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DIFFERENTIAL REGULATION OF MOUSE MAMMARY TUMOR VIRUS-BACTERIAL CHLORAMPHENICOL ACETYLTRANSFERASE CHIMERIC GENE BY HUMAN MINERALOCORTICOID HORMONE-RECEPTOR COMPLEXES

MANJAPRA V. GOVINDAN,* STEEVE LECLERC, RICHARD ROY, PALANISWAMI RATHANASWAMI and BOXUN XIE

Laboratory of Molecular Endocrinology, CHUL Research Center and Laval University, 2705 Laurier Boulevard, Quebec, Canada G1V 4G2

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Summary—The brain tissues of the rat and mouse express two types of corticosteroid binding proteins, the glucocorticoid (GR) and aldosterone (MR) receptors. Unlike the type II (GR) receptor, type I receptor has a high affinity for aldosterone (ALDO) and corticosterone and is structurally similar to the kidney mineralocorticoid receptor (MR). The results reported in this study provide direct evidence for the interaction of dexamethasone (DEX), triamcinolone acetonide (TA), dexamethasone-21-mesylate (DXM) and 11-deoxycorticosterone (DOC) with human MR expressed in cells by transient co-transfection of a hMR expression vector. The interactions of hMR with DEX, TA, DXM, DOC, promegestone (R5020) and methyltrienelone (R1881) were measured by trans-activation of mouse mammary tumor virus long terminal repeat fused to bacterial chloramphenicol acetyltransferase (MMTV-tk-CAT) in gene co-transfection experiments and by cell free hormone binding assay. The incubation of various steroid hormones in the presence of [^{3}H]ALDO in a competition assay with extracts prepared from HeLa cells co-transfected with hMR expression vector, showed that hMR expressed under these conditions has a high relative affinity for DEX which is similar to ALDO, TA and DOC. Incubation with DXM under these conditions showed very little competition, as was observed with R1881 and R5020. Incubation of the co-transfected cells with DEX, ALDO, DOC, R5020, TA, R1881 and DXM demonstrated that the level of trans-activation did not reflect the previously observed order of binding affinity for the hMR. The level of trans-activation was always higher with DEX and TA compared to ALDO and DOC. Analysis of the binding of labeled glucocorticoid regulatory element (GRE) and hMR incubated with DEX, ALDO and DXM by gel shift analysis demonstrated that the trans-activation of MMTV-tk-CAT by hMR is a result of the interaction of hMR with GRE in the MMTV-LTR.

INTRODUCTION

Adrenal steroids play an important role in regulating neuroendocrine, neurophysiological, neurochemical and behavioral functions through their interaction with corticosteroid receptors [1]. The hippocampus plays a central role in feedback regulation of hypothalamic–pituitary–adrenal axis [2–7]. Within the brain, there are two defined types of receptors implicated in corticosteroid action, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) which are typified as type I and type II receptors, respectively [8]. Luttge *et al.* [9] demonstrated that the type I receptor from mouse brain had a greater affinity for dexamethasone (DEX) than type II [9]. Recent reports by Arriza *et al.* [10] in co-transfection studies of type I receptor expression vector into CV-1 monkey kidney cells show that it requires comparatively high concentrations of DEX (100 nM) to induce the transcription of a mouse mammary tumor virus (MMTV)-luciferase reporter plasmid [10]. Recent reports by Funder *et al.* and Edwards *et al.* [11, 12] support the view that the target tissue specificity of mineralocorticoid action is mediated by the enzyme 11β-hydroxysteroid dehydrogenase, which converts both cortisol and corticosterone, but not aldosterone, to their 11-keto analogues.

*To whom correspondence should be addressed.

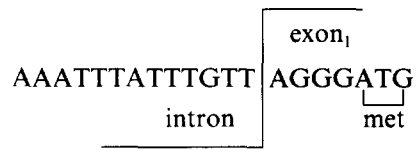
In these co-transfection experiments the required concentration of DEX differs from its previously reported concentration necessary for the induction of MMTV-long terminal repeat fused to bacterial chloramphenicol acetyltransferase (tk-CAT) chimeric plasmids by hMR expression vector [13].

Using hMR clones obtained from a human testis cDNA library, we have studied the competition of various steroid hormones for binding to hMR expressed by transient co-transfection into HeLa cells in the presence of DEX in a cell free binding assay. We report here the organization of the 5′ region of the largest exon of hMR (hMR_{1-984}), the transient co-transfection of hMR 1–984 and MMTV-tk-CAT chimeric plasmids, and comparison of induction of MMTV-tk-CAT chimeric plasmids by various steroid hormones. We show that the trans-activation of hMR follows the classical glucocorticoid regulatory element (GRE)-hMR interaction pattern known for the other counterparts of the nuclear receptor family.

MATERIALS AND METHODS

cDNA cloning and construction of hMR_{1-984}

We have previously reported the cloning of human androgen receptor clones using oligonucleotides complementary to hGR [14–16] from a human testis cDNA library (Clonetech, U.S.A.). Out of 214 positive clones isolated as described [14], 4 cDNA clones cross-hybridized with one another (hMR6, hMR7, hMR15 and hMR25). These hMR–cDNA clones 6, 7 and 15 contained an internal *Eco*RI site which was absent in hMR25. The open reading frame of hMR6, 7 and 15 contained coding information for $hMR_{330-984}$ and hMR25 (1800 bp) contained $hMR_{633-933}$, but differed in remaining sequences from that of hMR [10]. We used the *Eco*RI–*Eco*RI ($hMR_{330-516}$) fragment of hMR6 to screen the human chromosome 4 genomic library in λ phage constructed with the *Eco*RI digested human DNA to isolate 12 positive hMR genomic signals. The genomic clones contained an *Eco*RI fragment of 4.5 kb which was subcloned in M13mp18 and SK bluescript M13-vectors and sequenced using deletion mutants generated by restriction digestion with *Hind*III, *Kpn*I, *Xba*I, *Sac*I and *Pst*I. This genomic fragment contained an open reading frame of hMR_{1-516} until the internal *Eco*RI site at amino acid residue 516 of hMR [10]. The intron–exon junction at the amino terminus:



suggested that the 5′ non-translated region is encoded in separate exon(s) as was found in the organization of the hGR 5′ non-translated region [17].

