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Identification and Tissue Distribution of a Novel Variant of 11β-Hydroxysteroid Dehydrogenase 1 Transcript

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A novel variant of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) mRNA was identified from the ovine liver by reverse transcription–polymerase chain reaction (RT/PCR), and was named 11β-HSD1C mRNA. Sequence analysis of the RT–PCR product revealed that 11β-HSD1C mRNA was the product of an alternative exon-splicing within the 11β-HSD1 gene in which exon 5 was spliced out. Although it caused a deletion of 48 amino acids in the deduced 11β-HSD1 protein, this alternative splicing did not result in a shift within the predicted open reading frame of 11β-HSD1 cDNA. Thus, 11β-HSD1C mRNA was predicted to code for a protein of 244 amino acids. Using RT–PCR, we also examined the expression of 11β-HSD1C mRNA in ovine fetal organs and in maternal myometrium, endometrium, chorion, amnion and placenta. The 11β-HSD1C mRNA was expressed ubiquitously, similar to 11β-HSD1A mRNA, but at a lower abundance. Furthermore, since levels of 11β-HSD1C mRNA were directly related to those of 11β-HSD1A mRNA, there is no tissue-specificity for this shorter transcript and the only factor regulating its production appears to be 11β-HSD1A mRNA itself. To determine whether 11β-HSD1C mRNA encoded a functional enzyme, we inserted the cDNA into the expression vector pRc/CMV, and transfected the construct into Chinese hamster ovary cells. The transfected cells expressed a mRNA of expected size but contained no detectable 11β-HSD activity. When combined with cellular extracts of 11β-HSD1A cDNA transfected cells, they also did not alter either the dehydrogenase or reductase activity. The functional significance of the 11β-HSD1 transcript lacking exon 5 (11β-HSD1C mRNA) remains to be determined.

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INTRODUCTION

It is now widely accepted that at least two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 2) are present in mammals [1–6]. 11β-HSD1 is a reversible NADP(H)-dependent enzyme, and is expressed ubiquitously, with liver being the major site [7–13]. Although it has been proposed that 11β-HSD1 regulates the intracellular level of bioactive glucocorticoids, its precise physiological role in individual organs remains speculative [14]. The enzyme was first purified from the rat [15], and most recently from the mouse liver microsomes [16]. It is a glycoprotein with an apparent molecular weight of 34 kDa [15, 16]. The cDNAs encoding 11β-HSD1 have been cloned from a number of species, including rat [7], human [11], sheep [13], squirrel monkey [17] and mouse [18]. The gene for the human 11β-HSD1 has also been cloned, and consists of six exons [11]. Recently, a variant of 11β-HSD1 mRNA has been identified in kidneys of rat [19] and sheep [13]. It is a truncated form of 11β-HSD1 mRNA, and results from differential promoter usage by the 11β-HSD1 gene [20]. In order to make clear distinctions between these two forms of 11β-HSD1 mRNA, the original full length mRNA has been named 11β-HSD1A mRNA and the truncated form 11β-HSD1B mRNA [21]. However, the functional significance of 11β-HSD1B mRNA remains elusive as the expressed product in both CHO [22] and COS [23] cells is enzymatically inactive.

In a previous study, we have cloned the ovine 11β-HSD1 cDNA, and also identified 11β-HSD1B mRNA in the ovine kidney [13]. More recently, we have demonstrated the presence of 11β-HSD1 and 2

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activities in ovine liver and kidney, respectively [6]. In the present study, we identified a novel variant of 11β-HSD1 mRNA, 11β-HSD1C mRNA, and examined its expression in ovine fetal organs and in the maternal myometrium, endometrium, chorion, amnion and placenta. In addition, we expressed 11β-HSD1A and 11β-HSD1C cDNAs in CHO cells, and assessed 11β-HSD enzyme activity of the expressed proteins.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA modifying enzymes were purchased from Gibco/BRL (Burlington, Ontario) and Boehringer Mannheim (Laval, Quebec), and the eukaryotic expression vector pRc/CMV from Invitrogen (San Diego, CA). [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [1,2,6,7-^{3}H(N)]cortisol (70 Ci/mmol) were obtained from New England Nuclear. [1,2,6,7-^{3}H(N)]cortisone was prepared from [1,2,6,7-^{3}H(N)]cortisol in our laboratory as described previously [6].

