Functional Effects of Polymorphisms in the Human Gene Encoding 11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1): A Sequence Variant at the Translation Start of 11β-HSD1 Alters Enzyme Levels

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Regeneration of active glucocorticoids within liver and adipose tissue by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) may be of pathophysiological importance in obesity and metabolic syndrome and is a therapeutic target in type 2 diabetes. Polymorphisms in *HSD11B1*, the gene encoding 11β-HSD1, have been associated with metabolic phenotype in humans, including type 2 diabetes and hypertension. Here, we have tested the functional consequences of two single nucleotide polymorphisms located in contexts that potentially affect tissue levels of 11β-HSD1. We report no effect of allelic variation at rs846910, a polymorphism within the 5′-flanking region of the gene on *HSD11B1* promoter activity *in vitro*. However, compared with the common G allele, the A allele of rs13306421, a polymorphism located two nucleotides 5′ to the translation initiation site, gave higher 11β-HSD1 expression and activity *in vitro* and was translated at higher levels in *in vitro* translation reactions, possibly associated with a lower frequency of "leaky scanning." These data suggest that this polymorphism may have direct functional consequences on levels of 11β-HSD1 enzyme activity *in vivo*. However, the rs13306421 A sequence variant originally reported in other ethnic groups may be of low prevalence because it was not detected in a population of 600 European Caucasian women. **(*Endocrinology* 151: 195–202, 2010)**

The microsomal enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) generates active glucocorticoids (cortisol, corticosterone) from intrinsically inert 11-keto substrates (cortisone, 11-dehydrocorticosterone), thus amplifying glucocorticoid action in cells and tissues in which it is expressed (1, 2). Recent evidence has indicated a pathogenic role for 11β-HSD1 in metabolic disease. A number of studies have demonstrated a strong association in humans between the level of 11β-HSD1 expression in adipose tissue and body mass index (reviewed in Refs. 2 and 3). Moreover, 11β-HSD1 expression in omental adipose tissue correlates with fat cell size independently of obesity (4). A causative role is suggested by the phenotype of transgenic mice which overexpress 11β-HSD1 in adipose tissue (5). These mice develop all the major features of metabolic syndrome, including central obesity, insulin resistance, dyslipidaemia, and hypertension (5, 6). Mice overexpressing 11β-HSD1 in liver also show insulin resistance and hypertension but remain lean (7). Conversely, 11β-HSD1 inhibition increases hepatic insulin sensitivity in humans (8), and its deficiency or inhibition ameliorates the metabolic consequences of obesity, increasing insulin sensitivity and reducing blood glucose levels in obese or diabetic mice (9–13).

Sequence variation in *HSD11B1*, the human gene encoding 11β-HSD1, has been linked with cardiovascular risk fac-

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Abbreviations: C/EBPα, CCAAT/enhancer-binding proteinα; CHO, Chinese hamster ovary; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; PCOS, polycystic ovary syndrome; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism.

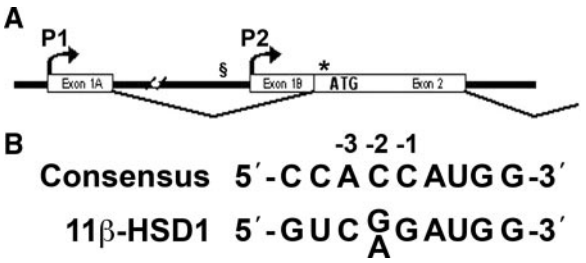

FIG. 1. Locations of relevant SNPs: rs846910 at −2937 with respect to the P2 promoter and rs13306421, close to the translation start of the human *HSD11B1* gene. A, Schematic representation of the 5′ end of the *HSD11B1* gene. Exons 1A and 1B (and the associated P1 and P2 promoters, respectively) (15) are indicated, as is exon 2, containing the translation start of 11β-HSD1. The position of SNP rs846910 is indicated by a *section sign*, and SNP rs13306421 is indicated by an *asterisk*. B, SNP rs13306421 is located at −2 (numbering with respect to the AUG translation start codon at +1), within the ribosome binding site of 11β-HSD1 mRNA. The ribosome binding site of *HSD11B1* is a poor match to the consensus sequence (21). When a pyrimidine replaces the preferred purine at position −3, translation becomes more sensitive to changes at other positions, including −2 (21).

tors associated with obesity in adults, although not with obesity *per se* (14). *HSD11B1* is transcribed from two promoters (Fig. 1), with the P2 promoter predominating in metabolically active tissues, where it is potently regulated by the transcription factor CCAAT/enhancer-binding proteinα (C/EBPα) (15). Polymorphisms in the P2 promoter region (rs846910) and an intronic enhancer (rs12086634) are associated with type 2 diabetes and/or hypertension in three different populations (16–18), and the G allele of rs12086634, associated with lower 11β-HSD1 transcriptional activity *in vitro* (19), may be protective against obesity among patients with polycystic ovary syndrome (PCOS) (20). Moreover, the combination of less common allelic variants at rs846910 and rs12086634 is associated with higher levels of 11β-HSD1 mRNA and activity in adipose tissue in Southern European Caucasian women with and without PCOS (Gambineri, A., F. Tomassoni, A. Munarini, R. H. Stimson, U. Pagotto, R. Mioni, K. E. Chapman, R. Andrew, R. Pasquali, and B. R. Walker, submitted for publication). However, with the exception of rs12086634, the functional relevance of these and other nonexonic polymorphisms has not been reported.

