In vivo footprinting of the human 11beta-hydroxysteroid dehydrogenase type 2 promoter: Evidence for cell-specific regulation by Sp1 and Sp3

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SUMMARY

11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) is selectively expressed in aldosterone target tissues, where it confers aldosterone selectivity for the mineralocorticoid receptor (MR) by inactivating 11β–hydroxyglucocorticoids with a high affinity for the MR. The present investigation aimed to elucidate the mechanisms accounting for the rigorous control of the 11βHSD2 gene in humans. Using DMS in vivo footprinting via ligation-mediated PCR we identified potentially important regions for 11βHSD2 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 11βHSD2 transcription in cells with high (SW620), intermediate (HCD, MCF-7 and HK-2) or low 11βHSD2 mRNA levels (SUT). Moreover, gel shift assays with nuclear extracts from these cell lines revealed that decreased 11βHSD2 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 to be 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 11βHSD2.

INTRODUCTION

The intracellular access of glucocorticoids to mineralocorticoid or glucocorticoid receptors is modulated by the 11β-hydroxysteroid dehydrogenase enzymes (11βHSD), which interconvert
biologically active 11β-hydroxyglucocorticoids and inactive 11-ketosteroids (1-4). Two kinetically distinct isoforms, type 1 and type 2 (11βHSD1 and 11βHSD2), differentiate in cofactor specificity, substrate affinity and directionality of the reaction (2, 4). The type 2 isoform (11βHSD2) catalyses the dehydrogenation of 11β-hydroxyglucocorticoids, has a nanomolar Km 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 non-selective mineralocorticoid receptor from promiscuous activation by 11β-hydroxyglucocorticoids (4, 5, 7, 8, 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’ (AME) has been attributed to mutations in the 11βHSD2 gene (5, 9). The phenotype is characterised by increased tubular sodium retention with low renin, low aldosterone hypertension. Moreover, some studies suggest that variations in 11βHSD2 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 as well as their
transcription activation status. With respect to the 11βHSD2 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 analysed using in vitro techniques, including EMSA, DNase I footprinting and transient transfections of promoter reporter constructs (12). In the present study we investigated the 11βHSD2 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 11βHSD2 gene, in order to uncover the transcriptional mechanisms which 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. Radiochemicals were obtained from Amersham Pharmacia Biotech. Oligonucleotides were synthesised by Microsynth (Switzerland). Vent polymerase was from New England Biolabs; other enzymes were obtained from Roche Diagnostics, Gibco BRL or Promega. TLC plates (G-25 fluorescence indicator UV 254) 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 (DMEM) supplemented with 10% fetal calf serum (Gibco). Human
cortical collecting duct cells (HCD) were a generous gift from P. M. Ronco (13). HCD cells were
cultured in HAM’s F12 nutrient mix/DMEM (1:1 v/v) supplemented with 2% new born calf
serum (Sigma), 20 mM HEPES (Gibco), 5 µg/ml transferrin, 5 ng/ml sodium selenate, 5 µg/ml
insulin and 50 nM dexamethasone (all from Sigma). A human proximal tubule derived cell line
(HK-2) was purchased from ATCC and grown in keratinocyte serum-free medium (K-SFM,
Gibco) supplemented with 5 ng/ml epidermal growth factor and 40 µg/ml bovine pituitary extract
(14). The cell lines were complemented with 2 mM glutamine, penicillin (100 units/ml) and
streptomycin (100 µg/ml) and maintained in a 37°C/5% CO₂ humidified atmosphere.

RNA extraction and reverse transcription—Total RNA was extracted using the TRIZOL
reagent (Gibco) and was further treated with RNase free DNAse I for 15 min at 37°C. The RNA
concentration was determined by measuring the absorption at 260 nm and quality was estimated
by the ratio to the absorption at 280 nm. Reverse transcription (RT) was performed in RT buffer
containing 50 mM Tris-HCl (pH 8.2), 6 mM MgCl₂, 10 mM DTT, 100 mM NaCl, 250 nM
dNTPs, 2 units of ribonuclease inhibitor RNAsin, 1 unit avian myeloblastosis virus reverse
transcriptase, 2 µl of random hexanucleotide mix (10-fold concentrated) and 2 µg of total RNA
in a volume of 20 µl for 1h at 42°C.