Steroid standards were from Steraloids (Wilton, NH). Cofactors (NAD, NADH, NADP and NADPH) were from Sigma Chemicals Co (St Louis, MO). CHO cells were from American Type Culture Collection (Rockville, MD). Oligonucleotides were synthesized using a Pharmacia Gene Assembler and purified using NAP-5 columns (Pharmacia Canada) according to the manufacturer's instructions. Random Primed DNA Labeling Kit and T7 Sequencing™ Kit were purchased from Pharmacia, and Superscript™ Preamplification System from Gibco/BRL.

Cloning of 11β-HSD1C cDNA

11β-HSD1C cDNA was initially identified by a standard RT–PCR as described below using total hepatic RNA extracted from a pregnant sheep as the template. The RT–PCR product was cloned into pBluescript KS (Stratagene Cloning Systems, La Jolla, CA). Double-stranded plasmid DNA was obtained and sequenced using the dideoxy-chain termination method [24].

RT–PCR analysis of 11β-HSD1C mRNA expression

To determine tissue distribution of 11β-HSD1C mRNA, total cellular RNA was extracted by the lithium chloride/urea method [25] from fetal liver, lung, kidney, heart, pituitary, adrenal, thyroid, large intestine and skeleton muscle, and also from maternal myometrium, endometrium, chorion, amnion and placentome. These tissues were collected from a pregnant ewe and its fetus at day 140 of pregnancy (term = 145 days). Because of its extremely low abundance, the expression of 11β-HSD1C mRNA in the fetal and maternal tissues was determined by Southern blot analysis of the RT–PCR products using total tissue RNA as template. Single-stranded cDNA was synthesized by reverse transcription (RT) of 1 μg total RNA with oligo(dT)$_{12-18}$ as a primer in a total volume of 20 μl using the Superscript Preamplification System. To generate double-stranded 11β-HSD1A and 11β-HSD1C cDNAs, 2 μl of the single-stranded cDNA pools was used in PCR. The PCR tube (100 μl) also contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol/primer and 2.5 U TaqDNA polymerase. The two primers used were two oligonucleotides corresponding to the immediate 5′ (5′-GGAA AAGCTT CATCCCTGTC TGATGGCT) and 3′ (5′-GGAA TCTAGA CCAAGCTCCC TTTATGAT) non-coding portions of the ovine 11β-HSD1 cDNA [13], and each containing *Hind*111 or *Xbal* restriction site (underlined). The PCR conditions were 30 cycles (94°C, 45 s; 60°C, 45 s; 72°C, 2 min) with an initial denaturation of 2 min at 94°C and a final extension of 7 min at 72°C.

In order to demonstrate the presence of 11β-HSD1C cDNA, an aliquot (10 μl) of the RT–PCR products was subjected to a standard Southern blot analysis as described previously [13]. The blot was probed sequentially with [^{32}P]-full length 11β-HSD1 cDNA and an antisense oligonucleotide probe complementary to bases 671–715 within the published oving 11β-HSD1 cDNA [13]. The ovine 11β-HSD1 cDNA [13] was labelled with [^{32}P]dCTP by random priming [26], and the oligonucleotide which was confined to exon 5 of the 11β-HSD1 gene [11] was end-labelled with [^{32}P]ATP as described previously [27]. The underlying rationale was that the cDNA probe should hybridize with both 11β-HSD1A and 1C cDNAs, while the oligo probe should only hybridize with 11β-HSD1A cDNA but not with 11β-HSD1C cDNA in which exon 5 was absent.

To assess the abundance of 11β-HSD1C mRNA relative to that of 11β-HSD1A mRNA, autoradiographic signals on the X-ray film were analyzed using a computerized image analysis system (Imaging Research, St Catharines, Ontario). For each RNA sample, the ratio of 11β-HSD1C mRNA signal to 11β-HSD1A mRNA signal was calculated, and expressed as a percentage.

Construction of expression vectors

11β-HSD1A cDNA was obtained by a standard PCR using the above two primers and the full-length ovine 11β-HSD1 cDNA as a template. The 11β-HSD1A cDNA and 11β-HSD1C cDNA in pBluescript KS were digested with *Hind*111 and *Xbal*, gel purified and subcloned into the eukaryotic expression vector pRc/CMV. Both cDNA constructs were fully sequenced to confirm their identity.

