Transcription Activation of Mouse Mammary Tumor Virus–Chloramphenicol Acetyltransferase: A Model To Study the Metabolism of Cortisol†

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ABSTRACT: The human 11β-hydroxysteroid dehydrogenase (h11β-HSD) inactivates the active corticosteroid cortisol to its inactive metabolite cortisone. We have developed transactivation analyses of the reporter chimeric gene mouse mammary tumor virus–chloramphenicol acetyltransferase (MMTV-CAT) to study the catalytic activity of h11β-HSD introduced by cotransfection into receptor and 11β-HSD deficient CV-1 cells. Assay of 11β-HSD expressed in CV-1 cells by cotransfection showed that the catalyzed dehydrogenation of cortisol to cortisone was 2-fold higher in the presence of NADP. The reductase activity was dependent on the coenzyme NADPH. The addition of increasing concentrations of the inhibitor carbenoxolone (CBX) in the incubates blocked the enzyme activity in a dose dependent fashion. In CV-1 cells cotransfected with expression vectors of either human glucocorticoid (hGR_{1-777}) or mineralocorticoid (hMR_{1-984}) and the reporter plasmid MMTV-CAT, dexamethasone (DEX), aldosterone (ALDO), cortisol, and corticosterone induction of CAT activity was dose dependent. Cotransfection of CV-1 cells transfected with 10 μg of 11β-HSD expression vector reduced the transactivation of MMTV-CAT by hGR or hMR in the presence of either cortisol or corticosterone to basal values. The concomitant addition of 100 nM cortisone and 1 μM NADPH to these transfectants elevated CAT activity. These data show that transactivation analyses can be used to study the 11β-HSD-catalyzed regulation of corticosteroid levels, which triggers physiological processes and in certain cases provides an alternative to animal experimentation.

The human glucocorticoid receptor (hGR[1]), mineralocorticoid receptor (hMR), and other members of the nuclear receptor superfamily are ligand-regulated, intracellular transcription factors that share common structural similarities. The discrete functional domains of these large proteins emerged from sequence alignments, deletion studies, site-directed mutagenesis, and domain swap experiments (Evans, 1988; Green & Chambon, 1988). The hMR has much in common with its counterpart, hGR (Hollenberg et al., 1985; Arriza et al., 1987). Previous studies demonstrated a high affinity of both receptors for cortisol, corticosterone, deoxycorticosterone, and the semisynthetic glucocorticoids dexamethasone, and triamcinolone acetonide (Arriza et al., 1988; Govindan et al., 1991). The hMR has a greater affinity for aldosterone (ALDO) than the hGR (Birmingham et al., 1979; Krozowski & Funder, 1983; Shepperd & Funder, 1987). However, *in vitro* studies show a loss of specificity of the receptor in its inability to distinguish between aldosterone and other potent glucocorticoids (Emadian et al., 1989; Luttge et al., 1989). To confer the ALDO selectivity of hMR, the biologically active cortisol is inactivated by the 11β-hydroxysteroid dehydrogenase (11β-HSD) system to cortisone in the target cells (Bush et al., 1968; Lakshmi & Monder, 1985a). Such a protective mechanism was first suggested by Ulick et al., who studied the physiopathology of the "syndrome of apparent mineralocorticoids excess", a congenital disorder characterized by a deficient 11β-HSD system (Ulick et al., 1979; Stewart et al., 1988). cDNA clones encoding both human and rat 11-HSD have been isolated recently, and their enzymatic activity was expressed in Chinese hamster ovary cells (CHO) by gene transfer (Agarwal et al., 1989; Tannin et al., 1991). These studies suggest that 11β-dehydrogenation and 11-oxoreduction are catalyzed by a single enzyme with dual activity.

In this study, we have examined the functional significance of 11β-HSD and corticosteroid receptor dependent transcription activation of a chimeric responsive gene by transient transfection. We have established a model to study the expression and specificity of human 11β-HSD in CV-1 cells by cotransfection with the 11β-HSD expression vector in the presence of either hGR or hMR expression vector and MMTV-CAT reporter plasmid and by treatment of the transfectants with a number of agonists. We have further defined the role of 11β-HSD in transcription enhancement mediated by both hGR and hMR by inhibiting the enzyme activity with carbenoxolone (CBX), a synthetic derivative of glycyrrhetinic acid (Stewart et al., 1987).

MATERIALS AND METHODS

Receptor Expression Vectors and Reporter Plasmids. hGR (Govindan et al., 1985; Govindan, 1990; Leclerc et al., 1991) and hMR (Govindan et al., 1991) cDNAs were subcloned into the *Eco*RI (hGR) or *Hin*dIII–*Eco*RV (hMR) site of the eukaryotic expression vector pcDNA 1 (Invitrogen, San Diego) to yield hGR_{1-777} and hMR_{1-984}, respectively. Construction of MMTV-CAT reporter plasmid was as described previously (Govindan, 1990; Govindan et al., 1991).