Quantitative PCR—PCR was performed using the TaqMan system from Applied Biosystems.
The reaction mixture contained one fold concentrated TaqMan universal PCR master mix, 500
nM each of the appropriate forward and reverse primers, 200 nM of specific TaqMan probe and
100 ng of reverse transcribed total RNA in a final volume of 25 µl. Primers and probe were
designed with the Primer Express 1.0 software (Applied Biosystems), optimized and validated.
They were complementary to the human 11βHSD2 cDNA (forward primer position 802-821,
reverse 850-869, probe 823-847). TaqMan ribosomal RNA control reagents were used to normalize for the S18 rRNA gene (20-fold concentrate, Applied Biosystems). Probes were labelled with a reporter fluorescent dye, 6-carboxyfluorescein (FAM) and with 6-carboxy-tetramethyl-rhodamine (TAMRA) as quencher. The pre-run cycling conditions were 2 min at 50°C and 10 min at 95°C. Thermal cycling involved 40 cycles at 95°C for 15 s and 1 min at 60°C (15, 16).

11βHSD2 activity assay—Approximately 150’000 trypsinized cells from each cell line were resuspended in reaction buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM MgCl₂, 100 mM NaCl and 250 mM sucrose and subjected to three freeze/thaw cycles. Enzymatic activity was determined in vitro by measuring the rate of conversion of corticosterone to 11-dehydrocorticosterone. Cellular lysates were mixed with 100 μM NAD, 5 nM corticosterone, and 1.26 nM [³H]corticosterone (specific activity 79 Ci/mmol) in 20 μl reaction buffer and incubated for different time points at 37°C. As controls, 0.2% Triton X-100 was added to inhibit 11βHSD2 activity. The reactions were stopped by the addition of an excess of corticosterone and dehydrocorticosterone (10 μg). Finally, the steroids were separated by thin-layer chromatography and analysed as described previously (3).

In vivo footprinting via LMPCR—Genomic footprinting analysis was carried out using dimethyl sulfate (DMS) methylation and the ligation-mediated PCR method (17, 18) as adapted by us for the analysis of extremely GC-rich regions (19). Nested primer sets were selected to cover about 500 bp of the 5’ flanking region and exon 1 of the 11βHSD2 gene. The primer sequences were as follows:

PA.-1: 5’-GGGCTTTCATAAGCTCG-3’, PA.-2: 5’-AGGGCGAGCGAGAAAGCGAGT-3’, PA.-3: 5’-AGGGCGAGCGAGAAAGCGAGTGTCCCTCT-3’
PB.1: 5’-TGGCACAGCCAGTCA-3’, PB.2: 5’-AGACGCAGGTCTGAGCGAGCA-3’,
PB.3: 5’-ACGCAGGTCTGAGCGAGCTGCAGCA-3’.
PC.-1: 5’-ATGCCGGTTGTGCTGCT-3’, PC.-2: 5’-CGAACAAGCGTGAGGGCATGTG-3’,
PC.-3: 5’-CAAGCGGTGAGTGCCATGGCTACCTGAG-3’.
PD.-1: 5’-TCCTCAGCCAGCAGCAA-3’, PD.-2: 5’-GCAACTTTGGGACTTTGTTCCGGC-3’,
PD.-3: 5’-CCGGCTTTTTTCAAATCGAATCTGGTGGAGGGG-3’.
PE.1: 5’-GGAGAGAGCTTCTAGG-3’, PE.2: 5’-AGAGGGACACTCGCTTTCTGCT-3’,
PE.3: 5’-TTTCTCTGCTCGCCCTCGGGCaGGCTTAT-3’.

Footprinting results were visualized on a PhosphorImager screen (Cyclone, Canberra Packard) and analysed with the ScionImage software.