For the construction of hMR expression vector the cDNA clones hMR6, hMR7 and the genomic clone fragment *Kpn*I–*Bam*HI (hMR_{1-351}) was subcloned into bluescript SKM13. The partial *Eco*RI digestion of hMR7 consisting of both 1100 and 500 bp inserts was digested with *Pst*I (hMR_{862}) and *Hind*III restriction enzymes to delete the overlapping 5′ region with hMR6 up to the *Pst*I site and to isolate a *Pst*I-*Hind*III carboxy terminal fragment flanking a *Hind*III site. In a final cloning step the *Kpn*I–*Bam*HI gene fragment was ligated together with the middle *Bam*HI (hMR_{351})–*Pst*I (hMR_{862}) from hMR6 (subcloned after partial *Eco*RI digestion into bluescript SKM13) and the carboxy terminal *Pst*I-*Hind*III fragment into bluescript SKM13 at *Kpn*I–*Hind*III site. The bluescript SK containing a 3500 bp insert was sequenced at the junctions and the fragment containing the hMR_{1-984} was blunt end ligated after filling in the *Eco*RI ends of the pKCR-2 expression vector using T4 polymerase catalysis. This expression vector is herein referred to as hMR_{1-984}. The construction of hGR_{1-777} used in this study has been described previously [14]. In order to study the trans-activation dependency of the cloned hMR on various steroids, transfection studies were performed using HeLa cells. Several groups have previously employed these cells for such experiments particularly because they are derived from a human source [18]. Before transfections were carried out, control experiments were executed to study the endogenous induction of MMTV-tk-CAT by DEX, since the presence of GR in HeLa cells has been recently reported [19]. To quantitate the endogenous levels of hGR present in these cells, we performed DNA–protein interaction studies with HeLa cell extracts in the presence and absence of 10^{-6} M DEX. As a positive control, extracts prepared from hGR_{1-777} transfected HeLa cells were also subjected to

DNA–protein interaction studies. For transfection experiments, we used the β-galactosidase expression vector CH110 (Pharmacia, Canada) in order to standardize the transfection efficiency, while for co-transfection 2.5 μg of reporter MMTV-tk-CAT [14], 5 μg of CH110 and 2.5 μg of hMR_{1-984}, hGR_{1-777} and human androgen receptor ($hAR_{244-910}$) pKCR-2 expression vectors were utilized. *In vitro* transcription, translation and fluorography were performed as described [14].

Hormone binding assay

HeLa cells were grown to 60% confluency in MEM medium supplemented with 10% fetal calf serum treated with charcoal in 250 ml Falcon flasks and were transfected with 20 μg of 2 × CsCl purified hMR_{1-984} by a calcium phosphate transfection procedure. The cells were collected (2 ml/flask) after 48 h and total cell extracts were prepared after homogenization with a tight fitting glass–glass homogenizer in buffer containing 400 mM KCl [18]. The high speed supernatant was prepared after centrifugation in a Beckmann ultracentrifuge using a Ti65 rotor at 45000 rpm for 1 h. Following the centrifugation, 25 μg (total protein) of cell extract were incubated overnight with 1 nM [^{3}H]aldosterone (ALDO) (sp. act. 40–60 Ci/mmol; Amersham, U.S.A.) in the presence and absence of $1 \times 10^{-7} - 1 \times 10^{-11}$ DEX, ALDO, 11-deoxycorticosterone, (DOC), methyltrienelone (R1881), promegestone (R5020), triameinolone acetonide (TA) and dexamethasone-21-mesylate in a final volume of 50 μl. An aliquot of 50 μl dextran coated charcoal (10%) was added, mixed well and centrifuged to remove the unbound steroids. Radioactivity was determined in a 75 μl aliquot by scintillation counting. The specific binding of [^{3}H]ALDO was 4800 cpm determined in a Beckman scintillation spectrometer with 50% efficiency. Trans-activation of MMTV-tk-CAT and gel shift analysis of DNA–protein interactions were performed as described [14].

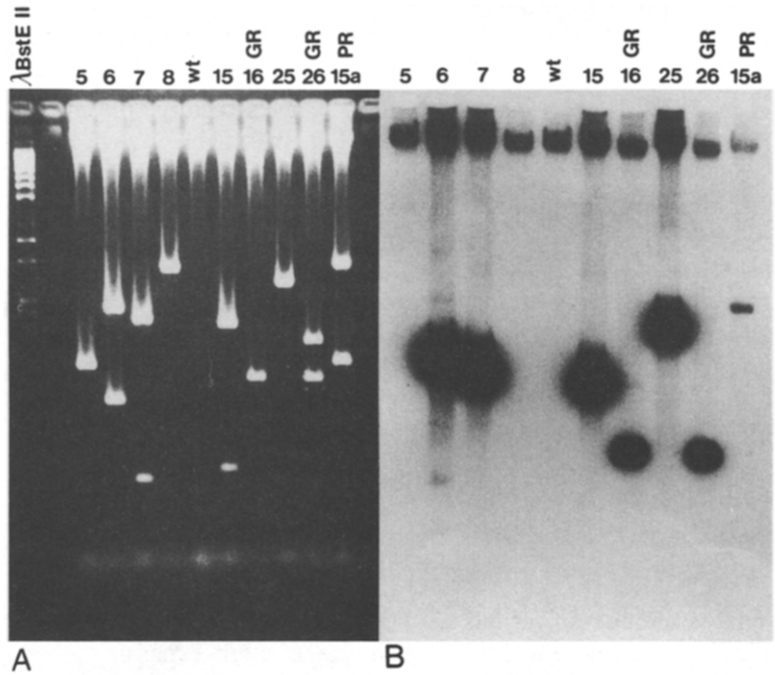


Fig. 1. Restriction mapping and Southern hybridization (A) and (B): analysis of human MR, GR and PR isolated from the human testis λgt11 cDNA library. 5 μg each of λ5, λ6, λ7, λ8, wild type λgt11, λ15, λ16, λ25, λ26 and λ15a purified phage DNAs were digested with *Eco*RI restriction enzyme and separated in a 1.2% agarose gel by electrophoresis. The ethidium bromide stained gel is shown in Fig. 1(A). The agarose gel was transferred onto Hybond N (Amersham, Canada) and hybridized with the nick-translated larger *Eco*RI fragment of clone 6. The hybridization was in 50% formamide at 42°C and the washings at 64°C in 2 × SSC and 0.1% SDS. The clones which gave rise to very strong hybridization signals were confirmed after DNA sequencing to be hMR [14] and cDNA clones λ16 and λ26 encoded hGR [15, 16], a cDNA clone λ25 both of which contained aberrant hMR sequences (MVG, unpublished) and finally λ15a which is a progesterone receptor clone containing codons for $PR_{356-933}$ with a long 3′ non-translated region [14].

