**In Vivo Footprinting of the Human 11β-Hydroxysteroid Dehydrogenase Type 2 Promoter**

EVIDENCE FOR CELL-SPECIFIC REGULATION BY Sp1 AND Sp3

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11β-Hydroxysteroid dehydrogenase type 2 is selectively expressed in aldosterone target tissues, where it confers aldosterone selectivity for the mineralocorticoid receptor by inactivating 11β-hydroxyglucocorticoids with a high affinity for the mineralocorticoid receptor. The present investigation aimed to elucidate the mechanisms accounting for the rigorous control of the HSD11B2 gene in humans. Using dimethyl sulfate in vivo footprinting via ligation-mediated PCR, we identified potentially important regions for HSD11B2 regulation in human cell lines: two GC-rich regions in the first exon (I and II) and two upstream elements (III and IV). The footprints suggest a correlation between the extent of in vivo protein occupancy at three of these regions (I, II, and III) and the rate of HSD11B2 transcription in cells with high (SW620), intermediate (HCD, MCF-7, and HK-2), or low HSD11B2 mRNA levels (SUT). Moreover, gel shift assays with nuclear extracts from these cell lines revealed that decreased HSD11B2 expression is related to a decreased binding activity with oligonucleotides containing the putative regulatory elements. Antibody supershifts identified the majority of the components of the binding complexes as the transcription factors Sp1 and Sp3. Finally, transient transfections with various deletion mutant reporters define positive regulatory elements that might account for basal and selective expression of 11β-hydroxysteroid dehydrogenase type 2.

The intracellular access of glucocorticoids to mineralocorticoid or glucocorticoid receptors is modulated by the 11β-hydroxysteroid dehydrogenase enzymes, which interconvert biologically active 11β-hydroxyglucocorticoids and inactive 11-ketosteroids (1–4). Two kinetically distinct isoforms, 11β-hydroxysteroid dehydrogenase type 1 and 11β-hydroxysteroid dehydrogenase type 2, differentiate in cofactor specificity, substrate affinity, and directionality of the reaction (2, 4). The 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) isoform catalyzes the dehydrogenation of 11β-hydroxyglucocorticoids, has a nanomolar $K_m$ for glucocorticoids, utilizes NAD$^+$ as a cofactor, and is localized in the endoplasmatic reticulum membrane with a cytoplasmic orientation of its catalytic domain (5, 6). The enzyme exhibits a remarkable cell-specific constitutive expression in mineralocorticoid target tissues, such as epithelial cells from the renal cortical collecting tubule and the distal colon, where its primary function is to protect the nonselective mineralocorticoid receptor from promiscuous activation by 11β-hydroxyglucocorticoids (4, 5, 7–9).

Impaired 11βHSD2 function leads to clinical symptoms in which cortisol is acting as a mineralocorticoid. A potentially fatal genetic disorder, the syndrome of “apparent mineralocorticoid excess,” has been attributed to mutations in the HSD11B2 gene (5, 9). The phenotype is characterized by increased tubular sodium retention with low renin, low aldosterone hypertension. Moreover, some studies suggest that variations in HSD11B2 gene expression may play a role in the development of essential hypertension and explain salt sensitivity of blood pressure in humans (10, 11).

Control of the constitutive pattern of gene expression in different cells and tissues occurs most commonly at the level of gene transcription. Typically, regulatory DNA elements located cis to the target gene, in cooperation with a distinct and sometimes gene-specific array of trans-acting DNA-binding proteins, control the processes of transcription initiation and elongation by the RNA polymerase II enzyme. The transcriptional activity is highly dependent on the presence and activity of these DNA-binding proteins, encompassing the number of molecules and their transcription activation status. With respect to the HSD11B2 proximal promoter sequence, little information is available on the basal and/or tissue-specific transcriptional regulation. The highly GC-rich promoter lacks a TATA-like element but contains several putative Sp1 binding sites. Two Sp1 elements at positions −80 and −145 were previously analyzed using in vitro techniques, including EMSA, DNase I footprinting, and transient transfections of promoter reporter constructs (12). In the present study, we investigated the HSD11B2 promoter by in vivo dimethyl sulfate (DMS) footprinting via ligation-mediated PCR (LMPCR). This technique allows a view of gene-regulatory regions during transcription in an intact chromosomal environment, so it may be the most relevant and informative method to locate regulatory sites. We compared protein binding events in a number of appropriate cell lines that differentially express the HSD11B2 gene to uncover the transcriptional mechanisms that may govern cell-specific regulation in vivo.