Electrophoretic mobility shift assays (EMSA)-Nuclear protein extractions were carried out as previously described (20). The sequences (top strand) of synthetic complementary oligonucleotides used were as indicated in Figures 9, 10 and 12 except for GSIV which was 5’-GCTCCTCGAGCCAGCAGAAAACTTTGGGACTTTGTTCC-3’. Nuclear extracts (3-4 µg) were incubated with 35 fmol of γ-[^32]P ATP labelled 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% NP40, 2 mM DTT and 1 µg of polydI/dC. Where indicated, specific binding was competed with unlabelled competitor oligonucleotides at a 100-fold molar excess. For antibody supershift experiments, 1 µl of the corresponding antibody solution (TransCruz Gel Supershift reagents, Santa Cruz Biotechnology) was added to the nuclear extracts and kept for 10 min on ice prior to mixing them with the labelled oligonucleotides. DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide/0.5 x TBE gels, dried and analysed on a PhosphorImager.
**Plasmid constructions**—The reporter plasmid p-400/+260 was obtained by PCR cloning. Genomic DNA was isolated from SW620 cells and amplified using forward primer 5’-CTCCTCGAGCGCAGCAACT-3’ and reverse primer 5’-TGGCACAGCCAGTCA-3’. The PCR product was subcloned into the pCRII-TOPO TA cloning vector (Invitrogen) and transferred into the pGL3-Basic vector (Promega) by *KpnI/EcoRV* digestion and ligation via *KpnI/SmaI* sites. Deletion mutants p-400/+260ΔGCI and p-400/+260ΔGCII are derivatives of plasmid p-400/+260 and were generated via PCR-based whole plasmid synthesis using Vent polymerase with proofreading activity and the following primers: forward 5’-CTGCAGCTGCTGCGCTCAG-3’, reverse 5’-AAGGCCAGCGCTCCATGG-3’ for deletion GCI and forward 5’-ATGGAGCGCTGGCCTTGGCC-3’, reverse: 5’-AGAGAGAGCTTCTAGGGCGG-3’ for deletion GCII. Parental templates were digested with the methylation sensitive restriction enzyme *DpnI* and the remaining PCR products were circularized with T4 DNA ligase.

**Transfections, luciferase and β-galactosidase assays**—Plasmid DNA was prepared using QIAfilter columns (QIAGEN). Transfections were performed with FuGene6 transfection reagent (Roche) following the manufacturer's recommendations. FuGene6 (1.2 µl) and 0.8 µg of plasmid DNA were incubated in serum-free medium for 15 min prior to application on subconfluent cells in 24-well plates. The reporter plasmids were co-transfected with 0.05 µg of a pCMV-LacZ control plasmid to correct for transfection efficiency. After 24 h incubation, the medium was removed, the cells were washed twice with PBS, 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 the MediatorsPhL luminometer (Mediators Diagnostic Systems). The normalized values from triplicate samples varied by less than 10%.
RESULTS

Transcriptional Activity of the 11\(\beta\)HSD2 Gene in Different Epithelial Cell Types-A selection of human, epithelial-like carcinoma cell lines were tested for their 11\(\beta\)HSD2 transcriptional activity by a real-time quantitative RT-PCR method (TaqMan) (15). The results are listed in Table I. The lowest 11\(\beta\)HSD2 mRNA levels were measured in the lung derived SUT cell line. Thus, we used SUT values as a reference for the calculation of the relative transcript number in other cell types. The SW620 colon carcinoma cell line contained the highest 11\(\beta\)HSD2 mRNA levels (~50-fold higher than SUT) and was therefore included for in depth analyses. A physiologically relevant locus for 11\(\beta\)HSD2 activity is the kidney; accordingly we selected two kidney cell lines, HCD derived from the cortical collecting duct and HK-2 from the proximal tubule. Both cell types expressed intermediate amounts of 11\(\beta\)HSD2 transcripts (~16-fold higher than SUT). Similar intermediate levels were found in the human breast cancer cell line MCF-7 (Table I).

Enzymatic Activity of 11\(\beta\)HSD2-To establish a correlation between the relative number of transcripts and the occurrence of functional proteins, we measured the oxidative activity of 11\(\beta\)HSD2 in lysates of the five cell types mentioned above. Conversion of radiolabelled 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 hours of incubation. HCD and HK-2 cells expressed a relatively small catalytic activity with up to 20% conversion. MCF-7 extracts converted about 52% in 4 hours. In contrast to the 11\(\beta\)HSD2 mRNA which was virtually absent, we measured 64% substrate conversion in SUT extracts.
Finally, residual oxidative activity of related enzymes like the 11βHSD type 1 isoform was established in the presence of 0.2% Triton X-100. Such treatment was observed to completely eradicate 11βHSD2, but not 11βHSD1 activity (21). Upon addition of Triton X-100, the conversion rate could be reduced to less than 10% in all cellular extracts except in those of SUT (Fig. 1, black bars), suggesting that the 11-beta dehydrogenase activity in SW620, MCF-7, HCD and HK-2, but not in SUT cells is attributable to the 11βHSD2 isoform. Therefore, the factual 11βHSD2 activity in SUT cells was estimated as the difference between the conversion rates in the absence and presence of Triton X-100 and was very low (around 10%).