RESULTS AND DISCUSSION

Cloning and expression

By using oligonucleotides complementary to the conserved regions of hGR [14, 16] we were able to isolate four cross-hybridizing cDNA clones from the human testis cDNA library which encompassed the region between amino acids 330 and 984 of hMR [Figs 1(A) and (B)]. We screened a λ charon phage library constructed with human chromosome 4 DNA digested with *Eco*RI, with the aim of isolating the promoter region of the hMR gene. The library was screened with a nick-translated hMR cDNA fragment. All of the 12 positive signals resulted in the isolation of a 4.5 kb *Eco*RI genomic fragment, which contained four nucleotides of the 5′ non-coding region and the codons for amino acids 1–516. The sequence was found to contain

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5′ AAATGCAATTTTAGAATGTC-
   TTTTAGAGTAATATTGCTATAACTGAC-
   TCTAATTTTTTAATGTAAATTT-

                exon    1   2   516
ATTTGTT | AGGG  ATGGAG-GTG
  intron |        met     val
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and the partial map of this hMR gene segment is shown in Fig. 2(A). We have constructed a hMR_{1-984} in pKCR-2 [14] which was subsequently used in transient co-transfection studies [Fig. 2(B)]. Prior to the construction of hMR_{1-984}, we characterized the hMR_{ORF} by *in vitro* transcription followed by *in vitro* translation in the presence of [^{35}S]methionine. After fluorography, the translation product demonstrated that the cDNA contained the entire hMR_{ORF} giving rise to a product of 110 kDa (Fig. 3, lane 2). The control experiment in the absence of RNA did not show any such translation product (Fig. 3, lane 1).

Hormone binding studies

To find the relative affinities of various steroid hormones capable of interacting with hMR, we performed competition binding experiments with cell free extracts incubated with 1 nM [^{3}H]ALDO in the presence of $10^{-7}-10^{-11}$ M concentrations of competitor non-radioactive DEX, ALDO, TA, DOC, R1881, R5020 and DXM. We found that hMR had a relative binding affinity for DEX, TA, ALDO and DOC within the nM range. DEX and TA were found to have similar relative binding affinities compared to those of ALDO and DOC (Fig. 4). The negligible level of binding observed in similar binding studies with R5020 and R1881 confirmed the ligand specificity of hMR. Incubations with DXM, an irreversible GR binder, demonstrated that the concentration required for saturation of competition is $>10^{-7}$ M revealing its poor relative affinity for hMR in the presence of [^{3}H]ALDO. These experiments demonstrate that hMR_{1-984} has a high affinity for both of the glucocorticoids DEX and TA while aldosterone has similar binding characteristics as DOC under these incubation conditions. So far, there are no direct experimental data available on the interaction of TA with type I receptor. We demonstrate here for the first time the interaction of TA with hMR_{1-984} produced in cells after co-transfection. The differences in affinities observed with hMR_{1-984} for DEX, TA, ALDO and DOC when compared to the previous reports [10] may be due to the fact that the present transient transfections were performed with human HeLa cells, thus indicating a possible tissue specificity of trans-activation by hMR.

Transcriptional regulation of MMTV-tk-CAT by hMR_{1-984}

The structure of hMR_{1-984} is shown in Fig. 2(B). Our transfection results varied by $<5\%$ by quantitation of the CAT assay autoradiogram by image analysis. The induction of MMTV-tk-CAT alone in the presence of CH110 into HeLa cells followed by treatment with 5 nM DEX [Fig. 5(A) and Fig. 6, lane 18] or 5 nM ALDO (Fig. 6, lane 19) showed the basal CAT activity in these control transfections. Control cDNA protein interaction studies carried out with HeLa cell extracts in the presence and absence of 10^{-6} M DEX are shown in Fig. 5(B), lanes 2–7. Extracts from hGR_{1-777} transfected HeLa cells, on the other hand, show an intense hormonal dependency of receptor DNA complex formation as illustrated in [Fig. 5(B), lanes 8–13]. The results of co-transfection experiments with hGR_{1-777} in the presence of MMTV-tk-CAT after treatment in the absence of hormone, demonstrated that the measured CAT activity did not result from the incomplete removal of hormones after charcoal treatment (Fig. 6, lane 1). Identical control experiments with hMR_{1-984} co-transfected cells in the absence of hormone treatment did not reveal any detectable CAT activity in the control cell extract (Fig. 6, lane 7). Additional

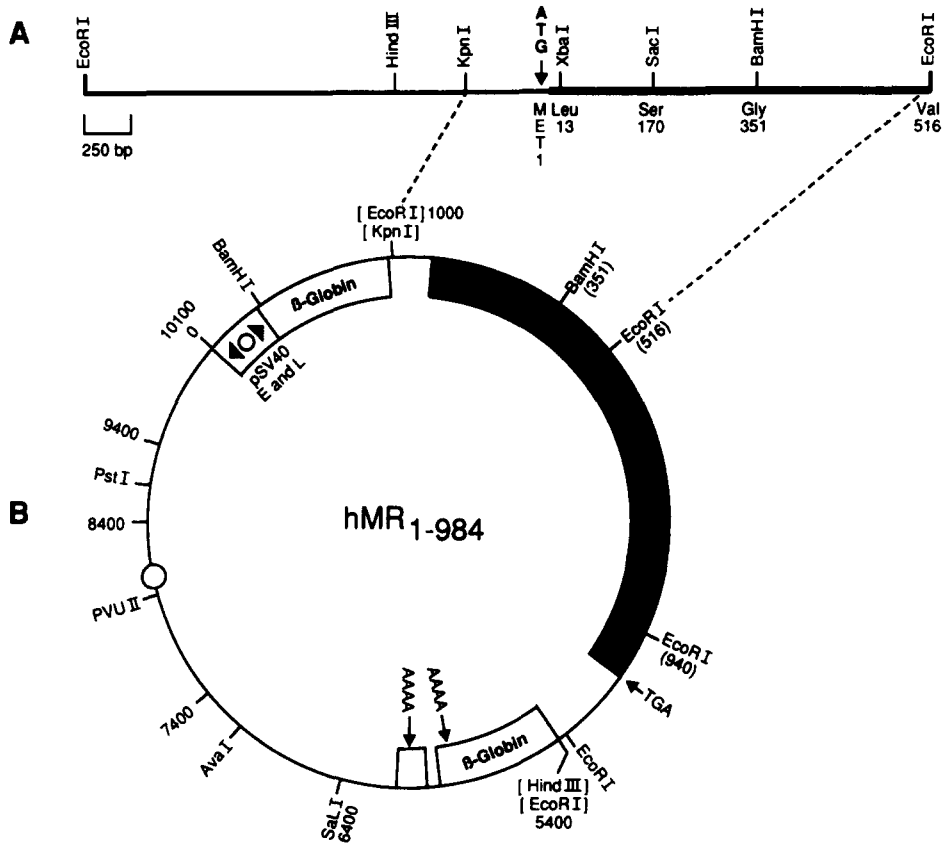


Fig. 2. (A): Partial map of hMR gene segment containing exon coding for hMR_{1-516}. (B): Structure of hMR_{1-984} in pKCR-2. The numbers in brackets refer to amino acid numbers. The position of nucleotide numbers is arbitrary. The *Kpn*I site at the 5′ end is the original *Kpn*I site found at the 5′ end of the genomic fragment.