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[1] Abbreviations: dexamethasone or DEX, 9a-fluoro-16a-methyl-11β, 17a,21-trihydroxy-1,4-pregnadien-3,20-dione; triamcinolone acetonide or TA, 9a-fluoro-11β,16a,17a,21-tetrahydroxy-1,4-pregnadien-3,20-dione 16,17-acetonide; ALDO, aldosterone; RU 486, 11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17a-(prop-1-ynyl)estra-4,9-dien-3-one (generic name, mifepristone); spironolactone, 7-(acetylthio)-17-hydroxy-3-oxo-pregn-4-ene-21-carboxylic acid γ-lactone; carbenoxolone or CBX, 3-(3-carboxy-1-oxopropoxy)-11-oxoolean-12-en-29-oic acid; hGR, human glucocorticoid receptor; hMR, human mineralocorticoid receptor; MMTV, mouse mammary tumor virus; CAT, bacterial chloramphenicol acetyltransferase; h11β-HSD, human 11β-hydroxysteroid dehydrogenase.

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Cell Culture and Transient Transfection. CV-1 cells were obtained from American Type Culture Collection. Cells were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide and air and grown in minimum essential medium (MEM) supplemented with 2% glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL gentamycin, and 5 μg/mL fungizone. The cells, grown to 70–80% confluency in 250 mL culture flasks, were collected by mild trypsinization, and 1.5×10^6 cells/100 mm Petri dish were plated. Two hours prior to transfection, the medium from the plates was aspirated, and 4 mL of DMEM supplemented with 10% FBS was added. Calcium phosphate-precipitated DNA (960 μL), 2.5 μg of receptor expression vectors (hGR or hMR), 2.5 μg of MMTV-CAT reporter plasmid, and 5 μg of CH110, the β-galactosidase expression plasmid used for standardization of transfection (Pharmacia, Montréal, Canada), were added dropwise to the medium (Herbommel et al., 1984). The calcium phosphate precipitate was left in contact with the cells for 12 h. Then, 2 mL of 15% glycerol in Hepes-buffered saline (HBS) was added to the medium and gently mixed. This step was found to be crucial for an efficient transfection and to quantitatively remove the calcium phosphate precipitate. After 3–5 min, the medium was removed by aspiration, the cells were washed twice with 4 mL of Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 140 mM KCl (TBS), and 4 mL of MEM supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) was added to the transfectants. Hormones were added as indicated from a 1000× concentrated stock solution in ethanol, and the incubation was continued for an additional 24 h.

The following day, the medium from the Petri dishes was collected in 15 mL plastic centrifuge tubes (Falcon), and the cells were washed twice with 4 mL of phosphate-buffered saline (PBS). Following brief trypsinization, the enzyme was blocked by the addition of the medium collected from each Petri dish. The cells were collected by centrifugation at 1500*g* for 10 min at room temperature, suspended in 500 μL of PBS, transferred into 1.5 mL reaction tubes, and centrifuged at 2500*g* for 5 min to collect the cells. The PBS was removed by aspiration, and the cells were resuspended in 100 μL of 0.25 M Tris-HCl (pH 7.8) containing 0.05 mM phenylmethanesulfonyl fluoride (PMSF). The cells were lysed by three cycles of freeze–thaw, and the cell extracts were collected by centrifugation at 15000*g* for 5 min at room temperature. The transfection efficiency was measured by determining the β-galactosidase activity in 10 μL of the cell extract (Herbommel et al., 1984). Extracts containing 10 units of β-galactosidase activity were used in assaying the CAT activity (Gorman et al., 1982). The results of three independent experiments varying by less than 5% were considered for evaluation.

Human 11β-HSD Expression Vectors. The human 11β-HSD cDNA was a gift from Dr. P. C. White (Cornell University Medical Center, New York). The cDNA was excised with *Bam*HI (3′ end) and *Hin*dIII (5′ end) and inserted into M13mp18 and -19 at the *Bam*HI and *Hin*dIII sites. The sequence of the cDNA was determined and found to contain a 79 nucleotide 5′ nontranslated region and codons for 292 amino acids (Tannin et al., 1991). We have deleted the internal *Eco*RI site between amino acid codons 26 and 27 by oligonucleotide-directed mutagenesis with the synthetic oligonucleotide 5′-CGAGGAGTTCAGACC-3′ and single-stranded DNA without changing the codons. The entire cDNA was sequenced using single-stranded DNA as a template and appropriate synthetic oligonucleotide primers with the Sequenase sequencing kit (version 2) from United States Biochemical (Cleveland, Ohio). The cDNA was then inserted at the *Eco*RI site of pcDNA 1 (Invitrogen) in both orientations. Plasmid DNAs for transfection were purified twice by CsCl gradient centrifugation.

Assay of 11β-HSD Activity. The CV-1 cells and 11β-HSD transfectants (transfected with 20 μg of 11β-HSD per 250 mL flask) were grown in MEM as described above. The 11β-HSD activity was assayed as described by Quirk et al. (1990) with minor modifications (Pagé et al., 1994a,b).

Synthesis of [^{3}H]Cortisone. Synthesis of [^{3}H]cortisone was performed with 11β-HSD-transfected CV-1 cells (5×10^6) in a final volume of 1 mL, with 50 μL of radioactive cortisol (specific activity, 250 GBq/mmol). The synthesis was as previously described (Pagé et al., 1994a).