Transcriptional Control of 11βHSD2 in Different Cell Lines—Because of the probable transcriptional nature of the control of 11βHSD2 gene expression, we investigated the potential of its upstream sequences to induce transcription in the five cell lines. Approximately 1800 bp of the 5’ flanking region of the 11βHSD2 gene were cloned in front of a luciferase marker gene and analysed using classical transient transfection assays. In addition, a set of progressive deletion mutants was generated and analysed. As expected, the overall luciferase activity was highest in SW620 cells, intermediate in MCF-7, and relatively low in HCD, HK-2 and SUT (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 11βHSD2 gene (data not shown) (22).

In Vivo Footprinting of the 11βHSD2 Gene—Dimethyl sulfate (DMS) in vivo footprinting was used to determine the regions, which contribute to the differential expression pattern in human tissues, in an in vivo setting. Using this method, we analysed chromosomal protein-DNA contacts occurring at the 11βHSD2 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 ligation-mediated PCR (LMPCR) allows for single copy gene control regions to be analysed from a whole genome. We have selected several sets of nested primers (PA, PB, PC, PD and PE), so as to map approximately 500 bp of the 11βHSD2 proximal promoter and exon 1 sequence (Fig. 2). A modified LMPCR procedure was employed for the amplification of highly GC-rich regions, such as are found in this sequence (19).

Throughout the 11βHSD2 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 and one hypersensitive guanines (nt +84, +88, +93 and nt +90, respectively) (Fig. 3). Furthermore, when amplifying the top strand with primer set PB we detected a protected G (nt +92), along with two hypersensitive G’s at positions +81 and +97 within region II (Fig. 4A). To better visualize these differences, the guanine ladders were analysed 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 G +92 and both hypermethylations G +81 and G +97 were clearly visible in SW620 cells (Fig. 4A and 4B; compare lane 1 and 2), which express high levels of 11βHSD2. Histograms from intermediate and low expressing MCF-7, HCD, HK-2 and SUT cells, respectively, exhibited a similar pattern, albeit less pronounced (Fig. 4B; compare lane 3 with lanes 4, 5, 6 and 7). Similar cell type dependency was seen with primer set PA where decreasing intensity of footprints was accompanied by decreasing 11βHSD2 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 11βHSD2 transcriptional start site, DMS in vivo footprints were detected in two regions: region III from -75 extending to -122 and region IV from -173 to -208. Indicative for protein binding, we found a strong footprint covering an Sp1 and an overlapping NFκB motif in region III, where a hypersensitive G (nt -89) was located next to two protected G 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. 5A and 5B). Additional evidence for protein occupancy in this region came from two neighbouring 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 G and A residues on the bottom strand (Fig. 5B; nt -186 to nt -199). These footprints were complementary to a single hypersensitive A (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 11βHSD2 genomic sequence is summarized in Figure 7. Putative transcription factor recognition sites are indicated according to the results of a Transfac Matrix Database search (24).

**Electrophoretic Mobility Shift Assays (EMSA) to Discover the Nature of Binding Proteins**—Our *in vivo* footprinting experiments suggested numerous proteins interactions with widespread regions along the 11βHSD2 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 radiolabelled probes matching the sequences around regions I to IV as described in Figure 2 (GSI, GSII, GSIII and GSIV).

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, since binding was inhibited in the presence of a 100-fold molar excess of unlabelled oligo (wtGSI). In addition, complex C3 partially disappeared upon the addition of the wild-type competitor (wtGSI) while another two, fast migrating bands were considered as unspecific bands (ns). Moreover, competition with an oligo 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 11βHSD2 expresser cells (SW620), compared to 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.
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).

Sp1 and Sp3 Protein Binding Activity to Multiple GC-rich Elements Present in the 11βHSD2 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 radiolabelled probe GSII are shown in Figure 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 oligo (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 mutABCD, was necessary to partially or completely inhibit competition (Fig. 9A).

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, the complex C5 emerged as a combination of both proteins Sp1 and Sp3, whereas complex C6 reacted only with Sp3-specific, but 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 these proteins having a similar potential for binding to such GC-rich sequence elements (data not shown).
strongly implicates Sp1 and Sp3 in the transcriptional control of the 11βHSD2 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 oligos, as has been observed elsewhere (26), or alternatively Sp1 and Sp3 may bind individually to the oligos, which might not be separated by EMSA analysis.

We also analysed Sp1 and Sp3 protein levels in our cell lines by immunoblotting, but 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 as well as in the nature of interactions with co-activator/co-repressor proteins to direct cell-specific expression of the 11βHSD2 gene.