control transfections demonstrated that even at 10^{-6} M DEX, the endogenous hGR did not trans-activate MMTV-tk-CAT to any considerable extent [Fig. 5(A)]. Following incubation of the hGR_{1-777} co-transfected in HeLa cells, the addition of 5 nM DEX resulted in the highest level of measurable CAT activity in these sets of experiments. There was no induction of MMTV-tk-CAT by hGR_{1-777} after treatment with 5 nM ALDO (Fig. 6, lane 3), nor with 5 nM R5020 (Fig. 6, lane 4) or with 5 nM R1881 (Fig. 6, lane 5). However, the incubation of an identical hGR_{1-777} and MMTV-tk-CAT co-transfectant showed some induction after incubation with 10^{-7} M DXM (Fig. 6, lane 6). We assume that this 5% CAT activity may be due to a previously described agonistic activity of DXM mediated by a reduced capability of DXM to covalently bind hGR [20, 21]. From these results, we conclude that ALDO, R5020 and R1881 have no measurable glucocorticoid activity in inducing MMTV-tk-CAT under our experimental conditions. These results, however, are not concordant with the recent report of Luttge *et al.* [9], which shows that the K_d of ALDO for type II receptor in mouse brain is in the range of 20 nM. Consequently, we should have observed at least some induction of MMTV-tk-CAT in the extracts prepared from hGR_{1-777} co-transfected HeLa cells treated with 5 nM ALDO. It must be taken into consideration that the affinity measurement by Luttge *et al.* was performed with crude cytosol from mouse brain which contains both type I and type II receptors which may have contributed to the results they obtained.

In our analysis with MMTV-tk-CAT and hMR_{1-984}, we wanted to compare the ability of DEX, TA, R5020, DOC, ALDO and R1881 to induce trans-activation by hGR_{1-777} and hMR_{1-984}. As can be seen in Fig. 6 (lanes 7–17), DEX has not only a very high affinity for the type II receptor measured in trans-activation of MMTV-tk-CAT, but also has comparatively the highest affinity for type I receptor (Fig. 6, lane 8 and Fig. 7, lane 6). When we compare the induction, 5 nM ALDO has 28% efficiency when compared to the 100% of 5 nM DEX with

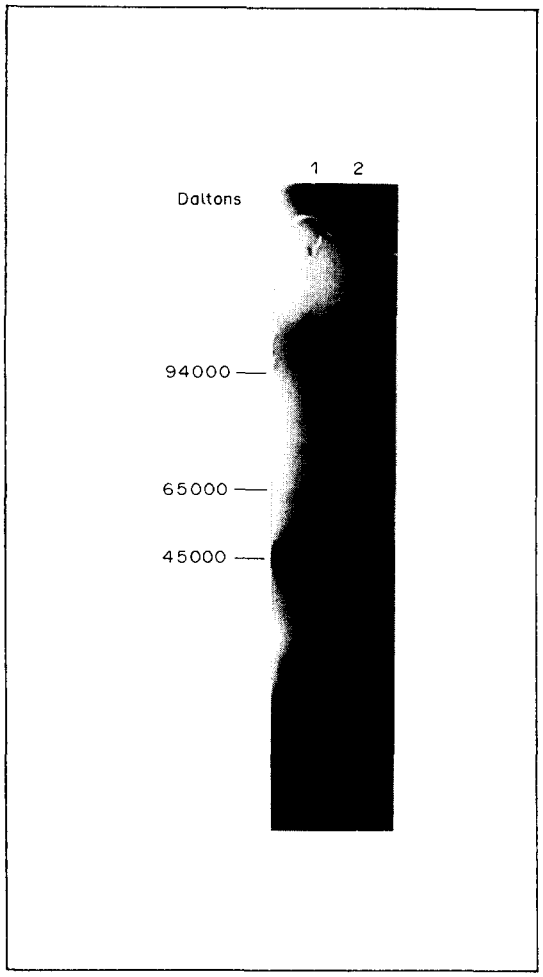


Fig. 3. *In vitro* translation product of hMR_{1-984} in bluescript SK. An aliquot of 5 μg of *Hind*III linearized hMR_{1-984} in bluescript SK was used in transcription as template with T7-RNA polymerase (Promega, Canada). 2 μg of the RNA was translated *in vitro* with nuclease treated rabbit reticulocyte lysate (N90, Amersham) in the presence of [^{35}S]methionine. The translation products were dissolved in SDS-sample buffer [25] and separated in a 10% SDS–polyacrylamide gel by electrophoresis. The translation products in the absence of any added RNA is shown in lane 1 and the products observed with the hMR_{1-984} in lane 2. The very highly labeled band at 47,000 Da is a protein present in the reticulocyte lysate being labeled by a decomposition product of ^{35}S methionine as described by the manufacturer. The high M_W band at 110 kDa (lane 2) in the hMR_{1-984} represents the expected size of hMR_{1-984}.

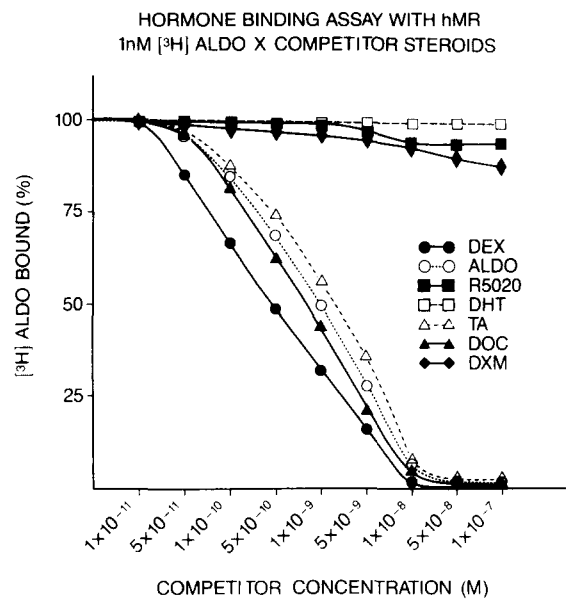
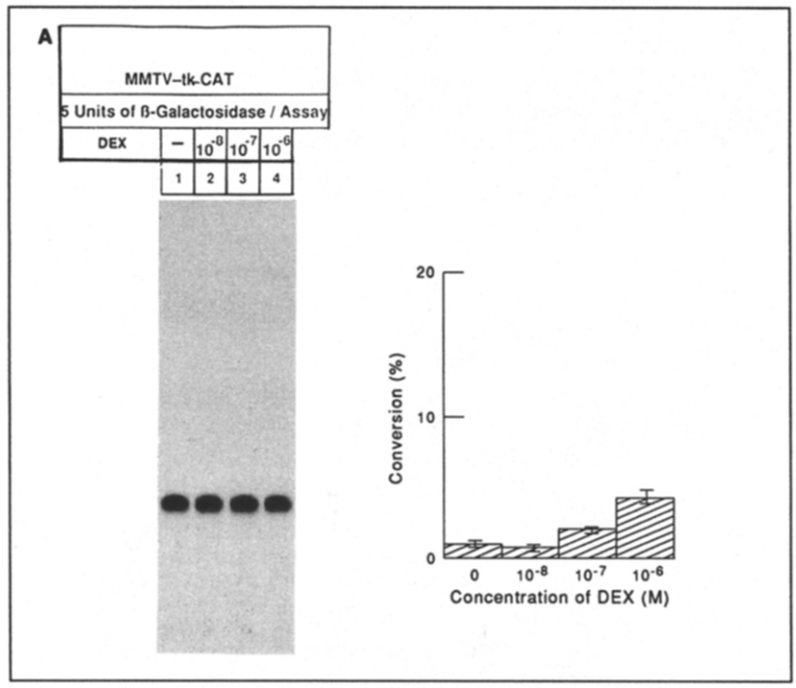