Cell Fractionation and Extraction of Nuclei. Cells were concentrated by centrifugation and resuspended in complete medium. After incubation with or without the addition of steroids for 30 min at 37 °C, the cells were harvested by centrifugation and washed in cold 20 mM potassium phosphate (pH 7.4 at 20 °C) containing 250 mM sucrose and 1 mM EDTA. Cells were lysed by two cycles of freeze–thaw in the above buffer, with the addition of 1.5 mM $MgCl_2$ and $CaCl_2$. Centrifugation at 5000*g* separated the crude nuclear and cytosolic fractions. The cytosol was cleared by centrifugation at 105000*g* in a Beckman TL-100 Ultracentrifuge TLA-100L rotor. The nuclear fraction was washed three times with the above buffer and then extracted with buffer containing 300 mM KCl for 30 min at 20 °C with occasional shaking. Following centrifugation, the protein concentrations and hormone binding activities were determined in an aliquot of the clear supernatant.

RESULTS

11β-HSD Activity in CV-1 Cells. The conversions of cortisol to cortisone and of cortisone to cortisol were assayed with nontransfected CV-1 cells, cotransfected CV-1 cells, the control pcDNA 1, and the pcDNA 1 containing the 11β-HSD insert in reverse orientation to the CMV (cytomegalovirus) promoter controls to establish the basal levels. The 11β-HSD activity was detectable only with the pcDNA-11β-HSD in the correct orientation in cotransfected CV-1 cells. All controls showed undetectable levels of 11β-HSD activity. The addition of 1 μM NAD or 1 μM NADP to the cells cotransfected with 11β-HSD expression plasmid revealed that the metabolic activity of the conversion of cortisol to cortisone was almost 2-fold higher in the presence of NADP as coenzyme (Figure 1). Conversion of cortisone to cortisol was effective in the presence of NADPH.

11β-HSD Inhibition by Carbenoxolone. To study the effect of 11β-HSD inhibition, we used CBX, a synthetic derivative of glycyrrhetinic acid. CBX, a known potent inhibitor of 11β-HSD both *in vivo* and *in vitro*, was used in this series of experiments to block the effect of 11β-HSD activity (Funder et al., 1990). Increasing concentrations of CBX inhibited the enzymatic activity in the incubates in a dose dependent fashion (Figure 2). CBX (1 μM) blocked the conversion of cortisol and corticosterone almost completely.

Transcriptional Regulation of MMTV-CAT by hGR_{1-777} and hMR_{1-984} with Various Steroids. CV-1 cells were transfected with MMTV-CAT and receptor expression vectors as described in Materials and Methods. Treatment of hGR_{1-777}-cotransfected CV-1 cells with DEX produced dose dependent transactivation of MMTV-CAT (Figure 3A), and maximal induction of MMTV-CAT was achieved at 10 nM

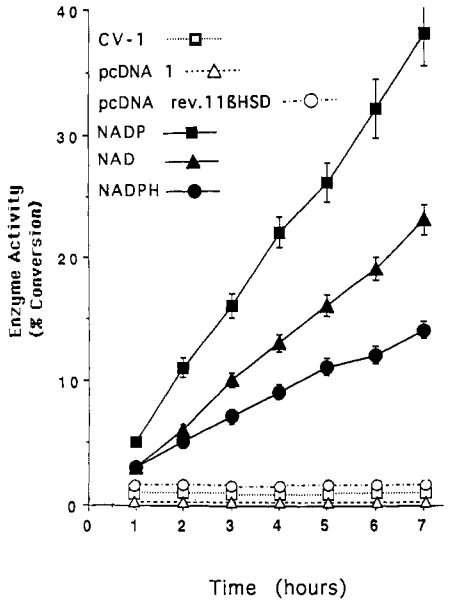

FIGURE 1: Expression of 11β-HSD in transfected CV-1 cells. CV-1 cells were transfected with control pcDNA 1 vector and pcDNA 1 containing h11β-HSD cDNA inserted in the reverse orientation, and 11β-HSD activity was measured as described in Materials and Methods. For the conversion of cortisol to cortisone, the enzyme activity was determined in cells transfected with h11β-HSD cDNA in the correct orientation in the presence of coenzymes NADP and NAD. The 11-oxoreductase activity was determined in cell extracts with radioactive cortisone and NADPH. The triplicates varied by less than 5%.