Expression in mammalian cells

CHO cells were cultured in T25 flasks in MEM supplemented with 10% fetal calf serum and penicillin-streptomycin 24 h prior to transfection.

Transfection was accomplished by incubating the cells in the presence of plasmid DNA (10 μg/ml), polybreme (12.5 μg/ml) and DMSO (15%). After 40 h incubation, the cells were passed into 100 × 20 mm dishes. Positive colonies were selected by using G-418 (1 mg/ml), and cultured for 14 days. For RNA analysis, the extraction buffer (3 ml) was added directly to the cells which were then collected and stored at −70°C until use. For enzyme activity assays and Western blot analysis, cells were harvested by trypsinization, and the cell pellets stored at −70°C.

Northern blot analysis of 11β-HSD1 mRNA produced by the transfected CHO cells

To determine whether the transfected cells produced 11β-HSD1 mRNA of the expected size, total cellular RNA was extracted and subjected to Northern blot analysis as described previously [13]. Briefly, denatured total RNA samples (20 μg) were subjected to agarose gel (1%) electrophoresis in the presence of formaldehyde, and transferred overnight by capillary blotting to a Zeta-Probe membrane (Bio-Rad Canada Ltd., Mississauga, Ontario). The RNA was fixed by UV cross-linking (Gene Cross-Linker, Bio-Rad) to the membrane which was then baked under vacuum at 80°C for 60 min. The blot was hybridized at 42°C for 16 h in the presence of formamide (50%) and [^{32}P]sheep 11β-HSD1 cDNA. The ovine liver 11β-HSD1 cDNA [13] was labelled with [^{32}P]dCTP by random priming [26]. We used a cDNA for mouse 18S rRNA as an internal control for gel loading and efficiency of RNA transfer. The same blot was stripped and reprobed with the radiolabelled 18S rRNA cDNA, as described previously [13, 28].

Western blot analysis of 11β-HSD protein produced by the transfected CHO cells

Western blot analysis was used to ascertain the presence of immunoreactive 11β-HSD1 protein in the transfected cells. Ten micrograms of the cell homogenates (protein concentrations were determined by the Bradford Method using a Bio-Rad protein assay kit with BSA as standard) were subjected to a standard 12% SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. 11β-HSD1 protein on the nitrocellulose filter was detected using a Bio-Rad Immun-Blot Assay Kit following the manufacturer's instructions. Briefly, the nitrocellulose filter was blocked with 5% Blotto in TTBS (0.05% Tween-20 in TBS) for 1 h at room temperature, and incubated with the affinity-purified primary antibody (diluted 1:200 in blocking solution) for 2 h at room temperature. The primary antibody was generated from rabbits using a synthetic peptide (Phe-Leu-Ser-Leu-Lys-Lys-Tyr-Asn-Met-Glu-Arg-Phe-Ile-Asn) corresponding to residues 278–291 of the predicted ovine 11β-HSD1 protein [13]. This synthetic peptide is encoded entirely by sequences corresponding to exon 6 of the human 11β-HSD1 gene [11]. The crude antiserum was purified by affinity chromatography using a modified method of Weigel *et al.* [29]. After 3 × 5 min washes with TTBS, the filter was incubated with Protein A-HRP conjugate solution for 1 h at room temperature, and developed in AP colour developing solution for 5–10 min.

Assay of 11β-HSD activity in the transfected CHO cell homogenates

To determine whether 11β-HSD1 protein produced by the transfected CHO cells was functional with respect to enzymatic activity, 11β-HSD activity in CHO cell homogenates was determined by a radiometric conversion assay as described previously [6, 30]. Both dehydrogenase and reductase activities were assessed. For dehydrogenase assay, homogenate (100 μg) was added to the assay mix containing approx. 100,000 cpm of the labelled cortisol, 0.2 μM non-radioactive cortisol, and 250 μM cofactor (NAD or NADP). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 8 vol of ethyl acetate. Steroids were extracted and analyzed by TLC as described. Percent conversion of the labelled cortisol to cortisone was calculated by dividing the dpm in the cortisone band from that of the total in the cortisol and cortisone combined bands, and the data were expressed as percent conversion per 30 min per cell homogenates containing 100 μg protein. The reductase activity was determined similarly except that cortisone was used as substrate, and NADH or NADPH as cofactor.