Fig. 4. Interaction of hMR_{1-984} expressed in HeLa cells with various steroid hormones. Semi-confluent HeLa cells in 250 ml culture flasks were transfected with 20 μg of hMR_{1-984} expression vector. Cells from 5 flasks were collected after 48 h with a rubber policeman, washed twice with phosphate buffered saline and homogenized in 5 ml of 200 mM Tris–HCl, pH 7.5, 1 mM EDTA, 460 mM KCl, 5 mM β-mercaptoethanol and 10% glycerol (v/v) using a tight fitting glass–glass homogenizer. The high speed supernatant was prepared by centrifugation 1 h at 45,000 rpm in a Beckman ultracentrifuge using T65 rotor. Hormone incubations were performed in duplicates in aliquots of 50 μl of cytosol with 1 nM [^{3}H]ALDO in the presence and absence of indicated concentrations of competitor steroids. The unbound radioactive [^{3}H]ALDO was removed by dextran coated charcoal treatment and the bound radioactivity was determined by scintillation counting.

type I receptor. In other words, hMR_{1-984} in the presence of 5 nM DEX, induced MMTV-tk-CAT 3.7-fold greater than with 5 nM ALDO under similar conditions (Fig. 6, lane 9). Whether the reduced levels induction observed with 5 nM ALDO and hMR_{1-984} is due to the inactivation of ALDO by conversion to an inactive metabolite during the course of incubation (24 h) remains unknown, although similar incubations with ALDO in co-transfection experiments were used by Arriza *et al.* [10, 13] as well as in animal experiments for longer periods of time by Luttge *et al.* [9]. Even though the inducibility of hMR_{1-984} by ALDO was relatively low, the induction was very specific for type I receptor with ALDO. As discussed in the previous section, the type II receptor (hGR_{1-777}) failed to respond to 5 nM ALDO. DOC induced MMTV-tk-CAT trans-activation by 1.5-fold (Fig. 6, lane 10) compared to 5 nM ALDO (Fig. 6, lane 9). Again, in these observations, the metabolism of these compounds was not taken into account. It appears that if ALDO and DOC are converted into inactive metabolites, ALDO is significantly more degraded than DOC, DEX or TA. The 5 nM TA incubated transfectants show a 2-fold increase in CAT activity when compared to ALDO and 50% when compared to DEX and hMR_{1-984} (Fig. 6, lane 12). The potent progesterone analogue R5020 did not induce MMTV-tk-CAT to any considerable level in the presence of hMR_{1-984} (Fig. 6, lane 11).



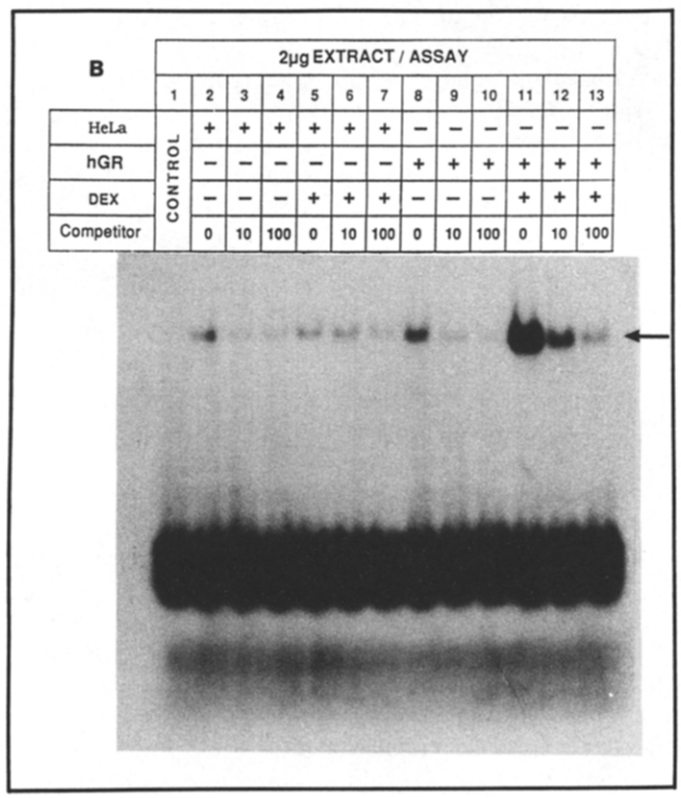


Fig. 5. (A) HeLa cells were co-transfected with 5 μg MMTV-tk-CAT and CH110. Cells were treated following transfection with 10^{-8}, 10^{-7} and 10^{-6} M DEX and extracts were prepared as described. Extracts corresponding to 5 U of β-galactosidase were used in CAT assay to determine the efficiency of endogenous GR in trans-activation in these cells. The histogram represents the quantity of acetylated ^{14}C–chloramphenicol localized in each of the radioactive spots. (B) Extracts were prepared from control HeLa cells grown in the presence (lanes 5–7) and absence (2–4) of 10^{-8} M DEX. For comparison, control gel shift assay was executed with extracts prepared from HeLa cells transfected with hGR_{1-777} and treated with (lanes 8–10) or without (lanes 11–13) 10^{-8} M DEX. Competition with 10 and 100-fold molar excess of non-labeled GRE was performed to determine the specificity of the retarded radioactive signals. The retarded complexes are shown here with an arrow.