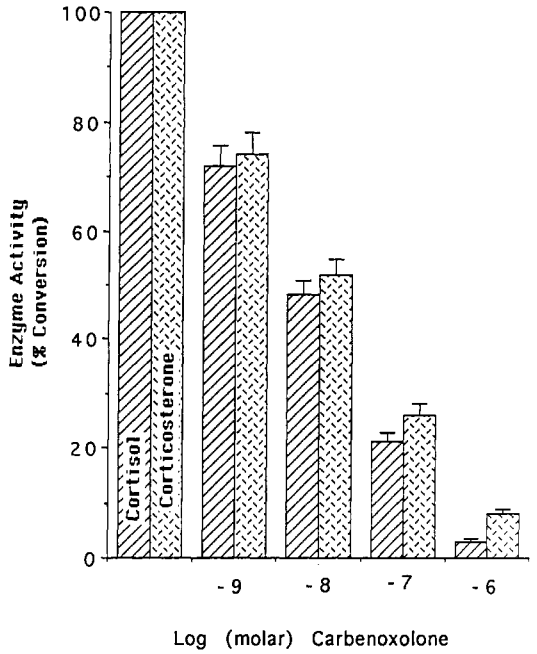

FIGURE 2: Inhibition of 11β-oxidation of overexpressed 11β-HSD in CV-1 cells. Aliquots of cell extracts transfected with h11β-HSD were incubated with [^{3}H]cortisol and [^{3}H]corticosterone in the presence of NADP and in the presence and absence of increasing concentrations of CBX at 37 °C for 3 h. The steroids were extracted with ethyl acetate, and the % conversion with respect to the control in the absence of CBX was determined. The variations in triplicate assays and the dose dependent inhibition of conversion are shown as a histogram.

DEX. Induction with cortisol and corticosterone was maximal at 100 nM, and relative CAT activity was about 85%. Cortisone had no effect on the transactivation of MMTV-CAT. Similar experiments with hMR_{1-984} and ALDO (Figure 3B) also showed dose dependent transactivation. Maximal

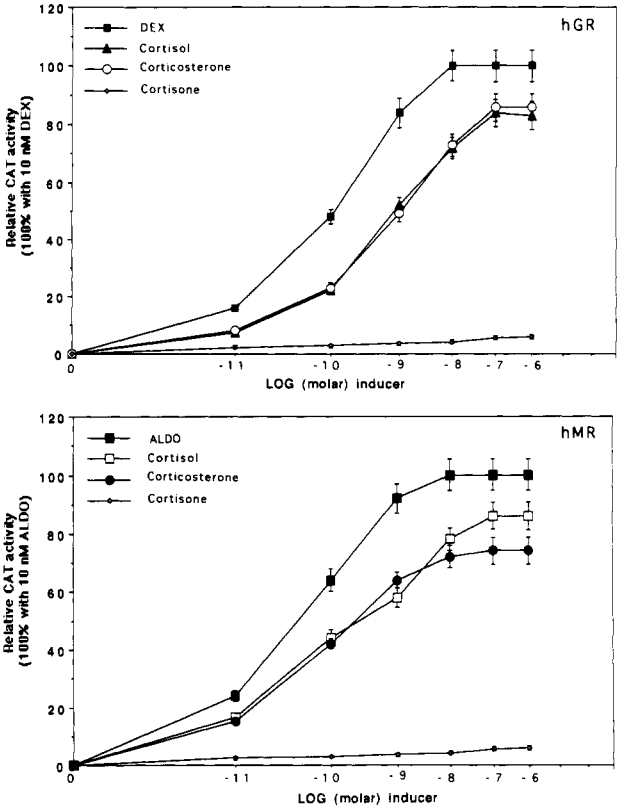

FIGURE 3: (A, top) Transcription activation of MMTV-CAT by hGR. CV-1 cells were plated at a density of 1×10^6 cells/plate. A mixture of 2.5 μg of hGR_{1-777}, 2.5 μg of MMTV-CAT, and 2.5 μg of the β-galactosidase expression vector CH 110/plate was coprecipitated in 960 μL as described in Materials and Methods, and the CAT activity in extracts containing 10 units of β-galactosidase was determined. Following autoradiography, the radioactive spots were localized with a transilluminator, and the radioactivity in each spot was determined by scintillation counting. Each assay shown in the curve is the average of triplicates varying by less than 5%. The CAT activity is expressed as a % of the activity determined in hGR and MMTV cotransfected CV-1 cells treated with 10 nM DEX. (B, bottom) Transcription activation of MMTV-CAT by hMR. The transfection procedure was essentially as described earlier, except that this time hMR_{1-984} was used as a transcription activator. The results are the average of triplicates that varied by less than 5%. The CAT activity is expressed as a % of the activity observed with hMR and MMTV-CAT cotransfected CV-1 cells treated with 10 nM ALDO.

induction of MMTV-CAT mediated by hMR_{1-984} was at 10 nM ALDO. Induction with cortisol and corticosterone was maximal at 100 nM, and the relative CAT activity was 85% and 75%, respectively. On the other hand, cortisone, in equimolar concentrations, had no effect on the transactivation of MMTV-CAT mediated by hGR_{1-777}.