Here, we have investigated the effect of two polymorphisms upon 11β-HSD1 transcription and translation: rs846910 located 2937 nucleotides 5′ to the transcription start of the *HSD11B1* P2 promoter, and rs13306421, a polymorphism situated at −2 with respect to the translation start site (Fig. 1). The translation start of *HSD11B1* lies in a suboptimal context, with deviation from the consensus ribosome binding site (21) (Fig. 1B) and with additional AUG codons located close downstream. When a pyrimidine occupies position −3 (as it does in *HSD11B1*), translation becomes sensitive to changes at other positions, including −2 (21). Moreover, even small departures from the consensus ribosome binding site allow nearby AUG codons to be reached by leaky scanning (22). Accordingly, we have tested the effect of the rs13306421 polymorphism on activity of 11β-HSD1 and its translation *in vitro*.

Materials and Methods

Plasmids

To test the effect of rs846910, the 5′-flanking region (−4643 to +88) of *HSD11B1* was amplified from BAC DNA (RP1-28O10, encompassing the *HSD11B1* gene, obtained from the Sanger Institute, Hinxton, UK) using primers 5′-TCCCTAGCAGAGGTTCTCCATGAGG-3′ and 5′-AGCTGGCCTGAAGACTCCTGTAGG-3′ and Accuprime Pfx (Invitrogen, Paisley, UK), a high-fidelity thermostable polymerase. Sequencing confirmed the presence of the more common G allele in the cloned product. The PCR product was subcloned into pSV0L (23), and the A allele of rs846910 introduced by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, creating plasmids pHSD11B1(−2937A)-Luc and pHSD11B1(−2937G)-Luc, differing only at the single nucleotide polymorphism (SNP) at −2397. Plasmids to test the effect of rs13306421, the translation start site SNP were constructed from pBS-SK$^+$-h11β-HSD1, encoding human 11β-HSD1 (24), a gift from Perrin White. pBS-SK$^+$-h11β-HSD1 was first digested with *Eco*0109I to remove a fragment of *lac* DNA originating from the λ vector used to clone the cDNA. Sequencing confirmed the presence of the G allele at −2 with respect to the translation start site; this plasmid was named pBS-h11β-HSD1(G). Site-directed mutagenesis to change the G allele to A was carried out (as above) to create pBS-h11β-HSD1(A). The two alleles of 11β-HSD1 were subcloned into pcDNA3.1(−) (Invitrogen) using standard techniques to generate pCMV-h11β-HSD1(G) and pCMV-h11β-HSD1(A). To test whether the translation start polymorphism could affect translation of a heterologous protein, we replaced the coding sequence of 11β-HSD1 with luciferase, creating pCMV-(G)Luc and pCMV-(A)Luc, as follows: The 11β-HSD1 leader sequence with either the G or the A allele of SNP rs13306421 was amplified by PCR from pCMV-h11β-HSD1(G) and pCMV-h11β-HSD1(A) using a T7 (forward) primer and a reverse primer; either 5′-ATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATCCGACAGGGAG-3′ (G allele; the translation start and −2 nucleotide are *underlined*) or 5′-ATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATCTGACAGGGAG-3′ (A allele). The product included an *Nhe*I restriction enzyme site from the polylinker in pCMV-h11β-HSD1(G)/(A) and a *Kas*I site in the reverse primer. pCMV-(G)Luc and pCMV-(A)Luc were then assembled in pcDNA3.1(−) from an *Nhe*I-*Kas*I fragment from the PCR product and a *Kas*I-*Spe*I fragment encoding luciferase from pGL3-basic (Promega, Southampton, UK). To create the N162Q glycosylation mutant, site-directed mutagenesis was used to change asparagine 162 of 11β-HSD1 to glutamine, as described above. All constructs were verified by sequencing.

Cell culture and transfection

The effect of rs846910 on *HSD11B1* promoter activity was tested in A549 cells, which express the endogenous *HSD11B1* gene (25). A549 cells were maintained and transfected as de-

scribed (25). Briefly, 1.5×10^5 cells per well of a six-well plate were transfected using lipofectamine 2000 (Invitrogen) with 250 ng of pHSD11B1(−2937A)-Luc or pHSD11B1(−2937G)-Luc and 250ng pRSV-*LacZ* (encoding β-galactosidase, as internal control). Where appropriate, 50 ng of pMSV-C/EBPα (gift from S. McKnight; University of Texas Southwestern Medical Center, Dallas, TX) was added. Forty-eight hours after transfection, cells were lysed and luciferase and β-galactosidase activity measured as previously described (15). Transfections were carried out in triplicate with at least two independent preparations of each plasmid and the experiment was repeated seven times.