Since the results with hMR_{1-984} and DEX showed that its affinity for hMR_{1-984} is comparable to its affinity for hGR, we performed the analysis of hMR induction or inhibition of MMTV-tk-CAT by DXM. By incubating the transfectants with 10^{-9}–10^{-6} M DXM, we observed that at higher concentrations DXM could induce MMTV-tk-CAT by hMR_{1-984}. The level of CAT activity reached 25% (Fig. 6, lanes 16 and 17) of that of the DEX incubated hMR_{1-984} transfectants. The quantitative evaluation of the co-transfection experiments is shown in Fig. 6(B). The differential induction of MMTV-tk-CAT transcription by various hormones and steroid hormone receptors produced by co-transfection performed in triplicate assay is shown for comparison in Fig. 7(A) and (B). This is solely to demonstrate that the variations observed in such assays under our conditions are not $>5\%$.

DNA–protein interaction

We have previously employed the analysis of receptor–DNA interaction by gel interaction techniques to demonstrate hormonal specificity [14]. We used a similar technique to study the interaction of hMR produced in HeLa cells after transfection with hMR_{1-984}. As a control in such interaction studies, we used extracts of hGR_{1-777} prepared from hGR_{1-777} transfected cells. HeLa cell extracts prepared from cells transfected with pKCR-2 vector devoid of any receptor coding sequences showed only negligible quantities of retarded complex in gel retardation analysis after incubation with ^{32}P-end labeled annealed synthetic oligonucleotides containing the palindromic GRE as shown in Fig. 8 (lane 2–4) and Fig. 9 (lanes 2–4). Incubation of the labeled DNA with HeLa cell extracts prepared from hGR_{1-777} transfected cells followed by treatment with 5×10^{-9} DEX resulted in specific retardation of GRE as seen in Fig. 8 (lanes 5–7). The retardation was found to be specific, as shown by the addition of a 10-fold or 100-fold molar excess of non-labeled competitor GRE which decreased the intensity in retarded labeled GRE. Control gel shift analysis with HeLa cell extracts prepared from control non-transfected and HGR_{1-777} transfected cells incubated with or without 10^{-6} M DEX showed only low levels of endogenous hGR present in the HeLa cells used in these experiments [Fig. 5(B)]. As shown in Fig. 8 (lanes 8–10), incubation of hGR extract with 5 nM DEX in the presence of 100 nM DXM similarly exhibited its interaction with GRE. This demonstrates the inability of 100 nM DXM to compete with hGR in the presence of 5 nM DEX. Treatment of the hGR co-transfected HeLa cell extract in the presence of 100 nM DXM alone showed a lower level of DXM–hGR interaction (Fig. 8, lanes 11–13), confirming that the increased signal of the retarded complex observed in the incubation containing 5 nM DEX and 100 nM DXM is indeed due to the binding of hGR–DEX complexes to GRE.

Similar binding studies with GRE were performed with hMR incubated with 5 nM DEX (Fig. 9, lanes 5–7), 5 nM ALDO (Fig. 9, lanes 8–10), 100 nM DXM (Fig. 9, lanes 11–13) and 5 nM ALDO in the presence of 100 nM DXM (Fig. 9, lanes 14–15). It can be seen that the DEX incubated hMR extract prepared from hMR_{1-984} transfected cells bound GRE very efficiently. Incubation of hMR_{1-984} with 5 nM ALDO resulted in similar signals as those observed for hMR incubated with GRE in the presence of DEX (Fig. 9, lanes 8–10). The interaction observed by gel retardation reflects the similar affinities of hMR for DEX and ALDO, indicating that the low level of induction of MMTV-tk-CAT observed in co-transfection experiments may be indeed related to the degradation of ALDO during a long period of incubation. Incubation of hMR extracts with 100 nM DXM showed some retardation of GRE (Fig. 9, lanes 11-13) but the signal was much more intense in the competition assay in the presence of 5 nM ALDO (Fig. 9, lanes 14–15).

CONCLUSION

We have cloned the hMR genomic clones containing an exon encoding amino acids 1–516. We constructed an hMR_{1-984} expression vector by fusing this exon segment with cloned hMR cDNA clones obtained from a human testis cDNA library. This hMR construct expressed a protein of 110 kDa following *in vitro* transcription and translation and is identical in size and sequence reported to that by Arriza *et al.* [10]. Introduction of hMR_{1-984} into HeLa cells resulted in the trans-activation of MMTV-tk-CAT following treatment with DEX, ALDO, DOC and TA. This trans-activation can be correlated to the interaction of hormone bound hMR with GRE, contained within MMTV–LTR (as shown by gel retardation analysis).

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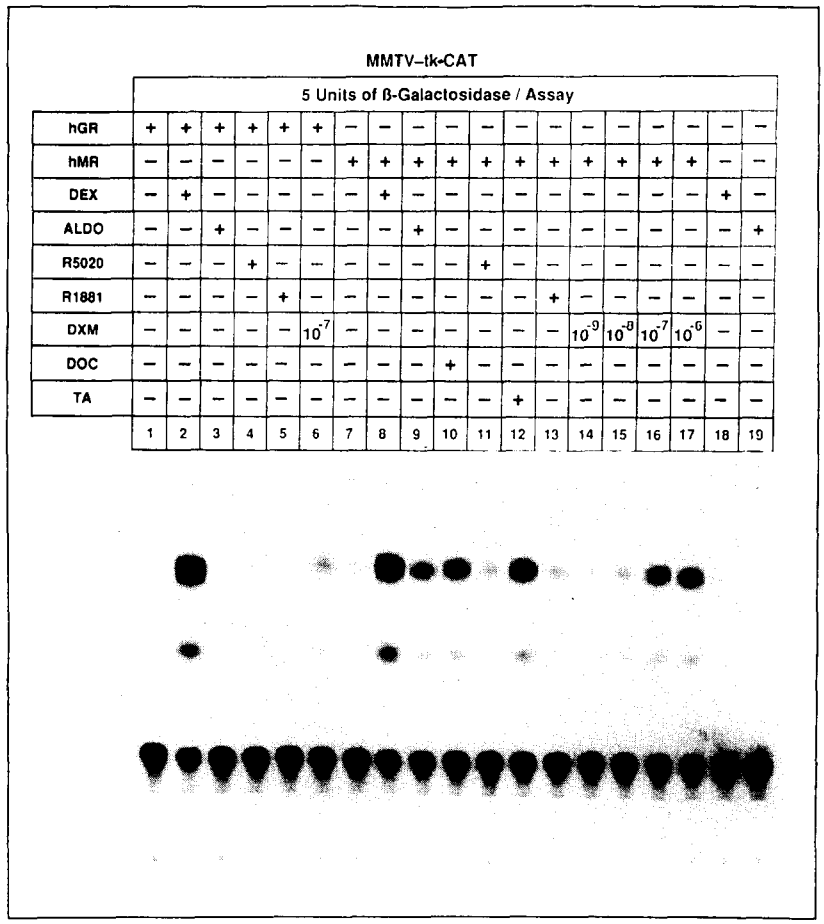

