' and *StAR*-mutprom-rev 5'-GTCTTAAATGTCCCC-CACTGAAGGCTGTGCATC-3') were designed according to the GenBank sequence U29098. The mutated nucleotide is underlined. Four separate clones were obtained and tested, which gave comparable results in the reporter gene assays.

Cell culture and transfections

The human adrenocortical carcinoma NCI H295R cell line was kindly provided by Dr W E Rainey (University of

Texas Southwestern Medical Center, Dallas, TX, USA) and maintained in a 1:1 mixture of Dulbecco's modified eagle medium (DMEM) and Ham's F12 medium containing pyridoxine, L-glutamine, and 15 mM HEPES (Life Technologies). The culture medium was supplemented with 1% insulin, transferin, selenium (ITS+, Becton Dickinson and Co. Labware, Bedford, MA, USA), and 2% Ultrosor (Ciphergen, Biosepra, France) as well as antibiotics (125 µg/ml streptomycin and 125 IU/ml penicillin). Transfections were performed using the Nucleofector Transfection system (Amaxa, Cologne, Germany) according to the instructions of the manufacturer. Briefly, 5×10^6 cells were transfected with 3 µg of either the wild type or the mutated pGL2-hStAR promoter construct. Cells were then plated on 12-well culture plates (3×10^5 cells/well). Following 48 h of post-transfection, the cells were stimulated with various agents for the indicated periods of time in serum-free medium. Cells were then harvested and luciferase assay performed using the Dual-Luciferase Reporter Assay System (Promega). The pRLSV40 vector was used as an internal control to normalise transfection efficiency.

The mouse adrenocortical cancer cell line Y-1 was obtained from Cell Line Service (Heidelberg, Germany). The adherent cells were maintained in DMEM supplemented with 10% fetal calf serum and 200 nM L-glutamine as well as with antibiotics as described elsewhere. The cells were plated on 12-well culture plates (1.5×10^5 cells/well) and transfections were performed using FuGENE 6 transfection reagent (Roche) according to the instructions of the manufacturer. Briefly, the cells were transfected with 1 µg/well of either the wild type or the mutated pGL2-hStAR promoter construct. The pRLSV40 vector was used as an internal control to normalise transfection efficiency. Forty-eight hours after transfection, cells were stimulated with various stimuli for the indicated periods of time in serum-free media. Cells were then harvested and luciferase assay was performed as stated above. Both cell lines were grown at 37 °C under an atmosphere of 5% CO₂–95% air.

Electrophoretic mobility shift assay (EMSA)

Double-stranded DNA corresponding to nucleotides –42 to –24 of the human StAR promoter according to the sequence published by Sugawara *et al.* (1997), was [α -³²P]dATP-labelled by Klenow fill-in (Promega). Wild-type sense 5'-gatcAGCCTTCAGCGGGGACAT-3' annealed with wild-type antisense 5'-gatcATGTCCCCGCTGAAGGCT-3' and mutated sense 5'-gatcAGCCTTCAGTGGGGGACAT-3' annealed with mutated antisense 5'-gatcATGTCCCCACTGAAGGCT-3'. Nuclear protein extracts (7 µg) were incubated for 20 min at room temperature with 30 000 c.p.m. of labelled probe and 2 µg of poly[d(IC)] in EMSA-binding buffer. The reaction

mixture was loaded onto a non-denaturing 5% polyacrylamide gel for 1 h 30 min at 150 V in a cold room. Gels were dried and exposed to autoradiography films (Kodak and Sigma) for 72 h. In supershift experiments, 1 µg of purified rabbit polyclonal anti-chicken ovalbumin upstream promoter-transcription factor-I (COUP-TFI) (kindly provided by Dr M.L. Dufau, National Institutes of Health, NICHD, Bethesda, MD, USA), anti-SF-1 (kindly provided by Prof. K Morohashi, Okasaki, Japan) or anti-DAX-1 (kindly provided by Dr E. Lalli, Strasbourg, France) antibody was added to the mixture 30 min prior to the labelled probe. In chase experiments, 100-fold excess of cold Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or SF-1 (5'-CAGCCTT-3') consensus sequence oligonucleotides were added to the mixture 30 min prior to the labelled probe.