The effect of rs13306421 on enzyme activity of 11β-HSD1 in mammalian cells was tested in Chinese hamster ovary (CHO) cells, which do not express endogenous 11β-HSD1 activity (26, 27). CHO cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Twenty-four hours before transfection, cells were seeded at 1.5×10^5 cells/well in a six-well plate and transfected with 500 ng of DNA using lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For assay of 11β-HSD1 activity, cells were transfected with 250 ng of H6PD expression plasmid (27) and 250 ng of pCMV-h11β-HSD1(G) or pCMV-h11β-HSD1(A). Controls contained pcDNA3.1(−) ("empty vector") instead of the H6PD plasmid, the pCMV-h11β-HSD1 plasmid, or both. 11β-HSD1 reductase activity was measured by incubating intact cells with 200 nM cortisone containing 5 nM [^{3}H]-cortisone tracer for 16–24 h (preliminary experiments established that conversion was linear up to at least 24 h and the time point used for each assay gave 20–40% conversion of added substrate). After incubation, steroids were extracted with ethyl acetate, separated by thin-layer chromatography using a mobile phase of chloroform and ethanol (92:8) and quantitated using a phosphorimager (Fuji FLA-2000; Raytek Scientific Ltd., Sheffield, UK). For assay of luciferase activity, cells were transfected with 250 ng of pRSV-*LacZ* (to monitor transfection efficiency) together with 250 ng of either pCMV-(G)Luc or pCMV-(A)Luc. Forty-eight hours after transfection, cells were lysed and luciferase and β-galactosidase activity measured as above. All transfections were carried out in triplicate and for each plasmid (A or G allele), at least three independent plasmid preparations were also used. Transfections were repeated four to six times. Data were normalized between experiments by arbitrarily setting the mean value of the G allele to 100%.

Immunoblotting was carried out on CHO cells transfected as above, but with 5×10^5 cells/well seeded in a six-well plate and 1 μg of pCMV-h11β-HSD1(G) or pCMV-h11β-HSD1(A) DNA (three independent preparations of each plasmid). Cells were harvested 48 h after transfection, lysed in Laemmli buffer, and proteins (50 μg) separated by electrophoresis on a discontinuous 15% sodium dodecyl sulfate (SDS)-polyacrylamide resolving gel. Proteins were transferred to Hybond nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK) at 200 mA for 2 h. Membranes were blocked with 5% milk block (Bio-Rad, Hemel Hempstead, UK) in Tris-buffered saline with Tween 20 [0.015 M Tris, 0.15 M NaCl, 0.1% Tween 20 (pH 7.4)] for 1 h at room temperature followed by overnight incubation at 4 C with 1:1,000 dilution of sheep antihuman 11β-HSD1 antibody (The Binding Site Ltd., Birmingham, UK). Membranes were washed in Tris-buffered saline with Tween 20, then incubated with Alexa Fluor 680-labeled donkey antisheep antibody (Invitrogen) used at 1:10,000 dilution. Immunolabeling was detected and quantified using an Odyssey infrared imager and Odyssey software (LI-COR Biosciences UK, Cambridge, UK).

In vitro translation

The mRNA encoding either the A or the G allele of rs13306421 was transcribed and translated *in vitro* from pBS-h11β-HSD1(G)/(A) or pCMV-h11β-HSD1(G)/(A) using the TNT T7 Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's instructions. Three independent plasmid preps for each allele were tested. Reactions (50 μl) contained 1 μg template DNA, 20 μCi ^{35}S-methionine (37.0 TBq/mmol; PerkinElmer, Beaconsfield, UK), 1 μl T7 TNT PCR enhancer (supplied with the kit), and 40 μl TNT Quick Master Mix (supplied with the kit). Positive controls contained 1 μg of a plasmid encoding luciferase (supplied with the kit); negative controls omitted DNA template. Reactions to investigate glycosylated 11β-HSD1 isoforms were carried out using pCMV-h11β-HSD1(A) or the N162Q mutant of 11β-HSD1 as template, with or without inclusion of 4 μl canine pancreatic microsomes (Promega). Proteinase K sensitivity was tested by incubating aliquots of the *in vitro* translation reactions with 0.2 mg/ml proteinase K on ice for 30 min, in the presence or absence of 0.5% Triton X-100. To inactivate proteinase K, 1 mM phenylmethylsulfonylfluoride was added, followed by heating at 100 C, 5 min in SDS loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% Bromophenol blue, 10% glycerol]. Proteins were separated by SDS-PAGE in either 15 or 10% discontinuous polyacrylamide gels. Gels were fixed for 30 min in 50% methanol, 10% glacial acetic acid followed by 5 min in 10% glycerol, then dried and exposed to autoradiographic film or to a phosphorimager screen. Quantification was carried out using a phosphoimager (Fuji BAS FLA-2000; Raytek Scientific Ltd.) and Aida 3.44 software (Raytek Scientific Ltd.).

Genotyping of SNP rs13306421 within a population of southern European women with and without PCOS

Three hundred unmedicated Caucasian women with PCOS, aged 18–45 yr, and 300 Caucasian controls recruited from the general population in Northern Italy and comparable for age and body weight were genotyped for rs13306421. PCOS was diagnosed according to the Rotterdam consensus conference criteria (28). Controls had no signs of hyperandrogenism and regular ovulatory menstrual cycles (29).

Blood samples for DNA extraction were collected in EDTA and stored at 4 C. DNA was extracted using a QIAamp DNA Blood Kit (QIAGEN Inc., Valencia, CA). Genotyping of rs13306421 was undertaken by single nucleotide primer extension and denaturing HPLC, adapted from (30). *HSD11B1* gene fragments were amplified by PCR for 35 cycles, each consisting of 30 s at 95 C, 30 s at 65 C, and 20 s at 72 C. Primers for rs13306421 were 5′-GCTGCCTGCTTAGGAGGTTGTAG-3′ (forward) and 5′-AACACATCTTGGTCCTCAGGAACAC-3′ (reverse). Reactions (10 μl) contained 25 pmol each primer, 40 ng of genomic DNA, 200 μM dNTPs, 2 mM $MgCl_2$, and 0.08 U AmpliTaq Gold (Applied Biosystems, Warrington, UK) in the buffer provided by the manufacturer. PCR products were incubated for 60 min at 37 C with 2 μl of Exo-SAP-IT (GE Healthcare Europe GmbH, Milan, Italy) to hydrolyze unincorporated nucleotides and degrade excess primers; the reaction was terminated by incubation at 80 C for 15 min. Primer extension reac-