**EXPERIMENTAL PROCEDURES**

Supplies—Chemicals were of a high quality commercial grade and were purchased from Sigma, Roche Diagnostics, Fluka, or Merck. Ra-
biochemicals were obtained from Amersham Biosciences. Oligonucleotides were synthesized by Microsynth. Vent polymerase was from New England Biolabs; other enzymes were obtained from Roche Diagnostics, Invitrogen, or Promega. TLC plates (G-25) coated with silica gel were from Macherey-Nagel.

**Cell Culture**

SW620, a human colon carcinoma cell line, MCF-7, a human breast adenocarcinoma cell line, and SUT, a human lung carcinoma cell line, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen). Human cortical collecting duct cells were obtained from the adrenal gland of pigs, GCTCCTGGACGCCGACATCTT-3' and GGGACTTTGTTCC-3'. Nuclear extracts (3-4 μg) were incubated with 35 fmol of (32P)ATP-labeled oligonucleotides for 10 min at room temperature in a final reaction volume of 10 μl. Binding buffer contained 4% Ficoll, 20 mM HEPES, pH 7.5, 35 mM NaCl, 60 mM KCl, 0.01% Nonidet P-40, 2 mM dithiothreitol, and 1 μg of poly(dI-dC). Where indicated, OX40 (Promega) and Rhodamine-123 (Molecular Probes) were added to the reaction, and then the extracts were kept on ice for 10 min before mixing with the labeled oligonucleotides.

DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide/0.5% Tri-borate EDTA gels, dried, and analyzed on a PhosphoImager.

**Plasmid Constructions**
The reporter plasmid pMCSF+260 was obtained by PCR cloning. Genomic DNA was isolated from SW620 cells and amplified using forward primer 5'-CTCTCTGGACGCCGACAACT-3' and reverse primer 5'-TGCGACCGCAGCTGA-3'. The PCR product was subcloned into the pCRII-TOPO TA cloning vector (Invitrogen) and then into the pGL3-Basic vector (Promega) for transfection and gel shift analyses. 18S cDNA probes were prepared using the reverse transcriptase RT-PCR method (TaqMan) (15) and were used as a positive control for transcription activity. The results are listed in Table 1.

**RESULTS**

**Transfections, Luciferase, and β-Galactosidase Assays**

Plasmid DNA was prepared using the QIAfilter columns (Qiagen). Transfections were performed with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) following the manufacturer’s recommendations. FuGENE 6 (1.2 μl) and 0.8 μg of plasmid DNA were incubated in serum-free medium for 15 min before application on subconfluent cells in 24-well plates. The reporter plasmids were co-transfected with 0.05 μg of pCMV-LacZ control plasmid to correct for transfection efficiency. After 24-h incubation, the medium was removed, and the cells were washed twice with phosphate-buffered saline, lysed in 100 μl of lysis buffer, and assayed for luciferase and β-galactosidase activity using the Dual-Light system (Tropix). Chemiluminescence was measured with a MediatorsPhl luminometer (Mediators Diagnostic Systems). The normalized values from triplicate samples varied by <10%.

**Footprinting results**

Footprinting results were visualized on a PhosphorImager screen (Cyclone; Canberra Packard) and analyzed with ScionImage software.