Analysis of data

Results are expressed as means $\pm$ S.E.M. The mean values were compared by ANOVA using Fisher's test. A value of $P < 0.05$ was considered as statistically significant.

Results

Identification of an SNP in the proximal StAR promoter

The initial screening of the 40 hypertensive and 20 control subjects identified a potentially functional C/T polymorphism located 33 bp upstream from the transcription start site of the human StAR gene promoter, 3 bp downstream of the SF-1 site and 9 bp upstream of the TATA box (Fig. 1). To date, this novel mutation is not described in public SNP databases (NCBI dbSNP or www.ensembl.org). The mutant allele was present in nine (2.2%) of the 406 subjects investigated. The prevalence of this novel mutation was 1.3% (4/308) in the non-diabetic population. When this polymorphism was investigated in the 98 type 2 diabetics a prevalence of 5.1% was found ($\chi^2 = 6.27$, $P < 0.01$ for diabetics versus non-diabetics). Four (11.1%) of the 36 hypertensive patients with raised ARR were carriers of the mutant T allele of the StAR gene promoter.

Reduced response of the mutated StAR promoter to cAMP messenger pathway activation in Y-1 cells

In order to determine whether the C/T substitution affects StAR promoter activity, we transiently transfected murine adrenocortical Y-1 cells with either the wild type or the mutated StAR promoter cloned into the pGL2 vector containing firefly luciferase as reporter gene (Fig. 2). Basal promoter activity did not differ between the wild type and mutant promoter. As expected,

Figure 2 Effect of the mutation on StAR promoter activity in Y-1 mouse adrenocortical cells. Transcriptional activity of the StAR promoter was determined with a luciferase reporter system as described under 'Materials and methods'. Y-1 cells were transiently transfected with either the wild-type StAR promoter or the mutated StAR promoter cloned into the pGL2 vector containing firefly luciferase as a reporter gene. Renilla luciferase under control of the SV40 promoter was used as an internal control. Forty-eight hours after transfection, the cells were treated for 6 h with or without 25 μ M forskolin (Fk) and luciferase activity was determined. Results are expressed as percentage of control. Values are means $\pm$ S.E.M. ($n=3$ separate experiments for each condition). * $P<0.05$ vs control.

As expected from previous studies (Sugawara *et al.* 1997, Zazopoulos *et al.* 1997, Sandhoff *et al.* 1998), SF-1 and DAX-1, an activator and a repressor of StAR expression, respectively, specifically bound the StAR promoter probe, as demonstrated by supershifts induced with specific antibodies (Fig. 4A, lanes 2–3 and 6–7, and Fig. 4B, lanes 2–3 and 4–5). Interestingly, the band that was supershifted with the SF-1 antibody was also supershifted with a purified antibody directed against COUP-TF (Fig. 4A, lanes 4–5 and 4'–5') suggesting an interaction of COUP-TF with the StAR promoter and/or SF-1. These results were similar, whether we used the wild type or the mutated 23-bp probe. In contrast, the mutated probe induced an additional DAX-1 shift as demonstrated in supershift experiments using the DAX-1 antibody (Fig. 4B, lanes 2'–3' and 4'–5').