tions were carried out in a 25 μl reaction containing the purified PCR products, 50 μM of the appropriate ddNTPs (ddATP, ddGTP, ddCTP), 200 pmol primer (5′-TTTTTTTTTTTTTTT-TGGAGTCTTCAGGCCAGCTCCCTGTC-3′), and 1.25 U Thermo Sequenase (GE Healthcare Europe GmbH) in the buffer provided by the manufacturer. Single nucleotide primer extension reactions were performed in a thermal cycler with 35 cycles each consisting of 30 sec at 94 C, 15 sec at 55 C, and 60 sec at 60 C, followed by 2 sec at 15 C. Primer extension products (10 μl) were loaded on SaraSep DNASep column (Transgenomic, Glasgow, UK) at 80 C and separated by denaturing HPLC on a WAVE System (Transgenomic) using a linear acetonitrile gradient [over 7 min from 20–37% acetonitrile in 0.1 M triethylamine acetate buffer (pH 7)] at a constant flow rate of 0.9 ml/min. Data were acquired using a UV-detector at 260 nm.

Results

SNP rs846910, located 2937 nucleotides 5′ to the translation start of HSD11B1, does not influence HSD11B1 promoter activity

A549 (human lung epithelial) cells in which the endogenous *HSD11B1* promoter is active (15) were used to test the effect of allelic variation at −2937. Luciferase reporter plasmids encoding −4643 to +88 of *HSD11B1*, differing only by A/G at −2937, showed similar promoter activity in A549 cells (Fig. 2A). Moreover, cotransfection of C/EBPα, a transcription factor important for expression of 11β-HSD1 in some tissues (15) but absent from A549 cells (25), did not differentially regulate the A and G alleles of rs846910 (Fig. 2B), although it did markedly increase *HSD11B1* promoter activity (fold induction with C/EBPα, mean ± SEM: A allele, 8.1 ± 1.0 *vs.* G allele, 8.9 ± 1.8; $P = 0.7$, n = 7 experiments, each with more than or equal to six replicates), consistent with previous data (15).

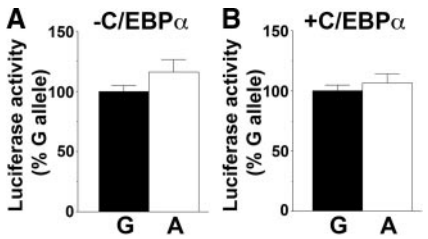

FIG. 2. Allelic variation at rs846910 does not affect *HSD11B1* P2 promoter activity. Panels A and B, Site-directed mutagenesis was used to change G at −2937 (with respect to the transcription start site of the P2 promoter, +1) to A, generating reporter plasmids pHSD11B1(−2937G)-Luc and pHSD11B1(−2937A)-Luc. After transfection into A549 cells, there was no difference in luciferase activity directed by pHSD11B1(−2937G)-Luc encoding the G allele (*black bars*) and pHSD11B1(−2937A)-Luc, encoding the A allele (*white bars*), either in the absence (Panel A) or in the presence (Panel B) of C/EBPα. Data are expressed relative to the more common G allele, arbitrarily set to 100% for each experiment and are means ± SEM from seven independent transfections, each carried out in triplicate and with more than or equal to two independent plasmid preps of either the A allele or the G allele. Significance was tested with unpaired Student's *t* test.

The A allele of SNP rs13306421 is associated with higher 11β-HSD1 activity and confers higher expression upon a heterologous protein

Expression plasmids encoding 11β-HSD1 cDNA, differing only by A/G at −2 with respect to the translation start site, were each cotransfected into CHO cells (which lack endogenous 11β-HSD1 activity) (26, 27) together with an expression plasmid encoding H6PD, required for reductase activity of 11β-HSD1 (27). Empty vector or H6PD alone gave no 11β-HSD1 reductase activity in transfected cells (data not shown). 11β-HSD1 reductase activity was higher when encoded by the A allele than the G allele (Fig. 3A), suggesting that the leader sequence of *HSD11B1*, including the Kozak sequence, determines the translation efficiency of 11β-HSD1. Consistent with this, immunoblotting of transfected cells showed more 11β-HSD1 protein expressed from the A allele compared with the G allele (Fig. 3, B and C). To test whether translation of a heterologous protein was affected by the polymorphism, the leader sequence of 11β-HSD1, including the translation start, was used to drive luciferase expression. Higher luciferase activity was produced from the A allele than the G allele (Fig. 3D).