Transactivation of the 11βHSD2 Promoter Through a GC-rich Element in Exon 1-In order to substantiate our findings on the importance of the exon 1 sites in the tissue specific regulation of 11βHSD2 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 11βHSD2 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 11βHSD2 gene expressing cell line, SW620. The transactivation efficiency, calculated as the ratio of the experimental vector to a constitutive β-galactosidase activity as internal standard, was about 2 times lower in MCF-7 cells and approximately 4 times less 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 transcriptional enhancing activity (Table II). Deletion
of region I slightly impaired the promoter activity in high 11βHSD2 expressing SW620 cells and surprisingly, to a similar extent in low expressing SUT and HK-2 cells (~30% and ~40%, respectively) whereas activity in MCF-7 remained unchanged and increased ~1.5 fold in HCD cells (p-400/+260ΔGCI). While 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/+260ΔGCII vector was transfected into the cells. Taken together, these data suggest that sites in the first exon and upstream elements may act co-operatively to regulate 11βHSD2 expression.

**EMSA Analysis of Putative Regulatory Motifs in the 11βHSD2 Upstream Region**-Initial studies focussed on the role of exon 1 sites in the control of 11βHSD2 transcription, therefore, to complete these studies, *in vitro* protein binding characteristics were analysed using probes GSIII and GSIV. These EMSA probes encompass *in vivo* footprinted areas upstream to the transcriptional start site. Specific retarded bands (C7, C8 and C9) were detected using probe GSIII, containing overlapping Sp1 and NFκB elements, and nuclear extracts under conditions as reported above (Fig. 11). Intercellular variations existed, as seen with probes GSI and GSII, in the binding activity of Sp1 and Sp3 using nuclear extracts from the different cell types (Fig. 11A). Protein-DNA interactions were directed via the Sp1 sites, but no evidence for NFκB binding was found by competition (Fig. 11A) or supershift assays (Fig. 11B). The identity of the components of complex C9 remains unclear, since it did not react with specific antibodies against NFκB subunits p65, p50 or c-Rel, nor Sp2, Sp4, Ap2 or Ikaros-2 proteins (Fig. 11B and data not shown).
Finally, weak binding activity was found using the GSIV probe covering an Ikaros-2 and a RFX1 consensus motif and nuclear extracts from all analysed cell lines. The Ikaros zinc finger family of transcription factors plays a role in hematopoietic cell differentiation (27). RFX proteins are ubiquitously expressed in mammalian cells and are essential for transactivation of MHCII and some viral promoters (28). However, competition was not inhibited adding an excess of unlabelled oligos that were mutated in either of the two binding sites, hence we did not further analyse region IV (data not shown).

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 expression of the enzyme is required (4, 5, 7, 9). In order to elucidate the molecular mechanism underlying this selective expression we performed for the first time an in vivo genomic footprinting analysis of the human 11βHSD2 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 a set of nuclear protein factors that repress or induce 11βHSD2 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. 11βHSD2 transcripts levels ranged over 50 fold in cell lines originating from human kidney (HCD and HK-2), colon (SW620) or 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 11βHSD2 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 11βHSD2 gene. Each of these elements contains one or more homologous Sp1 binding motifs and indeed, was shown to bind Sp1 and Sp3 proteins with high affinity under in vitro conditions such as in EMSA and supershift assays (Fig. 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, since 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 11βHSD2 promoter (Table II). Similar cooperative regulation of neighbouring Sp1 binding sites has been reported previously (29).

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 11βHSD2 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 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 \textit{in vitro} does not necessarily reflect the transcriptional involvement and occupation of a binding site \textit{in vivo} (30, 31). The DMS \textit{in vivo} footprinting technique cannot be criticised, as can classical \textit{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 the 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 11\textbeta{}HSD2, thus reflecting an altered availability of these sequences for protein binding \textit{in vivo} (Fig. 3 and 4). Differential binding of Sp1 and Sp3 proteins to the 11\textbeta{}HSD2 promoter is also observed \textit{in vitro}, whereby the binding activity appears to correlate positively with 11\textbeta{}HSD2 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 cannot alone 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, 34, 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 11\textbeta{}HSD2 transcription repressing activity of Sp1 or Sp3 in our system, although we cannot rule out this possibility because our data for this were obtained \textit{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, 40, 41). iii. Post-translational modifications. Sp1 is known to be phosphorylated (42, 43, 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. iv. Interaction with methylated CpG sites. The 11βHSD2 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 11βHSD2 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 canine kidney cells MDCK (50) and Sp1 and Sp3 binding regulate the Na,K-ATPase α1 subunit in rats (51). Furthermore, rat type A natriuretic peptide receptor
human endothelial nitric oxide synthase (53) and thromboxane synthase, a vasoconstriction involved gene, have been linked to Sp1 activity (54).