**EMSAs**

Nuclear protein extractions were carried out as described previously (20). The sequences (top strand) of synthetic complementary oligonucleotides used were as indicated. As a positive control for transcription activity, the probe was hybridized with the plasmid DNA, GCTCCTGGACGCCGACATCTT-3' and GGGACTTTGTTCC-3'. Nuclear extracts (3-4 μg) were incubated with 35 fmol of (32P)ATP-labeled oligonucleotides for 10 min at room temperature in a final reaction volume of 10 μl. Binding buffer contained 4% Ficoll, 20 mM HEPES, pH 7.5, 35 mM NaCl, 60 mM KCl, 0.01% Nonidet P-40, 2 mM dithiothreitol, and 1 μg of poly(dI-dC). Where indicated, OX40 (Promega) and Rhodamine-123 (Molecular Probes) were added to the reaction, and then the extracts were kept on ice for 10 min before mixing with the labeled oligonucleotides. DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide/0.5% Tri-borate EDTA gels, dried, and analyzed on a PhosphoImager.
Conversion of radiolabeled corticosterone into 11-dehydrocorticosterone was assayed as a function of various incubation periods at constant protein concentrations (Fig. 1). In SW620 cells, high mRNA expression levels were translated into a high rate of substrate conversion, which reached about 80% after 4 h of incubation. HCD and HK-2 cells expressed a relatively small catalytic activity with up to 20% conversion. MCF-7 extracts were localized to a region between 210 to 217 was sufficient for maximal protection or hypermethylation, respectively. Such genomic footprints were visualized by comparing samples of HSD11B2 gene were cloned in front of a luciferase marker gene and analyzed using classical transient transfection assays. In addition, a set of progressive deletion mutants was generated and analyzed. As expected, the overall luciferase activity was highest in SW620 cells, intermediate in MCF-7 cells, and relatively low in HCD, HK-2, and SUT cells (data not shown). Consistent with previous observations (12), a fragment ranging from −210 to +117 was sufficient for maximal promoter activity, and critical transcriptional-enhancing elements were localized to a region between −220 and −45 relative to the kidney transcription start site of the HSD11B2 gene (data not shown) (22).

**In Vivo Footprinting of the HSD11B2 Gene**—DMS in vivo footprinting was used to determine the regions that contribute to the differential expression pattern in human tissues in an in vivo setting. Using this method, we analyzed chromosomal protein-DNA contacts occurring at the HSD11B2 locus in intact cells. In principle, the small molecule DMS diffuses rapidly into the nuclei of living cells and methylates guanine residues in the major groove of DNA and, to a lesser extent, adenine residues in the minor groove (17, 23). Transcription factors binding to the DNA inhibit or stimulate guanine methylation, leading to protection or hypermethylation, respectively. Such genomic footprints were visualized by comparing samples of DNA that have been exposed to the methylating agent DMS in the living cell (in vivo) with samples treated with this agent, in the absence of proteins, after the DNA has been extracted from the cells (in vitro). Using LMPCR allows for single copy gene control regions to be analyzed from a whole genome. We have selected several sets of nested primers (PA, PB, PC, PD, and PE) to map −500 bp of the HSD11B2 proximal promoter and exon 1 sequence (Fig. 2). A modified LMPCR procedure was employed for the amplification of highly GC-rich regions, such as those found in this sequence (19).

Throughout the HSD11B2 upstream region and the first exon, we detected multiple differences in the guanine-specific DNA ladders obtained from different cell types. Initially, using primer set PA to amplify the bottom strand, we identified a prominent hypersensitive band within a GC-rich region referred to as region I (nt +153) (Fig. 3). Moving from 3′ to 5′, we detected an assembly of footprinted guanines within a second highly GC-rich region (designated region II). Region II encompasses several overlapping consensus binding sites for proteins from the Sp1 transcription factor family. In region II, protein occupancy was discovered by three protected guanines and one hypersensitive guanine (nt +84, +88, and +93 and nt +90, respectively) (Fig. 3). Furthermore, when amplifying the top
strand with primer set PB, we detected a protected guanine (nt +92), along with two hypersensitive guanines at positions +81 and +97 within region II (Fig. 4A). To better visualize these differences, the guanine ladders were analyzed by densitometry (histograms in Fig. 4B). Variations in the methylation pattern were discovered not only between in vitro- and in vivo-treated DNA but also within the different cell types. For example, protection at guanine +92 and both hypermethylations guanine +81 and guanine +97 were clearly visible in SW620 cells (Fig. 4, A and B; compare lanes 2 and 3), which express high levels of HSD11B2. Histograms from cells expressing intermediate (MCF-7, HCD, and HK-2) and low levels (SUT) of 11βHSD2 exhibited a similar, albeit less pronounced, pattern (Fig. 4B, compare lane 3 with lanes 4–7). Similar cell type dependence was seen with primer set PA, where a decreasing intensity of footprints was accompanied by decreasing HSD11B2 transcript levels (Fig. 3). These observations strongly suggest cell line-specific differences in the quantity and/or the quality of the factors binding to these sites.