The A allele of SNP rs13306421 directs more efficient translation of full-length 11β-HSD1 *in vitro*

To determine whether the A allele confers higher enzyme activity through increased translation, mRNA encoding either the A allele or the G allele of rs13306421 was transcribed and translated *in vitro*. Four products were obtained with both the A and the G allele, with the largest and most abundant migrating with a relative molecular size of approximately 29 kDa (Fig. 4, see also Fig. 5), smaller than the predicted Mr of 32.4 kDa, but corresponding to the size previously observed for *in vitro*-translated human 11β-HSD1 (31). This suggested that truncated 11β-HSD1 was synthesized in addition to the full length, giving rise to the smaller products. Addition of unlabeled methionine together with ^{35}S-labeled methionine had no effect on the pattern of labeled products (data not shown), indicating that they were not due to C-terminal truncation because of limiting amounts of methionine in the reactions. The N terminus of 11β-HSD1 determines the location and orientation of 11β-HSD1 in the endoplasmic reticulum (31), and N-terminally truncated proteins would not be predicted to translocate into microsomes. To test this, *in vitro* transcription-translation was carried out in the presence of microsomes, which results in glycosylation of 29-kDa human 11β-HSD1 at three sites, N123, N162, and N207 (31). Addition of microsomes resulted in the appearance of three products of approxi-

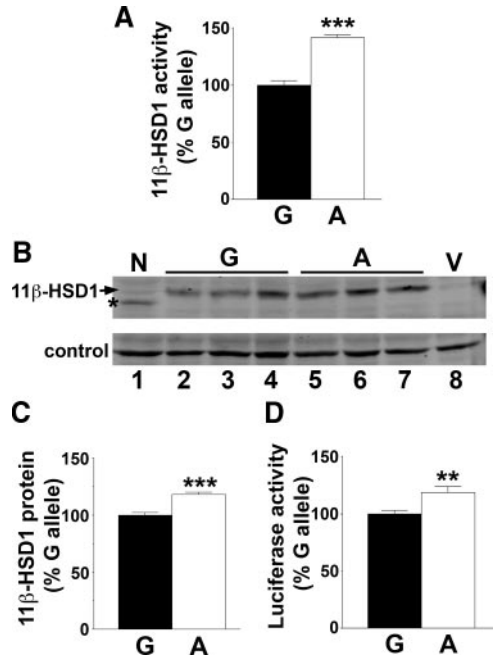

FIG. 3. The A allele of SNP rs13306421 is associated with higher enzyme activity. Panel A, Site-directed mutagenesis was used to change G at −2 of 11β-HSD1 (with respect to the ATG translation start) to A. 11β-Reductase activity was measured by conversion of 200 nM [^{3}H]-cortisone to cortisol following cotransfection of CHO cells with expression plasmids encoding H6PD and either the G (*black bar*) or A allele (*white bar*) of 11β-HSD1. 11β-HSD1 activity was normalized between experiments by arbitrarily setting the mean conversion of the G allele plasmids to 100% for each experiment. Panel B, Representative Western blot analysis showing levels of 11β-HSD1 protein encoded by three independent preparations of pCMV-h11β-HSD1(G) (lanes 2–4), pCMV-h11β-HSD1(A) (lanes 5–7), 11β-HSD1 N162Q (lane 1; a mutation at one of the glycosylation sites of human 11β-HSD1), or pcDNA3.1 vector (V, lane 8). Fifty micrograms of G protein were loaded per lane. The membrane was incubated with sheep antihuman 11β-HSD1 antibody. A cross-reacting band of approximately 58 kDa, present in untransfected cells, was used as a convenient loading control. Panel C, Quantification of immunoblot shown in panel B. Levels of 11β-HSD1 are expressed relative to the loading control, with the G allele arbitrarily set to 100%. Data are means ± SEM from three independent transfections, with three independent plasmid preps of either the A allele or the G allele. Significance was tested with unpaired Student's *t* test; ***, $P < 0.001$. Panel D, The A allele directs higher expression of a heterologous protein, luciferase. The leader sequence of luciferase (to the ATG translation start site) was replaced with the 11β-HSD1 leader sequence, either the A or G allele. Luciferase activity was measured in transfected CHO cells. To normalize between experiments, the mean luciferase activity of the G allele plasmid was arbitrarily set to 100% for each experiment. Data are means ± SEM from four to six independent transfections, each carried out in triplicate and with three independent plasmid preps of either the A allele or the G allele. Significance was tested with unpaired Student's *t* test; **, $P < 0.01$; ***, $P < 0.001$.