Of potential interest for the understanding of the co-localization of 11βHSD2 and mineralocorticoid receptors is the observation of Zennaro et al. who showed Sp1 binding to defined sequences of the promoter of the human mineralocorticoid receptor (55). 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.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

FIG. 1. **Enzymatic activity of 11βHSD2 in selected human cell lines.** Equal numbers of HCD, HK-2, SW620, MCF-7 or SUT cells were lysed and measured for their endogenous 11βHSD2 activity. The oxidative activity was determined as the percent conversion of corticosterone into 11-dehydrocorticosterone at 30 minutes, 1 hour and 4 hours after addition of [3H]corticosterone, corticosterone and NAD. Control samples from each cell line were incubated for 4 hours in presence of Triton X-100 to abrogate 11βHSD2 activity (black bars). The data are shown as the means + S.D. from triplicate samples of a representative experiment.

FIG. 2. **Localization of LMPCR primer sets and EMSA probes to map 11βHSD2 regulatory regions.** The 5’ flanking region and parts of the first exon of the 11βHSD2 gene are indicated. Numbers refer to the position relative to the transcription initiation site of the kidney transcript (asterisk). Arrows indicate the relative positions of LMPCR primer sets PA, PB, PC, PD and PE used to analyse the top or the bottom strand, respectively. The locations of EMSA probes GSI, GSII, GSIII and GSIV are shown.

FIG. 3. **DMS in vivo footprints in different cell types visualized by LMPCR.** Intact SW620, MCF-7, HCD, HK-2 and SUT cells were treated in vivo with DMS and subsequently the genomic DNA was isolated. In parallel, deproteinated, naked DNA was methylated in vitro to obtain control DNA samples. Both in vitro and in vivo methylated DNA were treated with piperidine to introduce strand cleavage at methylated guanine or adenine residues. Resulting fragments were amplified by LMPCR and radioactively end-labelled using primer set PA. The
fragments were separated on a 5% sequencing gel and exposed to a Phosphor-Imager screen. Results were confirmed with at least two separate DNA preparations and three LMPCR reactions. *Numbers* indicate the nucleotide position relative to the 11βHSD2 transcriptional start site. Protected and hypermethylated nucleotides are marked by *open* or *filled arrowheads*, respectively. Areas indicative of protein-DNA interactions and regions of putative transcription factor binding are summarized as regions I and II (*boxed*).

**FIG. 4.** *Top strand analysis of regions I and II within the first exon of 11βHSD2.* LMPCR of the top strand using primer set PB. *A.* Comparison of SW620, MCF-7, HCD, HK-2 and SUT cells (lanes 2, 4, 5, 6, 7) treated with DMS *in vivo* to genomic DNA treated in the absence of proteins (SW620 and MCF-7, lanes 1 and 3). Experimental procedures were as described for Figure 3. *Open arrowhead:* protected nucleotide; *Filled arrowhead:* hypermethylated nucleotide; *B,* Histogram analysis of the PhosphorImager scan. Profiles correspond to the intensity of each represented guanine residue around region II.

**FIG. 5.** *In vivo footprinting data from the 5’ flank of the 11βHSD2 gene.* *A,* Bottom strand analysis from nucleotide -30 to -280 using primer set PC; *B,* Bottom strand analysis from -150 to +5 using primer set PD. Legends are as for Figure 3. SW620, MCF-7, HCD, HK-2 and SUT genomic DNA was treated in the absence (*in vitro*) and in the presence (*in vivo*) of nuclear proteins and footprints were visualized using LMPCR. *Open arrowhead:* protected nucleotide; *Filled arrowhead:* hypermethylated nucleotide.
FIG. 6. **11βHSD2 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 (nt -200). Experimental procedures were as described before.

FIG. 7. **Summary of DMS in vivo footprints on the 11βHSD2 gene.** Partial nucleotide sequences around the 11βHSD2 transcriptional start site are shown. Motifs with strong homology to known transcription factor binding sites are underlined. Protected nucleotides are marked by *open arrowheads* and hypermethylated nucleotides are specified by *filled arrowheads* as detected by DMS in vivo footprinting. Putative regulatory elements are summarized and designated regions I to IV (*boxed*).