RESULTS

Sheep 11β-HSD1C cDNA sequence

Sequence analysis of 11β-HSD1C cDNA revealed that it was identical to the published ovine 11β-HSD1 cDNA except that 11β-HSD1C cDNA lacked bases 584–728. This missing region corresponded to exon 5 of the human 11β-HSD1 gene (unpublished data from the author's laboratory indicate a similar gene structure in the sheep), indicating that 11β-HSD1C mRNA was the product of an alternative exon splicing within the 11β-HSD1 gene in which exon 5 was spliced out (the entire exon was spliced out at the intron/exon boundary). When aligned with the published 11β-HSD1 cDNA, it was clear that this alternative splicing did not result in a shift within the predicted open reading frame of 11β-HSD1 cDNA. Thus, 11β-HSD1C cDNA contains a 735 bp open reading frame that encodes a protein of 244 amino acids. The deduced ovine 11β-HSD1C polypeptide has a molecular weight of 27 kDa.

Tissue distribution of 11β-HSD1C mRNA

The tissue distribution of 11β-HSD1C mRNA, as reflected by the presence of 11β-HSD1C cDNA, is shown in Fig. 1. As expected, the full length 11β-

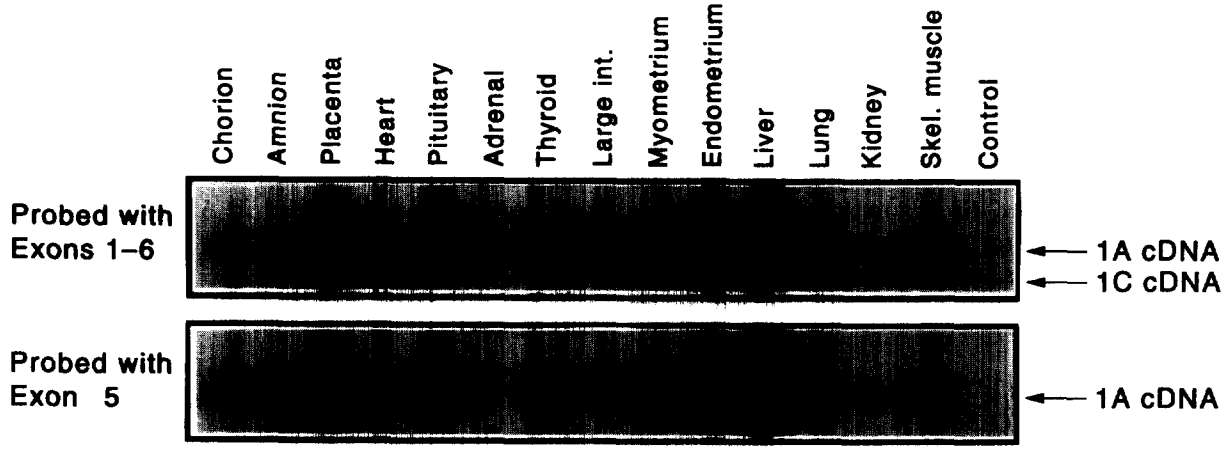

Fig. 1. Tissue distribution of 11β-HSD1C mRNA as reflected by the presence of 11β-HSD1C cDNA. Total RNA samples (1 μg) from various tissues were subjected to RT–PCR as described in the Materials and Methods. A fraction of the RT–PCR products was analyzed by Southern blotting. The top panel is an autoradiograph of the blot probed with [^{32}P]sheep 11β-HSD1 cDNA (1 h exposure time). The same blot was stripped and rehybridized with [^{32}P]sheep 11β-HSD1 oligonucleotide complementary to a portion of exon 5 sequence within the 11β-HSD1 gene (bottom panel; exposure time 1 h). To assess the abundance of 11β-HSD1C mRNA relative to that of 11β-HSD1A mRNA, autoradiographic signals shown in the top panel were analyzed as described in the Materials and Methods.