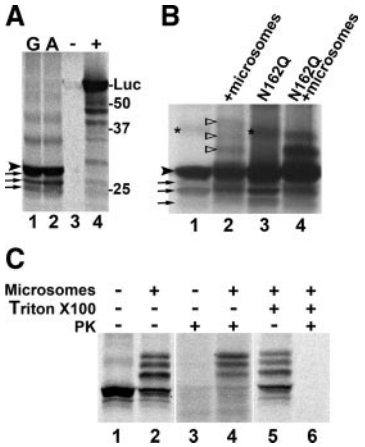

FIG. 4. The A allele of rs13306421 produces more full-length 11β-HSD1 in coupled *in vitro* transcription-translation reactions. Panel A, Representative autoradiograph showing ^{35}S-labeled products of *in vitro* transcription-translation of 11β-HSD1. Reactions contained 1 μg of plasmid encoding the G allele (lane 1) or the A allele (lane 2) of *rs13306421,* vector (negative control, indicated by a *minus sign above the lane*) or luciferase (positive control, indicated by a *plus sign above the lane*). An *arrowhead* indicates full-length 11β-HSD1 migrating at approximately 29 kDa (relative to molecular weight markers, indicated at the *right* of the gel), and *arrows* indicate the three truncated products obtained from the 11β-HSD1 plasmids. Luciferase (Mr, 61 kDa) is indicated (Luc). Panel B, Representative autoradiograph showing ^{35}S-labeled products of *in vitro* translation reactions carried out with plasmids encoding 11β-HSD1 (lanes 1 and 2; A allele of *rs13306421*) or 11β-HSD1 with a mutation in one of the glyosylation sites, N162Q (lanes 3 and 4) in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of microsomes. An *arrowhead* indicates full-length 11β-HSD1, and *arrows* indicate the three truncated products obtained from the 11β-HSD1 plasmids. *Open arrowheads* indicate the positions of glycosylated 11β-HSD1. An *asterisk* indicates a nonspecific band. Panel C, Representative autoradiograph showing ^{35}S-labeled products of *in vitro* transcription-translation of 11β-HSD1 carried out in the presence or absence of microsomes, Triton X-100 and proteinase K (PK) (indicated *above the lanes*). Unglycosylated 11β-HSD1 was susceptible to PK digestion, whereas the glycosylated products were protected (lanes 3 and 4). Addition of Triton X-100 to solubilize microsomal membranes rendered all products susceptible to PK digestion (lanes 5 and 6).

mately 31, 33, and 35 kDa (Fig. 4B, lanes 1 and 2), consistent with previous reports of glycosylated products of 31, 33, and 35 kDa (31, 32). Moreover, when a mutant 11β-HSD1 in which one of the three glycosylation sites was mutated to glutamine (N162Q) was translated *in vitro*, only two additional products were produced in the presence of microsomes (Fig. 4B), consistent with the effect of the mutation in transfected cells (Fig. 3B, lane 1). The mutation had no effect on the products in the absence of microsomes. Whereas the 31-, 33-, and 35-kDa proteins were protected from proteinase K digestion, the 29-kDa and smaller products were fully digested by proteinase K (Fig. 4C, lanes 3 and 4), supporting their exclusion from microsomes. Addition of Triton X-100 to solubilize membranes rendered all the 11β-HSD1 products susceptible to proteinase K digestion (Fig. 4C, lanes 5 and 6). Quantification of *in vitro* transcription-translation products from three independent plasmid preps of each of the G allele and A allele of rs13306421 showed a significant increase in the amount of full-length 11β-HSD1 produced from the A allele, with a concomitant decrease in the amount of the largest truncated product (Fig. 5). Overall, the ratio of full-length 11β-HSD1 to total truncated 11β-HSD1 (amount of full length/the sum of the three truncated prod-

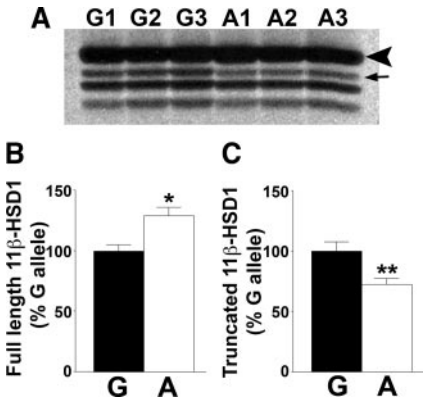

FIG. 5. The A allele of *rs13306421* generates a greater proportion of full-length 11β-HSD1 *in vitro*. Panel A, Representative autoradiograph showing ^{35}S-labeled products of *in vitro* transcription-translation of 11β-HSD1 carried out with three independent preparations of each of the A and G allele of *rs13306421* (indicated *above the lanes*; G1–G3 and A1–A3). An *arrowhead* indicates full-length 11β-HSD1, and the *arrow* indicates the largest of the truncated products. Panels B and C, Quantification by phosphorimager showing more full-length nonglycosylated 11β-HSD1 produced from the A allele (*white bars*) than the G allele (*black bars*) of *rs13306421* (B), but less of the largest truncated product from the A allele (C). Levels of the two smaller truncated products did not differ significantly between the A and G alleles. Data are means ± SEM (n = 3) and are expressed relative to the normal G allele, arbitrarily set to 100%. Significance was tested with unpaired Student's *t* test; *, $P < 0.05$, **, $P < 0.01$.

ucts) was significantly lower for the G allele than for the A allele of 11β-HSD1 (A allele, 3.29 ± 0.09 *vs.* G allele, 2.23 ± 0.13; $P < 0.05$).

The A allele of SNP rs13306421 is present at less than 1% in a southern European population of women with and without PCOS

The 600 women genotyped (300 PCOS and 300 controls comparable for age and body weight) were found to be homozygous for the G allele at rs13306421. Accordingly, the frequency of the A allele is considerably less than 1% in this population.

Discussion

We find no functional consequences of allelic variation at rs846910, located in the 5′ flanking region of the P2 promoter of *HSD11B1* in a context where it could alter promoter activity. Thus, despite its association with metabolic phenotype (16–18), this polymorphism does not appear to directly influence 11β-HSD1 expression, although it remains possible that it modifies response to stimuli not tested here, for example, TNFα. The lack of effect on promoter activity is consistent with our recent data showing no independent association of rs846910 with metabolic phenotype in the same Southern European population of women as examined here (Gambineri, A., Tomassoni, F., Munarini, A., Stimson, R. H., Pagotto, U., Mioni, R., Chapman, K. E., Andrew, R., Pasquali, R. and B. R. Walker, submitted for publication). SNP rs846910 is unlikely to affect the potential binding site of a transcription factor because no sites for mammalian transcription factors were predicted in this region (using the AliBaba2 program, which uses binding sites in TRANSFAC; www.gene-regulation.com). Moreover, rs846910 lies outside of the region that is highly conserved between human and mouse, so is unlikely to be in an important conserved regulatory region, although it may exert human-specific regulation. However, it may be in linkage disequilibrium with another, functional, polymorphism that accounts for the association with metabolic phenotype. Alternatively, the effect of the polymorphism on promoter activity may have been too small to detect in the current study but might become apparent in combination with other functional polymorphisms. Indeed, in our recent study, the combination of less common alleles at both rs846910 and at rs12086634, a polymorphism previously shown to influence transcription (19), was associated with more prevalent metabolic syndrome and higher adipose 11β-HSD1 activity (Gambineri, A., F. Tomassoni, A. Munarini, R. H. Stimson, U. Pagotto, R. Mioni, K. E. Chapman, R. Andrew, R. Pasquali, and B. R. Walker, submitted for publication).