FIG. 8. **In vitro binding activities of nuclear proteins on individual putative regulatory regions of the 11βHSD2 gene.** EMSA competition and supershift experiments were preformed using nuclear extracts from SW620, MCF-7, HCD, HK-2 and SUT cells in separate binding reactions. 35 fmol of radioactively labelled probe GSI containing sequence motifs of region I was assayed in the absence (-) or in the presence of a 100-fold molar excess of cold wtGSI probe or mutX probe as competitors. Complex formation was determined by migration retardation in non-denaturating 5% polyacrylamide gels and visualized by phosphorimaging. The positions of specific complexes (*C1, C2 and C3*) or non-specific bands (*ns*) are indicated. Supershift experiments were in the presence of Sp1 or Sp3 specific antibodies (*αSp1* or *αSp3*), combinations (*αSp1+αSp3*) or an unrelated anti-gelatinase antibody as non-specific control (*αNS*). Protein extracts obtained from SW620 nuclei were combined prior to the binding reaction with the respective antibodies for 10 minutes at 4°C.
FIG. 9. EMSA probe GSII binds nuclear factors Sp1 and Sp3. $^{32}$P labelled 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 or SUT cells. The position of specific binding (C5 and C6) and non-specific bands (ns) are indicated. A. The exact sequences of oligos used are indicated on the top. B. Antibodies have been included in the binding reactions as indicated. The location of complexes that reacted with $\alpha$Sp1 or $\alpha$Sp3 antibodies is referred to as supershifts. An anti-gelatinase antibody was used as control. Gel retardation assays were performed as described in “Experimental Procedures”.

FIG. 10. Scheme of 11βHSD2 deletion mutant reporter vectors. Schematic representation of deletion mutant reporter constructs. Parts of the 5'flanking sequence (white box) and the first exon (grey box) of the 11βHSD2 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 a cross. Roman numerals indicate GC-rich regions as described in the results. Graphic elements are as mentioned in Figure 2. Results from transient transfections with these vectors are given in Table II.

FIG. 11. EMSA analysis of Sp1 motifs in the upstream regions. EMSA assays with radiolabelled probe GSIII and nuclear extracts from various cell lines. A. Competition analyses were carried out with a 100-fold excess of unlabelled oligonucleotides as indicated on the top. The positions of specific (C7, C8 and C9) and non-specific (ns) bands are indicated. B.
Antibodies, included in the binding reactions and the location of supershifted complexes are shown (supershift). Anti-gelatinase antibody was used as non-specific control (αNS). Reaction conditions were as described in “Experimental Procedures”.
Table I. *Relative quantification of 11βHSD2 mRNA levels in different human cell types*

The relative amount of 11βHSD2 mRNA was detected by real-time quantitative RT-PCR as described in “Experimental Procedures”. The relative copy number was calculated according to the comparative Ct method, whereby the threshold cycle Ct corresponds to the PCR cycle number at which the amount of amplified template reaches a fixed threshold. PCRs were run in triplicates in separate tubes and the samples were normalized to ribosomal S18 rRNA as internal, endogenous control. The 11βHSD2 values were estimated in relation to SUT values.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Origin</th>
<th>11βHSD2 mean Ct</th>
<th>S18 rRNA mean Ct</th>
<th>11βHSD2 relative to SUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW620</td>
<td>colon</td>
<td>26.03±0.06</td>
<td>14.62±0.15</td>
<td>53.7 (48.1-60.0)</td>
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<tr>
<td>HCD</td>
<td>kidney, distal</td>
<td>27.98±0.06</td>
<td>14.98±0.06</td>
<td>17.8 (16.8-18.9)</td>
</tr>
<tr>
<td>HK-2</td>
<td>kidney, proximal</td>
<td>27.93±0.05</td>
<td>14.68±0.18</td>
<td>15.0 (13.2-17.0)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>breast</td>
<td>27.94±0.07</td>
<td>14.66±0.14</td>
<td>14.7 (13.2-16.5)</td>
</tr>
<tr>
<td>SUT</td>
<td>lung</td>
<td>31.98±0.19</td>
<td>14.82±0.18</td>
<td>1.0 (0.8-1.2)</td>
</tr>
</tbody>
</table>

\[^a\] 11βHSD2 values derive from the formula \(2^{-\Delta\Delta Ct}\), where \(\Delta\Delta Ct = \Delta Ct (= Ct 11\beta\text{-HSD2} - Ct S18)\) of the respective cell type minus \(\Delta Ct\) of the SUT cells.