In Vivo Nuclear Protein Interactions within the 5'-Flank—Upstream to the HSD11B2 transcriptional start site, DMS in vivo footprints were detected in two regions: region III from −75 to −122, and region IV from −173 to −208. Indicative for
protein binding, we found a strong footprint covering an Sp1 and an overlapping nuclear factor xB motif in region III, where a hypersensitive guanine (nt -89) was located next to two protected guanine residues (nt -86 and nt -91) (Fig. 5A). These differences could be best resolved with primer set PC for bottom strand analysis and were confirmed using primer set PD (Fig. 5, A and B). Additional evidence for protein occupancy in this region came from two neighboring adenine residues, which were hypermethylated (Fig. 5; nt -101 and nt -115).

Region IV is characterized by a consensus RFX1 and an overlapping Ikaros-2 motif and was identified by a series of hypermethylated guanine and adenine residues on the bottom strand (Fig. 5B; nt -186 to nt -199). These footprints were complementary to a single hypersensitive adenine (nt -200) that could be observed by top strand analysis with primer set PE (Fig. 6).

The sum of DMS in vivo footprints identified by our experiments and their exact position on the HSD11B2 genomic sequence are summarized in Fig. 7. Putative transcription factor recognition sites are indicated according to the results of a Transfac Matrix Data base search (24).

**EMSA to Discover the Nature of Binding Proteins—** Our in vivo footprinting experiments suggested numerous protein interactions with widespread regions along the HSD11B2 promoter. To further characterize these interactions and to determine the identity of the binding proteins, in vitro studies were necessary. EMSAs were carried out using nuclear extracts from the previously described cell types and radiolabeled probes matching the sequences around regions I to IV (GSI, GSII, GSIII, and GSIV) as described in Fig. 2.

Several abundant protein-DNA complexes were formed with probe GSI (Fig. 8). The most abundant complex, C1, and a faint complex, C2, proved to bind specifically because binding was inhibited in the presence of a 100-fold molar excess of unlabeled oligonucleotide (wtGSI). In addition, complex C3 partially disappeared upon the addition of the wild-type competitor (wtGSI), whereas another two fast-migrating bands were considered as unspecific bands (ns). Moreover, competition with an oligonucleotide in which the Sp1 site was point-mutated by the exchange of TA for GC bases (Fig. 8, mutX) abolished the ability to compete for complexes C1, C2, and C3 using extracts from SW620 cells. These results suggested a strong binding
activity through the GC-rich element as attributed to members of the Sp1 family of zinc finger DNA-binding proteins (25). Interestingly, the intensity of the major complex, C1, was significantly higher using extracts from high-level HSD11B2 expressing cells (SW620), compared with the other four cell types (MCF-7, HCD, HK-2, and SUT). Other intercellular variations were less pronounced (C2 and C3), with the exception of the weak complex C4 that repeatedly appeared using HCD extracts. 

Formation of complex C5 was predominant with SW620 extracts relative to the other four cell types (MCF-7, HCD, HK-2, and SUT). In supershift assays, complex C5 emerged as a combination of both proteins Sp1 and Sp3, whereas complex C6 reacted only with Sp3-specific antibodies and not with Sp1-specific antibodies (Fig. 9B). We did not find evidence for constitutive binding of Egr-1, Sp2, Sp4, or WT1 proteins to probe GSI or GSII, respectively, despite the fact that these proteins have a similar potential for binding to such GC-rich sequence elements (data not shown). This strongly implicates Sp1 and Sp3 in the transcriptional control of the HSD11B2 gene and probably rules out a role for Egr-1, Sp2, Sp4, or WT1 in the activation or repression of transcription of this gene at the constitutive level. Sp1 and Sp3 may both bind simultaneously to the oligonucleotides, as has been observed elsewhere (26), or, alternatively, Sp1 and Sp3 may bind individually to the oligonucleotides, which might not be separated by EMSA analysis.

We also analyzed Sp1 and Sp3 protein levels in our cell lines by immunoblotting, but we did not detect obvious variations in endogenous Sp1 and Sp3 protein content or in the ratios of Sp3 isoforms (data not shown). This may implicate differences in both the activation status and the nature of interactions with co-activator/co-repressor proteins to direct cell-specific expression of the HSD11B2 gene.