The reported polymorphism rs13306421, close to the translation start of 11β-HSD1, modulates translation of the enzyme, with the A allele resulting in greater enzyme expression in transfected cells and in *in vitro* translation reactions, with a higher ratio of full-length enzyme to truncated products synthesized *in vitro*. The G allele results in less full-length 11β-HSD1 translated *in vitro*, but more of the truncated products. The latter finding suggests that the A allele increases translation initiation at the ATG initiator codon, concomitantly decreasing "leaky scanning" where translation initiates at nearby internal AUG codons rather than the AUG translation start codon (22). The N terminus of 11β-HSD1 is required for its insertion into the endoplasmic reticulum membrane (31), and N-terminally truncated 11β-HSD1 is enzymatically inactive (33, 34). Accordingly, the truncated products of leaky scanning are predicted to be enzymatically inactive. The context of the initiator AUG dictates the extent to which leaky scanning occurs (22). The translation start of 11β-HSD1 is both in a suboptimal context (deviating from the consensus sequence at all three of the critical nucleotides immediately preceding the start codon) and has several nearby AUG codons (Met4, Met16, and Met31, being the closest). According to the Kozak rules (21), translation initiation is sensitive to the bp at −2 when the −3 nucleotide is a pyrimidine (as in *HSD11B1*) rather than the optimal pu-

rine. Thus, the A allele of rs13306421 may favor translation initiation at Met1, concomitantly decreasing initiation from downstream AUGs. Only the fully glycosylated full-length protein was detected in transfected cells. It is likely that any truncated proteins would be subject to rapid degradation.

Whether the polymorphism rs13306421 is truly a polymorphism or may more properly be called a mutation is an open question. We failed to find a single case of the A allele in our screen of 600 subjects. It is therefore highly unlikely that this allele contributes to the alterations in 11β-HSD1 activity associated with obesity and diabetes. Furthermore, although the A allele was reported at 0.5–1% in the original African American and Japanese populations (http://www.jmdbase.jp/snp_info.asp?targetkey=imcj-snp&keyword=JMDBase_000702), in a further study, genotyping of a total of 210 individuals in four different populations (Nigeria, Japan, China, and Europe) detected only the G allele (HapMap database). Thus, further studies in other populations are required to confirm whether the A allele is indeed a polymorphism. Nevertheless, our studies show the clear potential to influence levels of active 11β-HSD1.

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References

1. **Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, Stewart PM** 2004 11β-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. Endocr Rev 25:831–866
2. **Walker BR** 2007 Extra-adrenal regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1: physiological regulator and pharmacological target for energy partitioning. Proc Nutr Soc 66:1–8
3. **Seckl JR, Morton NM, Chapman KE, Walker BR** 2004 Glucocorticoids and 11β-hydroxysteroid dehydrogenase in adipose tissue. Recent Prog Horm Res 59:359–393
4. **Michailidou Z, Jensen MD, Dumesic DA, Chapman KE, Seckl JR, Walker BR, Morton NM** 2007 Omental 11β-hydroxysteroid dehydrogenase 1 correlates with fat cell size independently of obesity. Obesity 15:1155–1163
5. **Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS** 2001 A transgenic model of visceral obesity and the metabolic syndrome. Science 294:2166–2170
6. **Masuzaki H, Yamamoto H, Kenyon CJ, Elmquist JK, Morton NM, Paterson JM, Shinyama H, Sharp MG, Fleming S, Mullins JJ, Seckl JR, Flier JS** 2003 Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. J Clin Invest 112:83–90
7. **Paterson JM, Morton NM, Fievet C, Kenyon CJ, Holmes MC, Staels B, Seckl JR, Mullins JJ** 2004 Metabolic syndrome without obesity: hepatic overexpression of 11β-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proc Natl Acad Sci USA 101:7088–7093
8. **Walker BR, Connacher AA, Lindsay RM, Webb DJ, Edwards CRW** 1995 Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. J Clin Endocrinol Metab 80:3155–3159
9. **Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CRW, Seckl JR, Mullins JJ** 1997 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity or stress. Proc Natl Acad Sci USA 94:14924–14929
10. **Morton NM, Holmes MC, Fiévet C, Staels B, Tailleux A, Mullins JJ, Seckl JR** 2001 Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. J Biol Chem 276:41293–41300
11. **Alberts P, Engblom L, Edling N, Forsgren M, Klingström G, Larsson C, Rönquist-Nii Y, Ohman B, Abrahmsén L** 2002 Selective inhibition of 11β-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. Diabetologia 45:1528–1532
12. **Alberts P, Nilsson C, Selen G, Engblom LO, Edling NH, Norling S, Klingström G, Larsson C, Forsgren M, Ashkzari M, Nilsson CE, Fiedler M, Bergqvist E, Ohman B, Björkstrand E, Abrahmsen LB** 2003 Selective inhibition of 11β-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. Endocrinology 144:4755–4762
13. **Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ, Seckl JR** 2004 Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. Diabetes 53:931–938
14. **Draper N, Echwald SM, Lavery GG, Walker EA, Fraser R, Davies E, Sørensen TI, Astrup A, Adamski J, Hewison M, Connell JM, Pedersen O, Stewart PM** 2002 Association studies between microsatellite markers within the gene encoding human 11β-hydroxysteroid dehydrogenase type 1 and body mass index, waist to hip ratio, and glucocorticoid metabolism. J Clin Endocrinol Metab 87:4984–4990
15. **Bruley C, Lyons V, Worsley AG, Wilde MD, Darlington GD, Morton NM, Seckl JR, Chapman KE** 2006 A novel promoter for the 11β-hydroxysteroid dehydrogenase type 1 gene is active in lung and is C/EBPα independent. Endocrinology 147:2879–2885
16. **Nair S, Lee YH, Lindsay RS, Walker BR, Tataranni PA, Bogardus C, Baier LJ, Permana PA** 2004 11β-Hydroxysteroid dehydrogenase type 1: genetic polymorphisms are associated with type 2 diabetes in Pima Indians independently of obesity and expression in adipocyte and muscle. Diabetologia 47:1088–1095
17. **Franks PW, Knowler WC, Nair S, Koska J, Lee YH, Lindsay RS, Walker BR, Looker HC, Permana PA, Tataranni PA, Hanson RL** 2004 Interaction between an 11βHSD1 gene variant and birth era modifies the risk of hypertension in Pima Indians. Hypertension 44:681–688
18. **Morales MA, Carvajal CA, Ortiz E, Mosso LM, Artigas RA, Owen**