Sp1 and SF-1 strongly bind to the mutated StAR promoter

In order to further identify the additional DNA–protein interactions that occur on the mutated StAR promoter, we incubated nuclear extracts (NEs) incubated with

Figure 3 Effect of the mutation on StAR promoter activity in H295R human adrenocortical carcinoma cells. Transcriptional activity of the StAR promoter was determined with a luciferase reporter system as described under 'Materials and methods'. H295R cells were transiently transfected with either the wild-type StAR promoter or the mutated StAR promoter cloned into the pGL2 vector containing firefly luciferase as reporter gene. Renilla luciferase under control of the SV40 promoter was used as an internal control. Forty-eight hours after transfection, cells were treated for 6 h with 10 nM AngII and luciferase activity was determined. Results are expressed as percentage of control. Values are means $\pm$ S.E.M. ($n=3$ separate experiments for each condition). ** $P<0.01$ vs control.

excess cold probe of the consensus response element for either Sp1 or SF-1, two transcription factors known to positively regulate the *StAR* gene (Sugawara *et al.* 2000) (Fig. 5).

As shown in Fig. 5A (left), the major shift observed when an NE of H295R cells was incubated in the presence of the labelled wild-type StAR promoter probe was abolished by an excess cold Sp1 oligonucleotide (lanes 4 and 5). In contrast, three of the four shifts observed with the mutated StAR promoter disappeared when a chase was performed with the Sp1 cold probe, suggesting that the mutation promotes the formation of an additional Sp1–DNA complex (Fig. 5A, lanes 4' and 5'). The remaining protein–DNA complex observed with the mutant probe disappeared when competition was performed with a 100-fold excess of an SF-1 cold probe, indicating that the mutation promotes the reassembling of various crucial transcription factors over the proximal StAR promoter (Fig. 5B, lane 4). Also, the abundance of a slower migrating species was increased in the presence of excess unlabelled SF-1 oligonucleotide (Fig. 5B, lane 4). Although we have presently no explanation for this observation, it is

Figure 4 The 5'-flanking region of the mutated *StAR* promoter forms an additional DAX-1–DNA complex. (A) An oligonucleotide corresponding to region –42 to –24 of either the wild type or the mutated human *StAR* promoter and nuclear extract (NE) from H295R cells were used in EMSA experiments as described in 'Materials and methods'. Supershift assays were performed using specific antibodies (AB) against SF-1 and COUP-TF. (B) An EMSA with a specific DAX-1 antibody for supershift was performed with either the wild type or the mutated probe. Arrows indicate the bands corresponding to SF-1, to COUP-TF, to DAX-1 and to free probe. The figure shows a representative example from three separate experiments. C, control; AngII, angiotensin II.

Figure 5 Binding of additional factor(s) to the SF-1 response element of the mutated StAR promoter. (A) Mobility shift assay was performed using a probe corresponding to region -42 to -24 of either the wild type or the mutated human StAR promoter incubated with NEs from H295R cells. Competition was performed with a 100-fold excess of cold Sp1 response element (GGCGGG). (B) The same gel shift experiment was performed using a 100-fold excess of cold SF-1 response element. C, control; AngII, angiotensin II.

possible that, due to the availability of extra probe displaced by excess SF-1 oligonucleotide, there is increased binding of the probe to a non-specific complex.

Discussion

The search for functional polymorphisms in the promoter of the *StAR* gene was undertaken to investigate potential mechanisms underlying primary aldosteronism in hypertensive patients with increased ARR not explained by the homozygosity of –344T allele at the SF-1-binding site of CYP11B2 (Lim *et al.* 2002, Nicod *et al.* 2003). Homozygosity for the –344T allele of the CYP11B2 gene has been found to be associated with hypertension and increased aldosterone synthase activity as estimated by the ARR (Brand *et al.* 1998, Lim *et al.* 2002, Nicod *et al.* 2003). However, this genotype does not account for all subjects with raised ARR. Thus, it is possible that other proteins or enzymes involved in the cascade of aldosterone synthesis may be genetically altered to account for inadequately increased plasma aldosterone concentration for the prevailing circulating renin levels, in a subset of patients with primary aldosteronism. In the present study, 36 of 212 hypertensive patients had an ARR above the diagnostic cut-off level for primary aldosteronism (Ferrari *et al.* 2004). Genotyping of the *StAR* promoter showed that this novel *StAR* promoter mutation had a prevalence of 11.1% in these patients against the 1.7% prevalence in subjects with a normal ARR. On the other hand, 57.1% of subjects with the mutation had an increased ARR.