HSD1 cDNA probe hybridized with both 11β-HSD1 and 1C cDNAs while the exon 5 specific oligonucleotide probe only recognized 11β-HSD1A cDNA. This, therefore, not only demonstrated the presence of 11β-HSD1C mRNA in the ovine fetal organs and in the maternal myometrium, endometrium, chorion, amnion and placenta but also confirmed that the 11β-HSD1C mRNA produced by these tissues does not contain exon 5. As indicated by the presence of 11β-HSD1C cDNA, 11β-HSD1C mRNA was expressed ubiquitously, similar to 11β-HSD1A mRNA. Furthermore, the relative abundance of 11β-HSD1C mRNA always paralleled that of 11β-HSD1A mRNA (mean ± SEM, 30.0 ± 1.5%; Fig. 1).

Expression of 11β-HSD1 gene in CHO cells

As shown in Fig. 2, CHO cells transfected with 11β-HSD1A cDNA produced an identical sized 11β-HSD1 mRNA to that in the liver. As expected, a smaller size 11β-HSD1 mRNA was detected in the cells transfected with 11β-HSD1C cDNA, and no 11β-HSD1 mRNA was detectable in CHO cells transfected with the vector alone (Fig. 2). The efficiency of transfection as reflected in the 11β-HSD1 mRNA

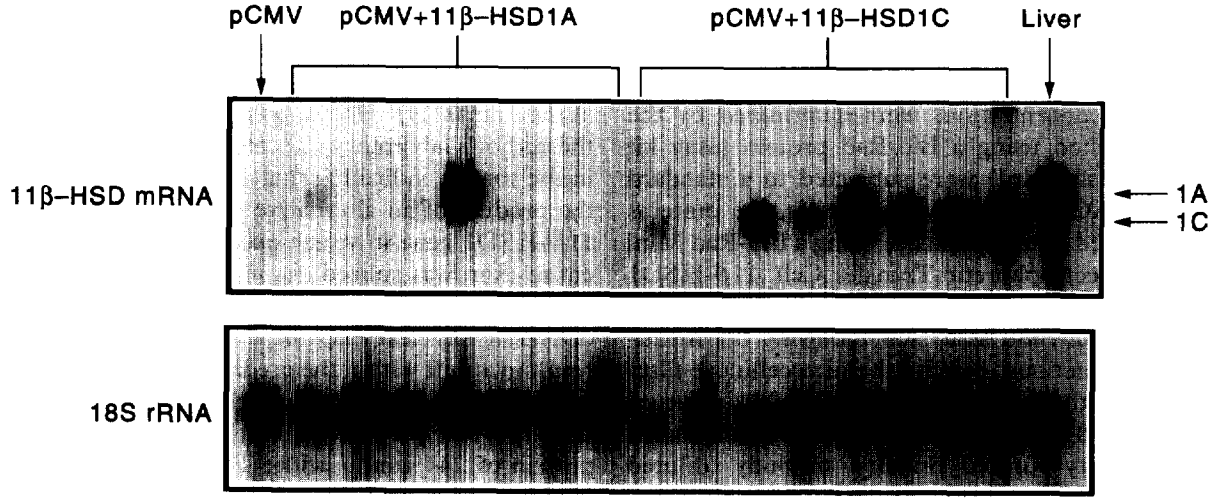

Fig. 2. Northern blot analysis of 11β-HSD1 mRNA in CHO cells transfected with 11β-HSD1A cDNA, 11β-HSD1C cDNA, or the vector alone. Total RNA samples (20 μg) were analyzed, and the top panel is an autoradiograph of the blot probed with [^{32}P]sheep 11β-HSD1 cDNA (20 h exposure time). As a control, the same blot was stripped and rehybridized with [^{32}P]mouse 18S rRNA cDNA (bottom panel; exposure time 2 h). Colony # 4 (lane 5) of 11β-HSD1A cDNA transfected and colonies # 5–8 (lanes 13–16) of 11β-HSD1C cDNA transfected were chosen for further analysis.