GI, Fardella CE 2008 Possible pathogenetic role of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) gene polymorphisms in arterial hypertension. Rev Med Chil 136:701–710

19. **Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W, Lavery GG, Bedendo O, Ray DW, Laing I, Malunowicz E, White PC, Hewison M, Mason PJ, Connell JM, Shackleton CH, Stewart PM** 2003 Mutations in the genes encoding 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. Nat Genet 34:434–439
20. **Gambineri A, Vicennati V, Genghini S, Tomassoni F, Pagotto U, Pasquali R, Walker BR** 2006 Genetic variation in 11β-hydroxysteroid dehydrogenase type 1 predicts adrenal hyperandrogenism among lean women with polycystic ovary syndrome. J Clin Endocrinol Metab 91:2295–2302
21. **Kozak M** 1986 Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283–292
22. **Kozak M** 1995 Adherence to the first-AUG rule when a second AUG codon follows closely upon the first. Proc Natl Acad Sci USA 92: 2662–2666
23. **de Wet JR, Wood KV, DeLuca M, Helsinki DR, Subramani S** 1987 Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7:725–737
24. **Tannin GM, Agarwal AK, Monder C, New MI, White PC** 1991 The human gene for 11β-hydroxysteroid dehydrogenase. J Biol Chem 266:16653–16658
25. **Sai S, Esteves CL, Kelly V, Michailidou Z, Anderson K, Coll AP, Nakagawa Y, Ohzeki T, Seckl JR, Chapman KE** 2008 Glucocorticoid regulation of the promoter of 11β-hydroxysteroid dehydrogenase type 1 is indirect and requires C/EBPβ. Mol Endocrinol 22: 2049–2060
26. **Agarwal AK, Monder C, Eckstein B, White PC** 1989 Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase. J Biol Chem 264:18939–18943
27. **Bujalska IJ, Draper N, Michailidou Z, Tomlinson JW, White PC, Chapman KE, Walker EA, Stewart PM** 2005 Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11β-hydroxysteroid dehydrogenase type 1. J Mol Endocrinol 34:675–684
28. **ESHRE/ASMR-sponsored PCOS consensus workshop group** 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41–47
29. **Nestler JE, Jakubowicz DJ, Evans WS, Pasquali R** 1998 Effects of metformin on spontaneous and clomiphene-induced ovulation in the polycystic ovary syndrome. N Engl J Med 338:1876–1880
30. **Hoogendoorn B, Owen MJ, Oefner PJ, Williams N, Austin J, O'Donovan MC** 1999 Genotyping single nucleotide polymorphisms by primer extension and high performance liquid chromatography. Hum Genet 104:89–93
31. **Odermatt A, Arnold P, Stauffer A, Frey BM, Frey FJ** 1999 The N-terminal anchor sequences of 11β-hydroxysteroid dehydrogenases determine their orientation in the endoplasmic reticulum membrane. J Biol Chem 274:28762–28770
32. **Blum A, Martin HJ, Maser E** 2000 Human 11β-hydroxysteroid dehydrogenase type 1 is enzymatically active in its nonglycosylated form. Biochem Biophys Res Commun 276:428–434
33. **Mercer W, Obeyesekere V, Smith R, Krozowski Z** 1993 Characterization of 11β-HSD1B gene expression and enzymatic activity. Mol Cell Endocrinol 92:247–251
34. **Obeid J, Curnow KM, Aisenberg J, White PC** 1993 Transcripts originating in intron-1 of the HSD11 (11β-hydroxysteroid dehydrogenase) gene encode a truncated polypeptide that is enzymatically inactive. Mol Endocrinol 7:154–160