Specificity of hGR_{1-777} and hMR_{1-984}. To assess the specificity of both receptors, 1, 2, 5, and 10 μg of the human 11β-HSD plasmid was cotransfected into CV-1 cells in similar studies. Following treatment with 10 nM DEX, the highest levels of CAT induction were unaffected in the presence of 1, 2, and 5 μg of cotransfected 11β-HSD expression plasmid (Figure 4, lanes 1–4). However, cotransfection with 10 μg of 11β-HSD expression plasmid, followed by similar treatment with 10 nM DEX, led to a decrease in the transactivation of MMTV-CAT (Figure 4, lane 5), indicating a possible 11β-dehydrogenation of DEX. hGR_{1-777}-induced transactivation by 100 nM either cortisol or corticosterone in the presence of increasing concentrations of the h11β-HSD expression vector resulted in a reduction in transactivation to basal values by the inactivation of cortisol to cortisone or of corticosterone to

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CV-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MMTV-CAT 2.5μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CH 110 2μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
hGR 1-777 2.5μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p11β-HSD μg	-	1	2	5	10	1	2	5	10	1	2	5	10	1	2	5	10	1	5
DEX 10nM	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cortisol 100nM	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Corticosterone 100nM	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Cortisone 100 nM	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
NADPH 1μM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

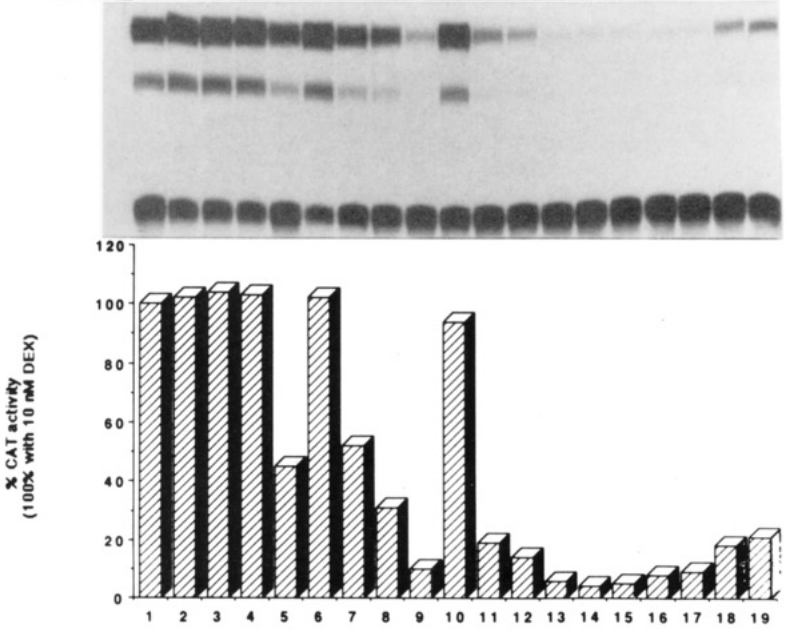

FIGURE 4: Transcription activation of MMTV-CAT by hGR. CV-1 cells were cotransfected with hGR, MMTV-CAT, CH 110, and 0 (lane 1), 1 (lanes 2, 6, 10, 14, and 18), 2 (lanes 3, 7, 11, and 15), 5 (lanes 4, 8, 12, 16, and 19), and 10 μg (lanes 5, 9, 13, and 17) of h11β-HSD expression plasmid. The transfectants were treated with 10 nM DEX (lanes 1–5), 100 nM cortisol (lanes 6–9), 100 nM corticosterone (lanes 10–13), and 100 nM cortisone (lanes 14–19). In addition, the transfectants in lanes 18 and 19 were treated with 1 μM NADPH. The CAT activity is expressed as a % of the activity observed with hGR-cotransfected CV-1 cells treated with 10 nM DEX. The histogram represents the mean activity from three independent experiments that varied by less than 5%.

dehydrocorticosterone (Figure 4, lanes 6–9 and 10–13, respectively). The addition of cortisone under identical experimental conditions did not result in an increase in CAT induction as expected for the inherent reductase activity of 11β-HSD (Figure 4, lanes 14–17). However, concomitant treatment of the transfectants with 100 nM cortisone and 1 μM NADPH showed a slight but measurable elevation (25%) of CAT activity compared to the control (Figure 4, lanes 18 and 19).

We conducted similar transcription activation analyses of MMTV-CAT mediated by hMR_{1-984}. Treatment with 10 nM ALDO did not affect transactivation in the presence of increasing quantities of 11β-HSD (Figure 5, lanes 1–5). hMR_{1-984}-induced transactivation with 100 nM either corticosterone or cortisol was reversed to almost basal levels by the addition of increasing quantities (1, 2, 5, and 10 μg) of human 11β-HSD expression vector (Figure 5, lanes 6–9 and 10–13, respectively). In the presence of 100 nM cortisone, hMR_{1-984}, and the 11β-HSD expression vector, there was only the basal level of CAT activity (Figure 5, lanes 14–17). However, the concomitant addition of 100 nM cortisone and 1 μM NADPH elevated CAT activity to 70% of the level observed with 10 nM ALDO (Figure 5, lanes 18 and 19). This suggests that although the 11β-HSD expressed in CV-1 cells by transient transfection catalyzes both the oxidation and the reduction reactions, as reported by others (Lakshmi & Monder, 1985a,b; Agarwal et al., 1989), the addition of NADPH was essential for 11β-HSD dependent reduction.