FIG. 6. HSD11B2 top strand footprinting analysis. The top strand of genomic DNA prepared from SW620 and SUT was amplified with primer set PE. The position of a hypermethylated adenine residue is shown (−200). Experimental procedures were as described for Fig. 3.

Transactivation of the HSD11B2 Promoter through a GC-rich Element in Exon I—To substantiate our findings on the importance of the exon 1 sites in the tissue-specific regulation of HSD11B2 gene expression, attention was directed to the two sites implicated by in vivo footprinting and EMSA analysis. We constructed a luciferase reporter vector under the control of an HSD11B2 genomic sequence extending from −400 to +260 (p−400/+260) (Fig. 10). The sequence included upstream regions (region II, III, and IV) as well as parts of the coding sequence of exon 1 (region I) and was found to have strong transcriptional activity in the HSD11B2 gene-expressing cell line SW620. The transactivation efficiency, calculated as the ratio of the experimental vector to constitutive β-galactosidase activity as an internal standard, was about 2 times lower in MCF-7 cells and -4 times lower in HCD, HK-2, and SUT cells than in SW620 cells (data not shown).

We then generated reporter vectors in which either region I or region II was disrupted, and these deletion mutants were tested for their transcription-enhancing activity (Table II). Deletion of region I slightly impaired promoter activity in high HSD11B2-expressing SW620 cells and, surprisingly, impaired promoter activity to a similar extent in low 11βHSD2-expressing SUT and HK-2 cells (~30% and ~40%, respectively), whereas promoter activity remained unchanged in MCF-7 cells.

Sp1 and Sp3 Protein Binding Activity to Multiple GC-rich Elements Present in the HSD11B2 Promoter—We next sought to determine whether Sp1 family members were able to interact with other GC-rich regions as exposed by in vivo footprinting experiments. Representative EMSAs with radiolabeled probe GSII are shown in Fig. 9. The probe encompasses three nested, homologous Sp1 binding sites. Indeed, abundant formation of protein-DNA complexes was discovered by the presence of an intense, retarded band C5. A faster-migrating band, C6, was specific but of low affinity, as determined by competition with an excess of cold oligonucleotide (Fig. 9A, wtGSII). Furthermore, point mutations designed to individually abrogate Sp1 binding sites were not sufficiently able to compete for binding, with the exception of an impaired competition when mutation B was introduced (mutB). The destruction of two or more of the Sp1 sites present, as realized by mutCD or mutABC, was necessary to partially or completely inhibit competition (Fig. 9A).

The only proteins implicated in the binding to probe GSI were the Sp1 and Sp3 members of the Sp1 family of zinc finger transcription factors. In supershift assays, nuclear extracts from SW620 cells were incubated with antibodies specific to Sp1 or Sp3 (Fig. 8, αSp1 and αSp3). These antibodies were able to considerably reduce C1 and C2 complex formation. Nuclear extracts from the other four cell types reacted in a similar manner (data not shown).
and increased ~1.5-fold in HCD cells (p~400/+260AGCII). Whereas the deletion of region I had miscellaneous effects in the different cell types, deletion of region II had a strong and uniform effect on the transactivation potential in all cell types.

Typically, the overall activity decreased to 20–30% relative to the wild-type construct when the p~400/+260AGCII vector was transfected into the cells. Taken together, these data suggest that sites in the first exon and upstream elements may act cooperatively to regulate HSD11B2 expression.

**DISCUSSION**

The best-established role of 11βHSD2 is the regulation of salt homeostasis by preventing access of glucocorticoids to the mineralocorticoid receptor in sodium-transporting epithelia (1, 2, 5, 8). To accomplish this task, a distinct tissue-specific ex-
retardation assays were performed as described under "Procedures.

to as nuclear protein factors that repress or induce regulation of the gene, rather than a specific individual or set of Sp1 and Sp3 transcription factors in the tissue-specific regulatory elements in the first exon, which were not identified performed for the first time an molecular mechanism underlying this selective expression, we

FIG. 9. EMSA probe GSII binds nuclear factors Sp1 and Sp3. 32P-labeled GSII probes covering three nested Sp1 consensus sites in region II were incubated with equal amounts of nuclear extracts from SW620, MCF-7, HCD, HK-2, and SUT cells. The position of specific binding (C5 and C6) and nonspecific bands (ns) is indicated. A, the exact sequences of oligonucleotides used are indicated on the top. B, antibodies have been included in the binding reactions as indicated. The location of complexes that reacted with aSp1 or aSp3 antibodies is referred to as supershift. An anti-gelatinase antibody was used as a control. Gel retardation assays were performed as described under "Experimental Procedures."