Herein, we report a newly identified C/T mutation located at position –33 from the transcription start site of the human *StAR* gene promoter, 3 bp downstream of the SF-1-binding site and 9 bp upstream of the TATA box. To our knowledge, this is the first description of a mutation within the *StAR* promoter. This polymorphism lies in a crucial region for adequate *StAR* gene activity that bears a major response element for SF-1 (Sugawara *et al.* 2000). The identified mutation in the human *StAR* promoter is associated with a reduced promoter activity. As the region of the mutation is critically involved in the regulation of *StAR* gene expression, this finding may be relevant for adrenal steroid response to physiological stimulators.

Since the *StAR* protein mediates the crucial rate-limiting step of steroidogenesis, i.e. the transfer of cholesterol from the outer to the inner mitochondrial membrane, its expression must be finely regulated. The polymorphism we observed in the present study is located in a region of the *StAR* gene known to bear important response elements for the activation of steroidogenesis (Caron *et al.* 1997, Manna *et al.* 2003). This polymorphism is associated with the marked

changes in promoter activity of the 1.3 kb 5'-flanking region of the *StAR* gene, as shown in two different adrenocortical cell lines, murine adrenocortical Y-1 cells and human H295R cells, with two different agonists.

Various mechanisms can concur to the observed reduction in *StAR* promoter activity. First, the mutation is located in close proximity to the most proximal response element for SF-1, an important—although not the sole—factor responsible for *StAR* gene expression (Manna *et al.* 2003). Secondly in the human promoter, the mutation is located within a nucleotide sequence which matches almost perfectly a GC box or consensus Sp1 box (GGCGGG, see Fig. 1) and Sp1 is a transcription factor known to activate the *StAR* gene by interacting with SF-1 (Sugawara *et al.* 2000). The presence of an Sp1-binding site in this proximal region of the *StAR* promoter was confirmed by the fact that a consensus GC box was able to compete with the labelled oligonucleotide probe and chase it in EMSA assays (see Fig. 5A). It is, therefore, conceivable that the binding of Sp1 to its response element might be hampered in the mutated promoter and, as a consequence, the binding of SF-1 to its neighbouring response element. Thirdly the orphan nuclear receptor DAX-1, a repressor of *StAR* gene expression (Zazopoulos *et al.* 1997, Lalli *et al.* 1998, Osman *et al.* 2002), binds to a hairpin structure located at position –61 to –27 of the *StAR* promoter, a sequence which includes the present mutation. Zazopoulos and co-workers have shown that the binding of DAX-1 is stronger when the hairpin loop is rich in thymines (Zazopoulos *et al.* 1997). Interestingly, in the C/T polymorphism we describe here, the hairpin loop is richer in thymines, a condition that should favour DAX-1 binding to the promoter and repression of the *StAR* gene. Indeed, when DNA–protein interactions were analysed, gel shift assays revealed striking differences in the binding pattern of the probe (Fig. 4). As expected, SF-1 and DAX-1 specifically bound the *StAR* promoter probe. Both factors were shown to be involved in adequate *StAR* promoter activity (Caron *et al.* 1997, Sugawara *et al.* 1997, Osman *et al.* 2002). However, when NEs were incubated with the mutated probe, an additional DAX-1–DNA complex was formed. This result strongly supports the hypothesis suggesting increased binding of DAX-1 to a thymine-rich hairpin structure and thereby providing an explanation for the decrease in *StAR* promoter activity observed in the luciferase assays with the mutated construct.

In addition, this same additional complex also disappeared when competition was performed in the presence of a cold Sp1-response element. Since the sequence of the hairpin structure also includes the mutated Sp1 response element or GC box, these data suggest that the mutation may lead to decreased binding of Sp1, in addition to the increase in binding affinity of DNA for DAX-1.