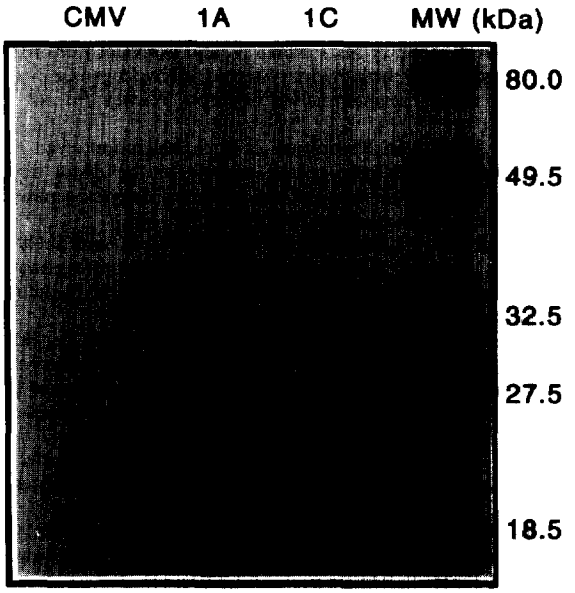

Fig. 3. Western blot analysis of 11β-HSD1 protein in homogenates from CHO cells transfected with 11β-HSD1A cDNA, 11β-HSD1C cDNA, or the vector alone. 10 μg of cell homogenates were subjected to Western blot analysis as described in the Materials and Methods. One representative blot is shown, and a total of four blots each with a different cell homogenate derived from the selected four colonies transfected with 11β-HSD1C cDNA, which were shown to contain the highest levels of 11β-HSD1C mRNA, were analyzed.

abundance differed greatly among different colonies transfected with the same 11β-HSD1 cDNA construct. Hence, the colonies expressing the highest levels of 11β-HSD1A mRNA (one colony) and 11β-HSD1C mRNA (four colonies) were chosen for the subsequent Western blot and enzyme activity analyses.

Western blot analysis of 11β-HSD1 protein

Western blot analysis using an ovine-specific antibody demonstrated the presence of a 34 kDa immunoreactive protein in homogenates from CHO cells transfected with 11β-HSD1A cDNA. However, there was no detectable immunoreactive 11β-HSD1 protein in 11β-HSD1C cDNA transfected cell homogenates (Fig. 3).

11β-HSD activity in the transfected CHO cells

Homogenates from CHO cells transfected with 11β-HSD1A cDNA displayed similar levels of 11β-HSD dehydrogenase and reductase activities which were clearly NADP(H)-dependent (Fig. 4). By contrast, no 11β-HSD activity could be detected in either direction in homogenates prepared from 11β-HSD1C cDNA transfected cells. Furthermore, when equal amounts of homogenates from 11β-HSD1A cDNA and 11β-HSD1C cDNA transfected cells were incubated together, the rate of conversion in both directions was similar to that obtained when homogenates from CHO cells transfected with 11β-HSD1A cDNA alone was used (Fig. 4). This indicated that the activity of 11β-HSD1C was not dependent on formation of a heterodimer with 11β-HSD1A protein.

DISCUSSION

The present results demonstrate, for the first time, the existence of another variant of 11β-HSD1 mRNA lacking exon 5, 11β-HSD1C mRNA. Evidence has also been presented to show that 11β-HSD1C mRNA, like 11β-HSD1A mRNA [13], is expressed ubiquitously in ovine fetal organs and in maternal myometrium, endometrium, chorion, amnion and placenta, but always at a lower abundance. Thus, there is no tissue-specificity for this shorter transcript, and the only factor regulating its production appears to be 11β-HSD1A mRNA itself. However, the functional significance of 11β-HSD1C mRNA remains obscure since *in vitro* transfection studies using CHO cells failed to demonstrate 11β-HSD enzyme activity. It is noteworthy that no immunoreactive 11β-HSD1C protein was detected by Western blot analysis. The precise reasons for this are not apparent, although it remains possible that