11β-HSD Inhibition by Carbenoxolone. We have shown previously that the addition of increasing concentrations of human 11β-HSD expression vector decreased hGR_{1-777} transactivation by 100 nM cortisol (Figure 4, lanes 6–9) to basal levels. The simultaneous treatment with increasing concentrations of CBX resulted in a progressive increase in transactivation, culminating in the highest induction levels of CAT activity at 75–85% (Figure 6A). Although, as shown previously, cortisone alone failed to induce CAT activity, the addition of increasing concentrations of NADPH between 0.01 and 1000 nM stimulated CAT activity to 60%. Comparable experiments with hMR_{1-984} showed that the decreased induction of transactivation by 100 nM cortisol (Figure 6B) was reversed by treatment with CBX. As with hGR, cortisone alone failed to induce CAT. However, the addition of increasing concentrations of NADPH stimulated the 11β-HSD reduction of cortisone, thereby increasing the CAT activity to 60% of basal levels.

Distribution of Activities in Transfected CV-1 Cells. CAT activity was measured in the cytosol and in the nucleosol fractions from CV-1 cells transfected with MMTV-CAT and hGR_{1-777} or hMR_{1-984}, in the presence and absence of 200-fold molar excess of non-radioactive competitors, to determine the specific binding (Figure 7). CAT activity was highest in

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CV-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MMTV-CAT 2.5μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CH 110 2μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
hMR1-984 2.5μg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p118-HSD μg	-	1	2	5	10	1	2	5	10	1	2	5	10	1	2	5	10	1	5
ALDO 10nM	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cortisol 100nM	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Corticosterone 100nM	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Cortisone 100 nM	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
NADPH 1μM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

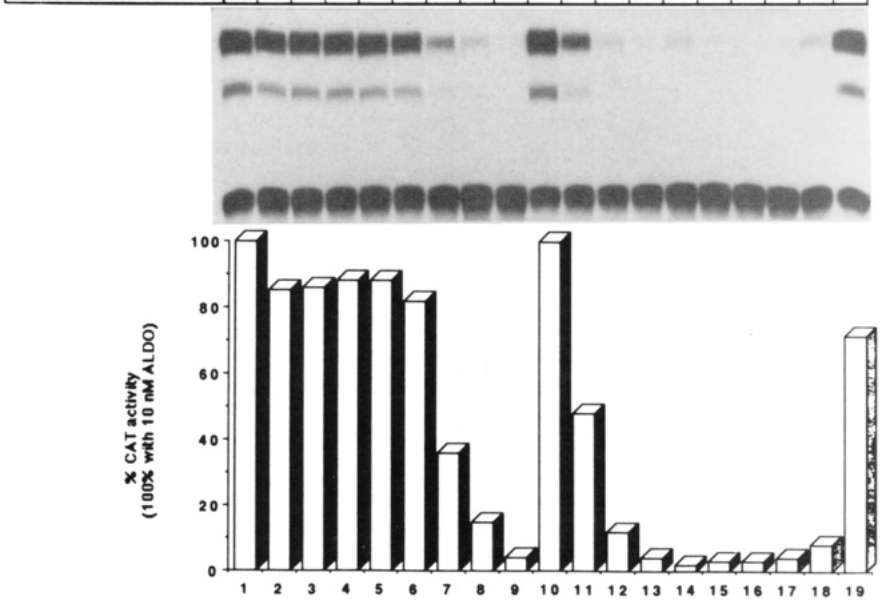

FIGURE 5: Transcription activation of MMTV-CAT by hMR. CV-1 cells were cotransfected with hMR, MMTV-CAT, CH 110, and 0 (lane 1), 1 (lanes 2, 6, 10, 14, and 18), 2 (lanes 3, 7, 11, and 15), 5 (lanes 4, 8, 12, 16, and 19), and 10 μg (lanes 5, 9, 13, and 17) of h11β-HSD expression plasmid. The transfectants were treated with 10 nM ALDO (lanes 1–5), 100 nM cortisol (lanes 6–9), 100 nM corticosterone (lanes 10–13), and 100 nM cortisone (lanes 14–19). In addition, the transfectants in lanes 18 and 19 were treated with 1 μM NADPH. The CAT activity is expressed as a % of the activity observed with hMR-cotransfected CV-1 cells treated with 10 nM ALDO. The histogram represents the mean activity from three independent experiments varying by less than 5%.

the cytosol fractions, while receptor binding was highest in the nuclear fractions.

DISCUSSION

In the present study, we have investigated the putative role of 11β-HSD *in vivo* by examining the selectivity of transactivation by various steroids. The 11β-HSD is a microsomal enzyme complex that catalyzes the reversible conversion of cortisol to cortisone in humans and that of corticosterone to dehydrocortisone in rats. The proportion of the reductase and dehydrogenase components (Lakshmi & Monder, 1985a) of this enzyme varies between tissues (Lakshmi & Monder, 1985b). In midgestational human fetal lung (HFL) *in vivo* or *in vitro* in explants, there were fewer epithelial cells than mesenchymal cells and the conversion of cortisol to cortisone predominated (Abramovitz et al., 1982). Mesenchymal cells grow as fibroblast-like cells in culture, and fibroblasts, on the other hand, convert cortisone to cortisol. The model study presented in this paper was conducted in CV-1 cells that are fibroblast-like.