In contrast, basal activity was not reduced in the mutated promoter. This may be due to the fact that repression of the StAR promoter is already maximal in the resting state. Indeed, we have recently shown that both DAX-1 (Osman *et al.* 2002) and COUP-TF (Buholzer *et al.* 2005), two repressors of the StAR promoter, are robustly expressed in adrenal glomerulosa cells and that overexpression of either factor does not affect basal promoter activity.

The second additional protein–DNA complex formed with the mutated probe disappeared when the NE was incubated in the presence of an excess of cold SF-1 consensus sequence and of the labelled mutated probe (Fig. 5B). However, a similar supershift could not be obtained with a specific antibody directed against SF-1 (Fig. 4A), suggesting that the mutation induces the binding of an yet unidentified factor to the SF-1 response element.

In addition to binding specific DNA sequences, Sp1, SF-1 and DAX-1 interact with each other and promote or counteract each other's binding to the promoter, bringing an additional degree of complexity to the regulation of the StAR proximal promoter (Ito *et al.* 1997, Sugawara *et al.* 2000, Babu *et al.* 2002, Suzuki *et al.* 2003). It is, therefore, likely that the change in binding affinity of one factor for the StAR promoter resulting from the mutation may affect the binding of its partner to this same promoter. Moreover, recent studies have demonstrated that the regulatory actions of SF-1 and Sp1 may be mediated by components of the chromatin environment such as the ubiquitous co-activator CBP/300 or histone deacetylases (Monte *et al.* 1998, Zhang & Dufau 2003, Hiroi *et al.* 2004). Thus, *in vivo*, the genomic environment may play an additional role in modulating the activity of the mutated StAR promoter.

Interestingly, a supershift was also observed with the antibody to COUP-TF for the same band that was also supershifted with the SF-1 antibody. This suggests an interaction with the StAR promoter and/or SF-1. COUP-TF is known to play a role as a repressor of steroidogenesis and, in particular, of *StAR* gene expression (Shibata *et al.* 2001, 2003, Buholzer *et al.* 2005). In addition, the competition between SF-1 and COUP-TF for a common binding site has been reported in the transcriptional regulation of the bovine *CYP17* gene (Bakke & Lund 1995) and modulation of the activity of the murine DAX-1 and aromatase P450 promoters (Zeitoun *et al.* 1999, Cooney *et al.* 2001). Our supershift assay results, however, do not suggest an involvement of COUP-TF in the effects induced by the mutation. Thus, we can conclude that a point mutation on the locus for a key regulator may induce a complete remodelling of the various transcription factors involved in the regulation of the *StAR* gene, and therefore, its activity.

We focused on the gene for the StAR protein because of its rate-limiting role in steroid biosynthesis and because an increased supply of steroid precursors could result in increased aldosterone production. Clearly, based on the *in vitro* expression studies, the functional effect of this novel mutation in the StAR promoter argues against the hypothesised mechanism. We would like to propose an alternative explanation for the present findings. The described StAR promoter mutation, the first of its kind, leads to reduced StAR promoter activity in response to adrenocorticotrophin (ACTH) and AngII. The main effect is likely to be a slight reduction in cortisol levels in response to ACTH. In turn, normal feedback regulation should result in a resetting of the hypothalamic–pituitary–adrenal axis such that cortisol levels are maintained. Consequently, there will be a subtle increase in the ACTH drive to the adrenal cortex. In the long term this is likely to cause hyperplasia of both zona fasciculata and zona glomerulosa of the adrenal cortex, resulting in increased synthetic capacity for both cortisol and aldosterone. In turn, this might lead to hypertension associated with altered regulation of aldosterone and possibly cause insulin resistance and increased predisposition to diabetes associated with altered cortisol secretion.

In view of the pleiotropic action of the StAR protein and because of the complex interactions between the various factors induced by this mutation, further experiments are needed to elucidate the exact role this polymorphism may play in states of altered function of the mineralocorticoid, glucocorticoid and sex hormone axes.