expression of the enzyme is required (4, 5, 7, 9). To elucidate the molecular mechanism underlying this selective expression, we performed for the first time an in vivo genomic footprinting analysis of the human HSD11B2 promoter and describe regulatory elements in the first exon, which were not identified earlier. Furthermore, we provide evidence for an involvement of Sp1 and Sp3 transcription factors in the tissue-specific regulation of the gene, rather than a specific individual or set of nuclear protein factors that repress or induce HSD11B2 transcription in different cell types.

We have based our studies on cellular models to unravel regulatory mechanisms at the level of DNA interacting with regulatory proteins in the context of an intact chromosomal architecture. HSD11B2 transcript levels ranged over 50-fold in cell lines originating from human kidney (HCD and HK-2), colon (SW620), breast (MCF-7), and lung (SUT) cancers (Table I). Thus, these cell lines appeared to be appropriate models to study basal and cell-specific transcriptional regulation of the HSD11B2 gene.

The DMS in vivo footprinting experiments pointed to the existence of DNA-protein interactions within three GC-rich DNA elements in the first exon (regions I and II) and upstream to the transcriptional start site (region III) of the HSD11B2 gene. Each of these elements contains one or more homologous Sp1 binding motifs and was indeed shown to bind Sp1 and Sp3 proteins with high affinity under in vitro conditions such as in EMSA and supershift assays (Figs. 8, 9, and 11). The functional roles of these regions were further evaluated by transfecting deletion mutant reporter vectors. Our results matched previous observations (12) suggesting the presence of strong transcription-enhancing elements between nucleotides −210 and −45 because the removal of these resulted in a significant loss of promoter activity (data not shown). However, deletion of downstream elements equally altered the promoter activity; removal of region II severely affected the transactivating potential (70−80% less active), whereas deletion of region I had moderate effects and was strongly dependent on the cellular background. These experiments indicated that both regions contain important cis-acting elements but are not fully capable of independently driving the HSD11B2 promoter (Table II). Similar cooperative regulation of neighboring Sp1 binding sites has been reported previously (29).

FIG. 10. Scheme of HSD11B2 deletion mutant reporter vectors. Schematic representation of deletion mutant reporter constructs. Parts of the 5′-flanking sequence (white box) and the first exon (gray box) of the HSD11B2 gene were cloned into the pGL3-Basic luciferase (LUC) reporter vector. Numbers indicate the position and the approximate length of the inserted fragments relative to the kidney transcription initiation site (asterisk). Internal deletions are specified with X. Roman numerals indicate GC-rich regions as described under "Results." Graphic elements are as defined in the Fig. 2 legend. Results from transient transfections with these vectors are given in Table II.

### Table II

Relative activity of luciferase reporters under the control of HSD11B2 promoter deletion mutants

| Cell type | p − 400/+260 | p − 400/+260+GCII | p − 400/+260+GCII |
|-----------|-------------|-----------------|-----------------|
| SW620     | 0.17 ± 0.09 | 0.71 ± 0.13     | 0.71 ± 0.13     |
| MCF-7     | 0.21 ± 0.02 | 1.08 ± 0.12     | 1.08 ± 0.12     |
| HCD       | 0.34 ± 0.08 | 1.63 ± 0.17     | 1.63 ± 0.17     |
| HK-2      | 0.14 ± 0.03 | 0.58 ± 0.09     | 0.58 ± 0.09     |
| SUT       | 0.27 ± 0.12 | 0.70 ± 0.01     | 0.70 ± 0.01     |

* The p − 400/+260 values are normalized to 1.

* Results are relative to p − 400/+260 and are the mean and S.E. of the mean of at least three independent experiments each performed in triplicate.
The footprints obtained in our study by in vivo footprinting differed from those described earlier by in vitro footprinting techniques, firstly through the identification of sensitive areas in the first exon of the HSD11B2 gene, and secondly through the inability to detect protein occupancy at an additional GC-rich site located between regions III and IV (12). This element was described previously as being capable of binding Sp1 proteins in vitro in nuclear extracts from a human choriocarcinoma cell line. We could not confirm this finding in our cell lines using DMS footprinting. It has been shown that protein binding in vitro does not necessarily reflect the transcriptional involvement and occupation of a binding site in vivo (30, 31). The DMS in vitro footprinting technique cannot be criticized, as can classical in vitro assays, for not taking into account the genomic architecture of a regulatory region.