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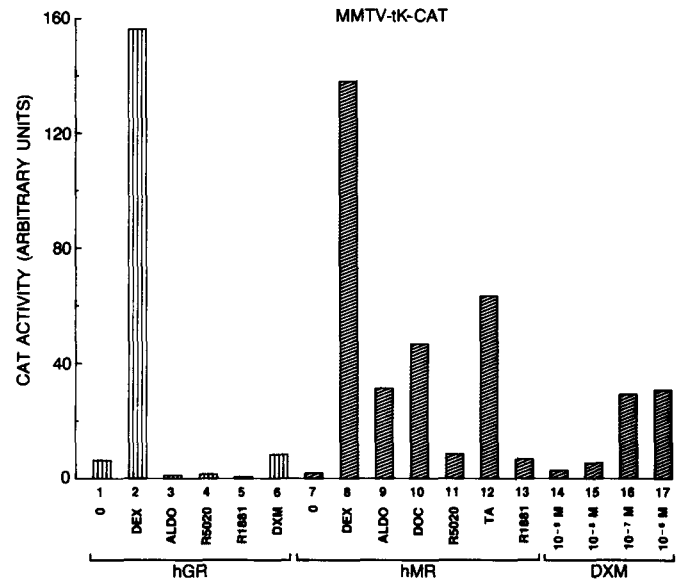
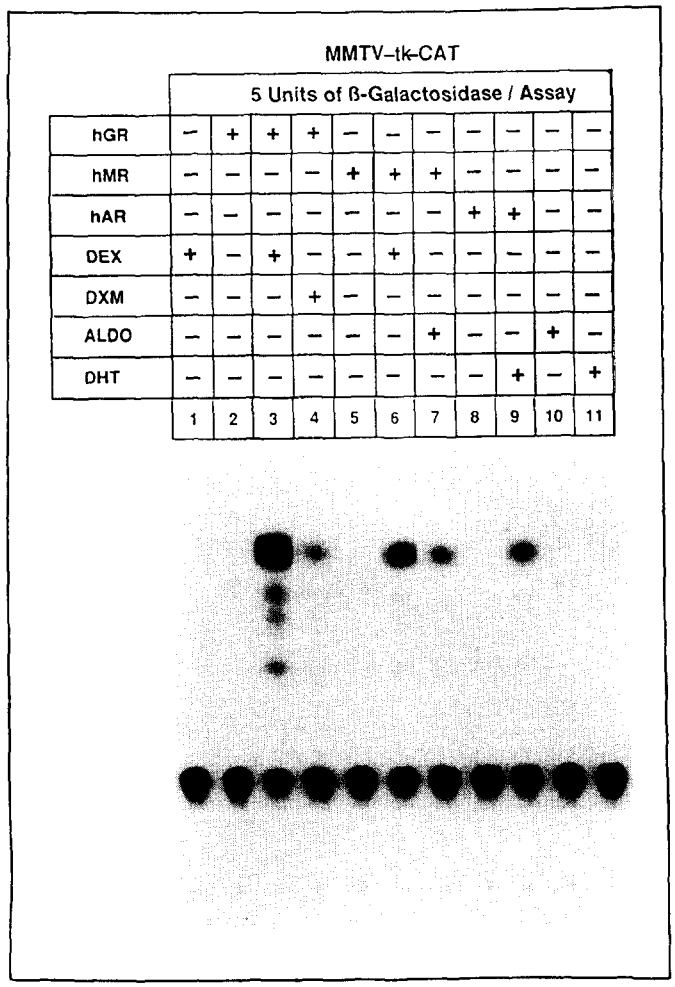


Fig. 6. (A) Differential regulation of MMTV-tk-CAT by hMR_{1-984} in the presence of various steroid hormones. Transfection of HeLa cells with MMTV-tk-CAT in the presence of hGR_{1-777} [14] and hMR_{1-984} expression vector followed by treatment with various steroid hormones as indicated directly on respective assays. Controls contained MMTV-tk-CAT co-transfectants treated with 5 nM DEX (lane 18) and 5 nM ALDO (lane 19), respectively, in the absence of any co-transfected steroid receptors. (B) Quantification of differential regulation in HeLa cells after co-transfection. The autoradiographs were scanned by an image analyzer and plotted as arbitrary CAT units [26]. The evaluations are the results of at least 4–5 identical transfection assays differing by $<5\%$.



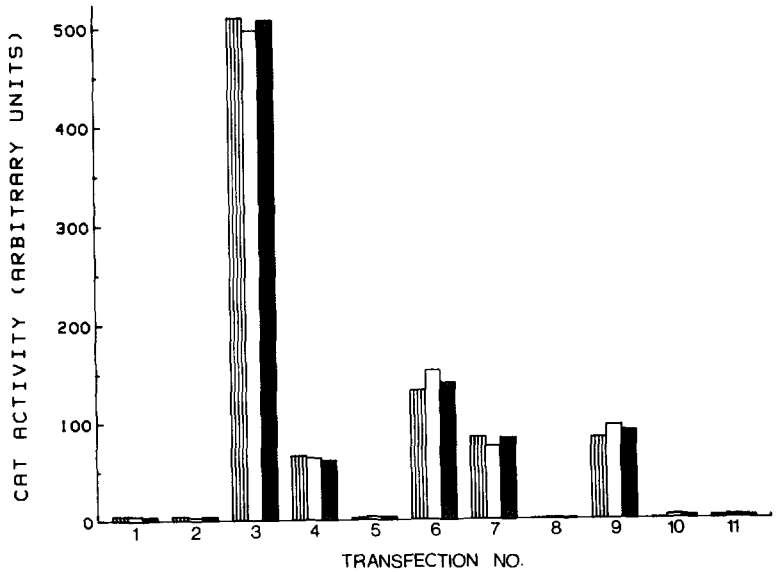


Fig. 7. (A) and (B) Differential regulation of MMTV-tk-CAT by hGR_{1-777}, hMR_{1-984} and $hAR_{244-910}$ in HeLa cells. HeLa cells were co-transfected with hGR_{1-777}, hMR_{1-984} and $hAR_{244-910}$ as described. The transfectants were treated with 5 nM concentrations of various steroid hormones as indicated directly in the legends. The quantification of this assay in triplicate transfection is shown in Fig. 6(B) solely to indicate the variations observed in such transfection studies.

We have shown here by DNA sequence analysis that the exon containing amino acids 1–516 of the A/B region of the hMR is separated from the exon containing the corresponding 5′ untranslated region. The intron–exon junction suggests that these two regions are encoded in separate exons. We concluded this from evidence obtained after sequence analysis wherein our data indicated that the sequence differed after 4 nucleotides 5′ to the ATG initiation codon from the sequence previously reported by Arriza *et al.* [10], strongly suggesting the possibility of a splice junction between nucleotides -5 and -4 upstream of the ATG initiation codon. This shows that the structural organization of hGR and hMR genes have several common features which are different from the organization of other steroid receptor genes [17, 22–24].