Acknowledgements

The authors are grateful to Manuella Rey for her excellent technical assistance. This work was supported by Swiss National Science Foundation grant no. 310A0-100797/1 (to AC) and the Ruth Mortimer Bequest (to PF). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Babu PS, Bayers DL, Beuschlein F, Shah S, Jeffs B, Jameson JL & Hammer GD 2002 Interaction between Dax-1 and steroidogenic factor-1 *in vivo*: increased adrenal responsiveness to ACTH in the absence of Dax-1. *Endocrinology* **143** 665–673.
- Bakke M & Lund J 1995 Transcriptional regulation of the bovine *CYP17* gene: two nuclear orphan receptors determine activity of cAMP-responsive sequence 2. *Endocrine Research* **21** 509–516.
- Brand E, Chatelain N, Mulatero P, Fery I, Curnow K, Jeunemaitre X, Corvol P, Pascoe L & Soubrier F 1998 Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* **32** 198–204.

- Buholzer CF, Arrighi JF, Abraham S, Piguat V, Capponi AM & Casal AJ 2005 Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) is a negative regulator of steroidogenesis in bovine adrenal glomerulosa cells. *Molecular Endocrinology* **19** 65–75.
- Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL & Clark BJ 1997 Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Molecular Endocrinology* **11** 138–147.
- Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.
- Cooney AJ, Lee CT, Lin SC, Tsai SY & Tsai MJ 2001 Physiological function of the orphans GCNF and COUP-TF. *Trends in Endocrinology and Metabolism* **12** 247–251.
- Crivello JF & Jefcoate CR 1980 Intracellular movement of cholesterol in rat adrenal cells. Kinetics and effects of inhibitors. *Journal of Biological Chemistry* **255** 8144–8151.
- Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C, Anderson NH, Fraser R & Connell JM 1999 Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene *CYP11B2*. *Hypertension* **33** 703–707.
- Ferrari P, Shaw SG, Nicod J, Saner E & Nussberger J 2004 Active renin versus plasma renin activity to define aldosterone-to-renin ratio for primary aldosteronism. *Journal of Hypertension* **22** 377–381.
- Garbers DL & Dubois SK 1999 The molecular basis of hypertension. *Annual Review of Biochemistry* **68** 127–155.
- Hiroi H, Christenson LK, Chang L, Sammel MD, Berger SL & Strauss JF 3rd 2004 Temporal and spatial changes in transcription factor binding and histone modifications at the steroidogenic acute regulatory protein (StAR) locus associated with StAR transcription. *Molecular Endocrinology* **18** 791–806.
- Ito M, Yu R & Jameson JL 1997 DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. *Molecular and Cellular Biology* **17** 1476–1483.
- Lalli E, Melner MH, Stocco DM & Sassone-Corsi P 1998 DAX-1 blocks steroid production at multiple levels. *Endocrinology* **139** 4237–4243.
- Lim PO, Macdonald TM, Holloway C, Friel E, Anderson NH, Dow E, Jung RT, Davies E, Fraser R & Connell JM 2002 Variation at the aldosterone synthase (*CYP11B2*) locus contributes to hypertension in subjects with a raised aldosterone-to-renin ratio. *Journal of Clinical Endocrinology and Metabolism* **87** 4398–4402.
- Lin D, Sugawara T, Strauss JF 3rd, Clark BJ, Stocco DM, Saenger P, Rogol A & Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828–1831.
- Lovati E, Richard A, Frey BM, Frey FJ & Ferrari P 2001 Genetic polymorphisms of the renin–angiotensin–aldosterone system in end-stage renal disease. *Kidney International* **60** 46–54.
- Manna PR, Wang XJ & Stocco DM 2003 Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids* **68** 1125–1134.
- Miller WL 1997 Congenital lipid adrenal hyperplasia: the human gene knockout for the steroidogenic acute regulatory protein. *Journal of Molecular Endocrinology* **19** 227–240.