We have recently purified the NAD dependent isoform from the human placenta to homogeneity and raised polyclonal antibodies against the 11β-HSD2 isoform (Warriar et al., unpublished results). In the human liver, the conversion of cortisone to cortisol predominated. Other organs change their net direction of metabolism during development (Murphy, 1981). The gene encoding 11β-HSD was recently cloned, and the fact that the enzyme synthesized *in vitro* from this cDNA mediated both dehydrogenase and reductase activities suggests the existence of one enzyme incorporating both activities, rather than two separate enzymes (Agarwal et al., 1989; Tannin et al., 1991). Recent physiological and biochemical evidence suggests a spatial separation of the oxidative and reductive activities. Variations in the interconversion of cortisol–cortisone from predominantly oxidase to reductase activity during fetal development have been observed in various human tissues (Giannopoulos et al., 1982; Murphy, 1978, 1981). Conclusively, these results indicate a distinct segmental heterogeneity of 11β-HSD. The heterogeneity of the 11β-HSD enzyme in the oxidoreduction process at C-11 is furthermore supported by clinical data from patients with 11β deficiency either in the oxidative or in the reductive direction (Harinck et al., 1984; Monder & Shakleton, 1984; Shakleton et al., 1985; Phillipou & Higgins, 1985). Numerous reports on 11β-HSD activity in rat and human tissues indicate a heterogeneous enzyme complex (Monder & Lakshmi, 1989; Sakai et al., 1989; Ghraf et al., 1975). In the face of this wealth of information and the consideration of the central role played by the 11β-HSD enzyme in the kidney in the control of local metabolism and function, we investigated the transactivation of MMTV-CAT as a model to study the control mechanism by transient transfection of CV-1 cells in culture.

We first established the conditions under which to assay the 11β-HSD activity with cortisol and cortisone as substrates

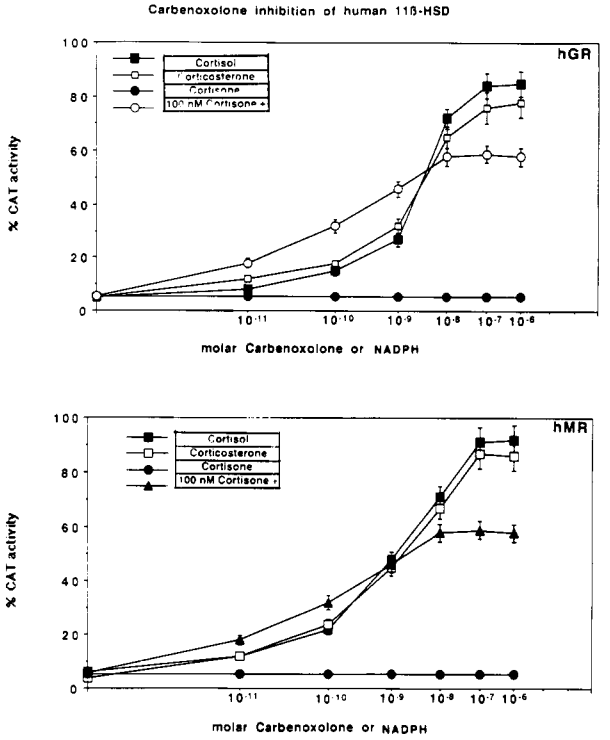

FIGURE 6: Effects of 11β-HSD inhibition with CBX and NADPH dependent conversion of cortisone to cortisol by 11β-HSD on transcription activation by hGR (A, top) and hMR (B, bottom). Transcription activation analyses were performed as described. The transfectants were treated with 100 nM cortisol, corticosterone, or cortisone in the presence of increasing concentrations of CBX. The CAT activity is expressed in % activity observed with hGR or hMR and MMTV-CAT-cotransfected CV-1 cells treated with either 10 nM DEX or ALDO. The results are the average of triplicates varying by less than 5%. 100 nM cortisone+ indicates the efficiency of conversion of cortisone to cortisol assayed in the presence of 100 nM cortisone and increasing concentrations of NADPH.

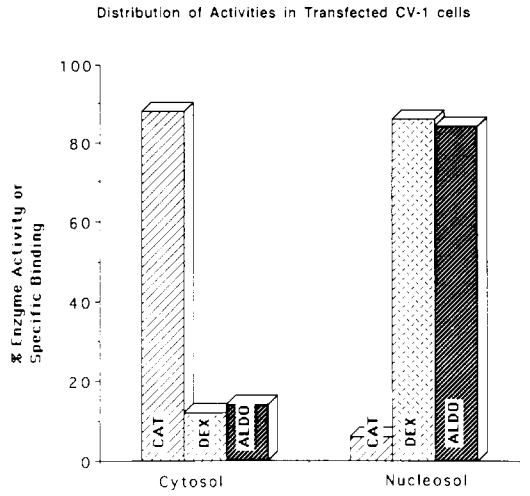

FIGURE 7: Distribution of enzyme and binding activities in transfected CV-1 cells. The hGR or hMR expression plasmid-transfected CV-1 cells were treated with radioactive steroids, as described in Materials and Methods. The CV-1 cotransfected with MMTV-CAT and hGR treated with 10 nM DEX were used for determining the distribution of CAT enzyme. The specific binding activities were determined by including a 200-fold molar excess of non-radioactive competitor in parallel incubation of the cells with the radioactive steroids. The results are the means of triplicate assays.