Hybridization of our isolated gene fragment with an oligonucleotide 5′ TCCTCTGAGCAGCCTGAAGTTGGCTCCC 3′ complementary to −184 to −212 of the previously described 5′ untranslated region of hMR [10] failed to demonstrate the presence of this sequence in the 4.5 kb genomic fragment while further attempts to localize this sequence in human chromosome 4 genomic DNA library (ATCC) were also unsuccessful. These results indicate that either the 5′ non-coding region found in our genomic fragment differs from the sequence previously reported or that this region may be encoded in a separate exon(s).

The physiological ligand for the type 1 receptor (hMR) is aldosterone. However, we have shown here that the synthetic glucocorticoids DEX and TA, which are widely used in receptor studies and have great importance in clinical applications as "pure" glucocorticoids, demonstrate a potent interaction with the hMR. Considering the high levels of circulating glucocorticoids, the specificity of mineralocorticoid

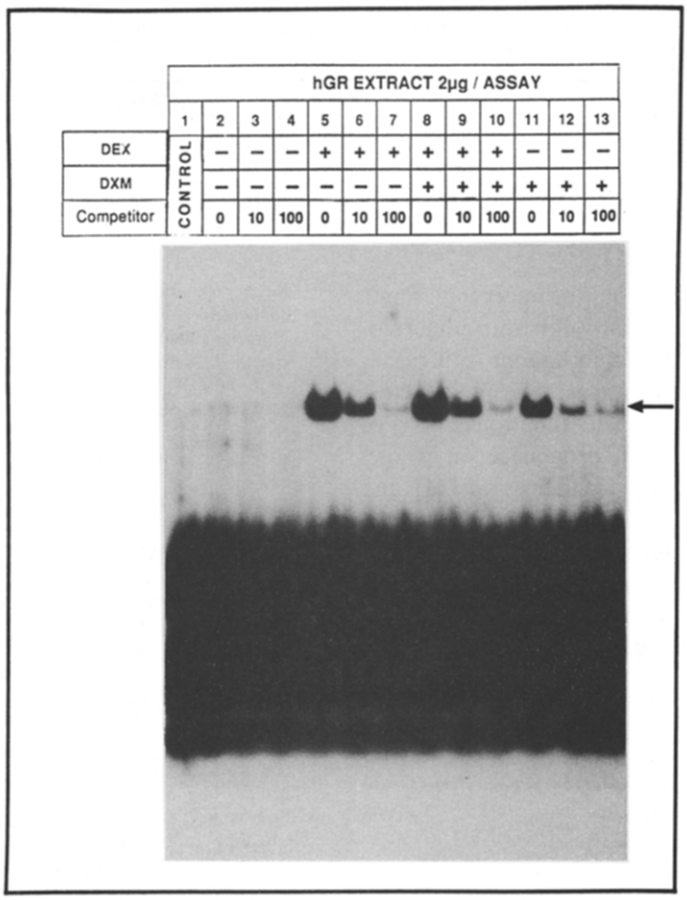


Fig. 8. Interaction of hGR_{1-777} with synthetic palindromic labeled GRE. An aliquot of 2.5 µg of the extracts prepared from cells co-transfected with pKCR-2 (lanes 2–4) or extracts containing hGR_{1-777} (lanes 5–15) incubated with 5 nM DEX (lanes 5–7), 5 nM DEX in the presence of 500 nM DXM (lanes 8–10) and 500 nM DXM (lanes 11–13) were incubated without any competitor (5000 cpm) or labeled GRE in the presence of 10-fold or 100-fold molar excess of non-labeled GRE and separated as described [14]. The arrow indicates the retarded complexes.

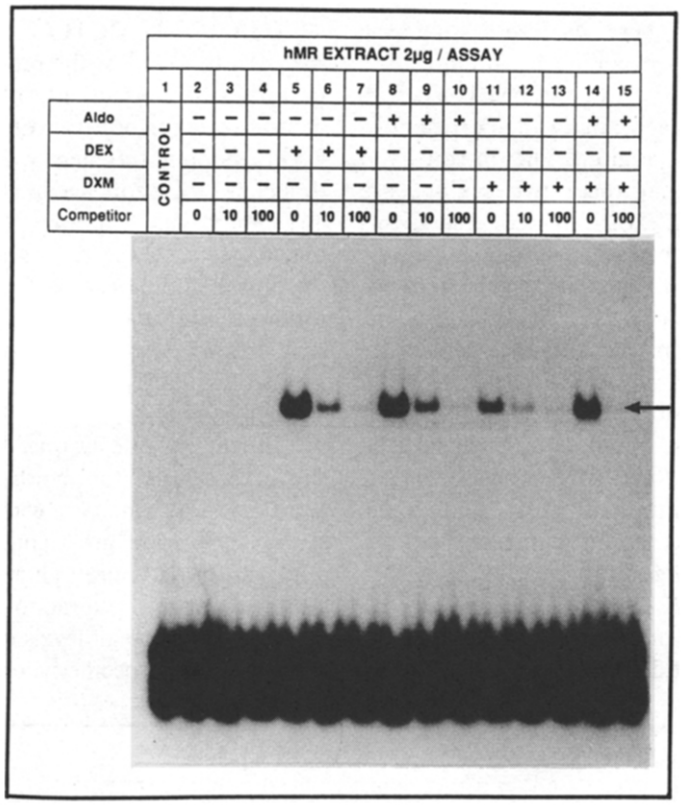


Fig. 9. Interaction of hMR_{1-984} with GRE. An identical binding experiment with hMR1–984 extract with GRE was performed. The extract was incubated with 5 nM DEX (lanes 5–7), 5 nM ALDO (lanes 8–10), 500 nM DXM (lanes 11-13) and 5 nM ALDO in the presence of 500 nM DXM (lanes 14 and 15), respectively prior to the incubation with 5000 cpm ^{32}P-end-labeled GRE in the absence or presence of 10-fold or 100-fold cold non-radioactive GRE. The complexes were separated on polyacrylamide gel, dried and autoradiographed. The arrow indicates the retarded complexes.

action rests not solely on the receptor, which shows a comparable affinity for both mineralocorticoid and glucocorticoid ligands, but rather on other known mechanisms. The most plausible of which includes the functional role of 11β-hydroxysteroid dehydrogenase in target tissues as suggested by Funder *et al.* [11] and Edwards *et al.* [12].

Further studies in this direction will open up greater possibilities to investigate the role of such intermediate regulatory pathways in defining the detailed mechanisms involved in the action of the MR.

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