- Monte D, DeWitte F & Hum DW 1998 Regulation of the human P450_{scc} gene by steroidogenic factor 1 is mediated by CBP/p300. *Journal of Biological Chemistry* **273** 4585–4591.
- Nicod J, Bruhin D, Auer L, Vogt B, Frey FJ & Ferrari P 2003 A biallelic gene polymorphism of *CYP11B2* predicts increased aldosterone to renin ratio in selected hypertensive patients. *Journal of Clinical Endocrinology and Metabolism* **88** 2495–2500.
- Osman H, Murigande C, Nadakal A & Capponi AM 2002 Repression of DAX-1 and induction of SF-1 expression. Two mechanisms contributing to the activation of aldosterone biosynthesis in adrenal glomerulosa cells. *Journal of Biological Chemistry* **277** 41259–41267.
- Paillard F, Chansel D, Brand E, Benetos A, Thomas F, Czekalski S, Ardaillou R & Soubrier F 1999 Genotype–phenotype relationships for the renin–angiotensin–aldosterone system in a normal population. *Hypertension* **34** 423–429.
- Privalle CT, Crivello JF & Jefcoate CR 1983 Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *PNAS* **80** 702–706.
- Reinhart AJ, Williams SC, Clark BJ & Stocco DM 1999 SF-1 (steroidogenic factor-1) and C/EBP beta (CCAAT/enhancer binding protein-beta) cooperate to regulate the murine StAR (steroidogenic acute regulatory) promoter. *Molecular Endocrinology* **13** 729–741.
- Sandhoff TW, Hales DB, Hales KH & McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139** 4820–4831.
- Shibata H, Ikeda Y, Mukai T, Morohashi K, Kurihara I, Ando T, Suzuki T, Kobayashi S, Murai M & Saito I 2001 Expression profiles of COUP-TF, DAX-1, and SF-1 in the human adrenal gland and adrenocortical tumors: possible implications in steroidogenesis. *Molecular Genetics and Metabolism* **74** 206–216.
- Shibata H, Kurihara I, Kobayashi S, Yokota K, Suda N, Saito I & Saruta T 2003 Regulation of differential COUP-TF–coregulator interactions in adrenal cortical steroidogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **85** 449–456.
- Stocco DM 2001 Tracking the role of a star in the sky of the new millennium. *Molecular Endocrinology* **15** 1245–1254.
- Sugawara T, Kiriakidou M, McAllister JM, Kallen CB & Strauss JF 3rd 1997 Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5′-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36** 7249–7255.
- Sugawara T, Saito M & Fujimoto S 2000 Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression. *Endocrinology* **141** 2895–2903.
- Suzuki T, Kasahara M, Yoshioka H, Morohashi K & Umesono K 2003 LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1. *Molecular and Cellular Biology* **23** 238–249.
- Takeda Y, Furukawa K, Inaba S, Miyamori I & Mabuchi H 1999 Genetic analysis of aldosterone synthase in patients with idiopathic hyperaldosteronism. *Journal of Clinical Endocrinology and Metabolism* **84** 1633–1637.
- White PC & Slutsker L 1995 Haplotype analysis of *CYP11B2*. *Endocrine Research* **21** 437–442.
- Zazopoulos E, Lalli E, Stocco DM & Sassone-Corsi P 1997 DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis. *Nature* **390** 311–315.
- Zeitoun K, Takayama K, Michael MD & Bulun SE 1999 Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same *cis*-acting element. *Molecular Endocrinology* **13** 239–253.
- Zhang Y & Dufau ML 2003 Dual mechanisms of regulation of transcription of luteinizing hormone receptor gene by nuclear orphan receptors and histone deacetylase complexes. *Journal of Steroid Biochemistry and Molecular Biology* **85** 401–414.

Received in final form 20 April 2006

Accepted 21 April 2006

Made available online as an Accepted Preprint 4 May 2006