Our results implicate a role for the Sp1 transcription factor and a closely related member of the same family, Sp3, in the control of transcription of the gene. Crucially, we observe variations in the protection patterns among cell lines that differentially express HSD11B2, thus reflecting an altered availability of these sequences for protein binding in vivo (Figs. 3 and 4). Differential binding of Sp1 and Sp3 proteins to the HSD11B2 promoter is also observed in vitro, whereby the binding activity appears to correlate positively with 11βHSD2 expression in our cells (Figs. 8, 9, and 11). Based on the virtually ubiquitous expression of Sp1 and Sp3 (29, 32), one might anticipate that these transcription factors alone cannot play a pivotal role in the regulation of tissue-specific expression. In fact, they have been implicated many times in the specific expression of a number of genes (33–35). Generally, Sp1 and Sp3 are assumed to be activators of transcription, although a repression domain has been identified in both (36, 37), and internally initiated translation sites in Sp3 mRNA can lead to smaller, inhibitory Sp3 species (38). There is no obvious support for an HSD11B2 transcription repressing activity of Sp1 or Sp3 in our system, but we cannot rule out this possibility because our data for this were obtained in vitro.

The specificity of expression of genes dependent on Sp1/Sp3 can be derived from a number of different mechanisms: (i) the relative abundance of Sp1 and Sp3 in the nucleus. Immunoblot data indicated that variations in Sp1 and Sp3 binding on EMSAs were not due to the quantity of these proteins in nuclear extracts (data not shown). However, previous reports have demonstrated that exogenous factors may influence Sp1 transcriptional function without affecting overall nuclear levels (35); (ii) interaction with enhancers or inhibitory nuclear proteins. Cell type-specific, direct protein-protein interactions might modulate the binding activity of Sp1 transcription factors (39–41); (iii) posttranslational modifications. Sp1 is known to be phosphorylated (42–44) and glycosylated (45). Increasing evidence exists for an influence of the phosphorylation status of Sp1 on its specific DNA affinity (35, 44). This may play a role in the different binding activities detected, although we cannot speculate further without additional data; and (iv) interaction with methylated CpG sites. The HSD11B2 gene sequence surrounding the transcriptional start site is extremely GC-rich and may function as a CpG methylation-free island, as has been shown to exist in many housekeeping but also tissue-specifically expressed gene promoters (46). Sp1 has been shown to protect CpG islands from methylation (47), although it may not be the only factor involved in vivo (48). On the other hand, binding of the methyl-CpG-binding protein MeCP2, which is thought to be responsible for the trans-repression of methylated promoters, can repress Sp1-activated transcription (49).

Although there still remains the possibility that other transcription factors and DNA recognition elements not detected in our current experiments play a role in the cell-specific expression of the HSD11B2 gene, the results from the present investigation add to a growing weight of evidence supporting the notion that Sp1 is associated with the transcriptional activation of a number of genes involved in control of sodium metabolism and hemodynamics. For instance, basal and hyperoxia-induced Na,K-ATPase β1 transcription has been shown to be mediated by Sp1 and Sp3 in Madin-Darby canine kidney cells.
and Sp3 binding regulate the Na,K-ATPase activity (54). Of potential interest for understanding of the co-localization of 11βHSD2 and mineralocorticoid receptors is the observation of Zennaro et al. (55), who showed Sp1 binding to defined sequences of the promoter of the human mineralocorticoid receptor. Although the large number of genes regulated by Sp1 and/or Sp3 probably precludes the pharmacological use of Sp1 effectors in the treatment of hemodynamic pathologies, information on exactly which genes are dependent on these factors is relevant for the understanding of the pathogenesis of these disease states. For instance, we and others have recently presented evidence for a reduced activity of 11βHSD2 in humans exhibiting a salt-sensitive blood pressure regulation (11, 56). The probability of identifying exonic mutations as a mechanism for this decreased 11βHSD2 activity is extremely low (57). Thus, alternative explanations, such as an altered transcriptional activity, should be considered.

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