and in the presence of NAD, NADP, or NADH as coenzyme. Our controls included CV-1 cells transfected with the vector pcDNA 1 and the vector with 11β-HSD cDNA inserted in the reverse orientation. There was no detectable 11β-HSD activity in any of our controls. We then expressed the human 11β-HSD in CV-1 cells and analyzed the ability of the enzyme to oxidize cortisol in the presence of coenzymes NAD, NADH, and NADP. Using the CV-1 cells, we have established both the oxidase and reductase activities of the human enzyme 11β-HSD. The overexpressed enzyme preferentially oxidized cortisol to cortisone in the presence of NADP. Conversion of cortisol to cortisone in CV-1 cells overexpressing 11β-HSD was linear with respect to cell number (data not shown). To analyze the 11-ketoreductase activity, we synthesized radioactive cortisone from radioactive cortisol with 11β-HSD overexpressed in CV-1 cells (Pagé et al., 1994a). Using this cortisone as a tracer, we measured the conversion of cortisone to cortisol in the presence of NADPH.

We then analyzed the specificity of agonistic glucocorticoid and mineralocorticoid ligands. The specific interaction of ALDO with the cloned receptor gene expressed in receptor deficient cells, subsequent to treatment with ALDO, supported the hMR dependent transactivation of MMTV-CAT. Cortisol and corticosterone failed to discriminate between hGR and hMR in transactivation assays, as previously described (Arriza et al., 1988). Both induced MMTV-CAT in a similar manner in hGR and hMR transfectants. Furthermore, in the transactivation studies, 11β-HSD showed a distinct preference for NADP. In the presence of this cofactor, the dehydrogenase activity of 11β-HSD was predominant. Finally, we have demonstrated that, in CV-1 cells, the CAT activity was highest in the cytosolic fractions and receptor binding was highest in the nuclear fractions.

Funder et al. (1990) showed that glucocorticoids, endogenous or administered, physiological or synthetic, produced a classical mineralocorticoid effect if they had access to MR or GR in the kidneys of rats. The presence of 11β-HSD in mineralocorticoid target tissues limited the access of active glucocorticoids (Edwards et al., 1988; Funder et al., 1988). Our results confirmed the data that cortisone had a lower affinity for MR and GR. In humans, inhibition of this enzyme or its deficiency results in the syndrome of apparent mineralocorticoid excess (Ulick et al., 1979), where sodium retention, hypokalemia, and hypertension occur in subjects with low levels of plasma and urinary aldosterone. Carbenoxolone clearly affected a variety of enzymatic processes *in vivo* (Latif et al., 1990), and administered glucocorticoids had a wide range of effects that altered renal electrolyte regulation as a secondary effect. CBX inhibited 11β-HSD both *in vivo* and *in vitro* (Monder et al., 1989; Latif et al., 1990). We have used appropriate steroid substrates (cortisol) to study the mechanism of CBX action. Likewise, we show that CBX inhibited 11β-HSD in transfected CV-1. Another study showed that potentiation of glucocorticoid action by CBX was exclusively due to inhibition of the dehydrogenase component of 11β-HSD in the liver and kidneys (Whorwood et al., 1993). A series of elegant studies in rabbit kidneys (Naray-Fejes-Toth & Fejes-Toth, 1994) convincingly demonstrated that this glucocorticoid effect takes place at the level of the cortical collecting tubule, a prime target for aldosterone action. Southern analysis of human genomic DNA indicated a single 11β-HSD gene, suggesting that other isoforms of 11β-HSD might have a significantly different structure. However, shorter transcripts originating at a different promoter region were found in the kidneys (Krozowski et al., 1992), liver, and lungs (Moisan et al., 1992). These shorter cDNAs had no 11β-HSD activity (Obeid et al., 1993).

The model outlined in this paper demonstrates the fact that glucocorticoids such as cortisol and corticosterone produce a classical mineralocorticoid effect through their interaction with hMR, followed by transcriptional activation of the responsive gene, MMTV-CAT. This method is most suitable for studying the metabolic activation or inactivation of the ligands by the cointroduced cDNA expression vector involved in the metabolic conversion of the ligand under investigation. The functional significance in metabolism involving the cloned enzymes can be scrutinized by eliciting ligand-regulated transcription of a model responsive gene as described in this report. Our results show the inability of ligand-bound hMR or hGR to discriminate between the responsive elements. Specificity in cellular response could arise from (i) the specificities of transcription factors rather than from hormones; (ii) the specificities of steroid-metabolizing enzymes in different tissues; and (iii) the availability of metabolites such as NAD, NADP, NADH, and NADPH. Hence, transactivation analyses can serve as a useful tool to investigate defined metabolic processes.

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