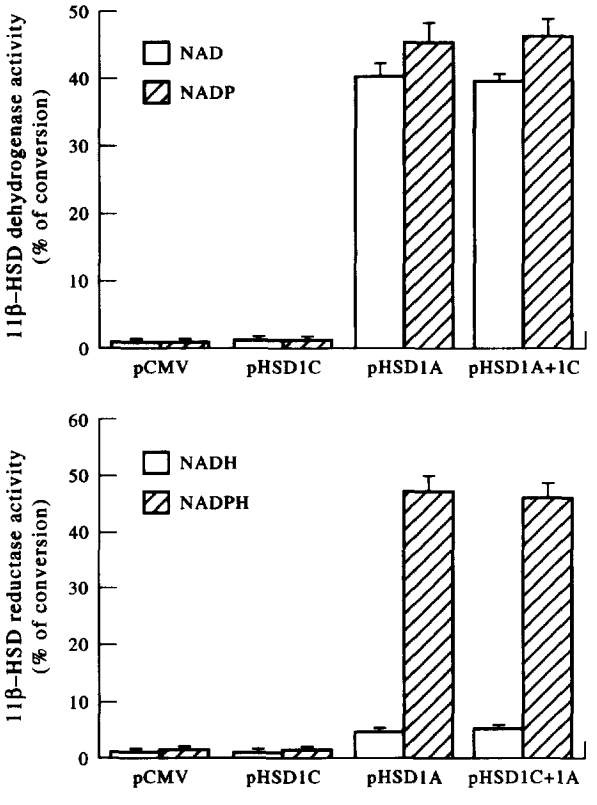

Fig. 4. 11β-HSD dehydrogenase (top panel) and reductase (bottom panel) activities in homogenates from CHO cells transfected with 11β-HSD1A cDNA, 11β-HSD1C cDNA, or the vector alone. Cell homogenates were incubated with 200 nM of cortisol in presence of NAD/NADP (for dehydrogenase) or with 200 nM of cortisone in the presence of NADH/NADPH (reductase) as described in Materials and Methods. Each bar represents the mean ± SEM of the four separate assays with homogenates from the same colony (transfected with 11β-HSD1A cDNA and the vector alone) or from the four different colonies (transfected with 11β-HSD1C cDNA).

11β-HSD1C mRNA has not been successfully translated. We feel that this may be unlikely since under identical experimental conditions, 11β-HSD1A was successfully translated and was shown to be functional with respect to the enzymatic activities. Furthermore, there is no shift in the open reading frame in 11β-HSD1C cDNA lacking exon 5, and the translational start and stop codons are intact. In addition, the whole construct of 11β-HSD1C cDNA was sequenced to verify that there were no sequence abnormalities. It is therefore more likely that the lack of the immunoreactive protein may represent decreased intracellular stability or poor recognition by the antiserum, perhaps due to abnormal conformation or lack of glycosylation. It is interesting to note that a much lower level of the immunoreactive protein was reported previously in CHO cells transfected with 11β-HSD1B cDNA lacking exon 1 [22]. It is also noteworthy that there was abundant 11β-HSD1B mRNA in the rat kidney but the truncated protein was undetectable, though the transfected COS cells did show a truncated protein [23]. This suggests that protein stability, at least in the case of 11β-HSD1B, may be cell specific.

It has been proposed that 11β-HSD regulates the bioavailability of glucocorticoids in target organs [12, 31, 32]. Multiple forms of 11β-HSD may carry out distinct functions in a tissue-specific manner [14]. Although like 11β-HSD1B mRNA (lacking exon 1) identified previously from kidney [22, 23], the newly-identified 11β-HSD1C mRNA (lacking exon 5) encodes a protein that is enzymatically inactive in CHO cells. It should be noted that in the rat 11β-HSD1B mRNA is only expressed in kidney, and in quantities comparable to that of 11β-HSD1A mRNA. In contrast, 11β-HSD1C mRNA is present in lower amounts everywhere 11β-HSD1A mRNA is expressed. We also addressed the question of whether the activity of 11β-HSD1C protein could depend on the presence of 11β-HSD1A protein by co-incubation. We found that this was unlikely because levels of both dehydrogenase and reductase activities were not altered.

Site-directed mutagenesis of the rat 11β-HSD1A cDNA has indicated that Tyr-179 and Lys-183, both of which are encoded by sequences within exon 5, are essential for the catalytic function of this enzyme [33]. The lack of enzymatic activity of the expressed product of 11β-HSD1C cDNA lacking exon 5 re-enforces this notion. It has also been suggested that the protein encoded by 11β-HSD1B mRNA (lacking exon 1) may function as a binding protein in glucocorticoid target organs [22]. It remains possible that 11β-HSD1C may function in a similar capacity.

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