\[^b\] The range given for 11βHSD2 relative to SUT was determined by calculating \(2^{-\Delta\Delta Ct \pm s}\) where \(s = \text{Standard Deviation (SD) of the difference of the Ct SD of both 11\beta\text{-HSD2 and S18}}\).

\[^c\] The results are representative of several experiments.
Table II. Relative activity of luciferase reporters under the control of 11βHSD2 promoter deletion mutants

A schematic diagram of the constructs is given in Figure 10. Different cell lines were transiently transfected with the respective reporter plasmid (0.8μg) along with a constitutive β-galactosidase expression vector (0.05μg) as internal control. The effect of deletions on the 11βHSD2 promoter activity is expressed as relative luciferase activity. For details see “Experimental Procedures”.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>p-400/+260 a:</th>
<th>p-400/+260ΔGCII:</th>
<th>p-400/+260ΔGCI b:</th>
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<td>SW620 1</td>
<td>0.17 ± 0.09</td>
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<td>MCF-7 1</td>
<td>0.21 ± 0.02</td>
<td>1.08 ± 0.12</td>
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<tr>
<td>HCD 1</td>
<td>0.34 ± 0.08</td>
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<tr>
<td>HK-2 1</td>
<td>0.14 ± 0.03</td>
<td>0.58 ± 0.09</td>
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<tr>
<td>SUT 1</td>
<td>0.27 ± 0.12</td>
<td>0.70 ± 0.01</td>
<td></td>
</tr>
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</table>

a The p-400/+260 values are normalized to 1.
b Results are relative to p-400/+260 and are the mean and standard error of the mean of at least three independent experiments each performed in triplicates.
LMPCR primer sets:

- PE
- PB

EMSA probes:

- GSIV
- GSIII
- GSII
- GSI
Nawrocki et al., Figure 3
A

<table>
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<tr>
<th></th>
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-57

* II

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+157

Nawrocki et al., Figure 4
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Nawrocki et al., Figure 5
wtGSI: CTTGGCCGTCGGCGGCGCCTGGCTGCTCGTGGCTGCC
mutX: -----------TTA-------------------------
A

\[
\begin{align*}
\text{wtGSII:} & \quad \text{CTCCCCGGGGCGCCCGGGCCCGCCCGGGCCCGGGCCCGGCCCAGCCC} \\
\text{mutA:} & \quad \text{-----------TTAA-----------------------} \\
\text{mutB:} & \quad \text{------------------------AA------------} \\
\text{mutC:} & \quad \text{-----------------------------AA-------} \\
\text{mutABCD:} & \quad \text{-----------TTAA----TT---TT---TT-------} \\
\text{mutCD:} & \quad \text{-------------------TT--------TT-------}
\end{align*}
\]

B

\[
\begin{align*}
\text{extract:} & \quad \text{none} \quad \text{SUT} \quad \text{HK-2} \quad \text{HCD} \quad \text{MCF-7} \quad \text{SW 620}
\end{align*}
\]
pGL3 | IV | III | II | I | LUC
---|---|---|---|---|---
-400 | +1 | +117 | +260 |  "*

- p-400/+260
- p-400/+260ΔGCI
- p-400/+260ΔGCI

11β HSD2 5’ sequence + exon1
wtGSIII: GTGAAGAAAGCCCCGCCCCCGCCCTCCCGCTC
mut.S: ----------------AA----AA----------
mut.N: -----------CC-------------------------
mut.SN: -----------CC----AA----AA----------

A

NfκB  Sp1  Sp1

comp:  / / / / / /  wGSIII  mutS  mutN  mutSN  control

C7
C8
C9
ns
ns
free probe

extract: none  SU  HK-2  HCD  MCF-7  SW620

B


supershift

C7
C8
C9
ns
ns
free probe

extract: MCF-7  SW620

Nawrocki et al., Figure 11
In vivo footprinting of the human 11beta-hydroxysteroid dehydrogenase type 2 promoter: Evidence for cell-specific regulation by Sp1 and Sp3
Andrea R. Nawrocki, Christopher E. Goldring, Radina M. Kostadinova, Felix J. Frey and Brigitte M. Frey

J. Biol. Chem. published online February 15, 2002

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