Cloning of Human Mineralocorticoid Receptor Complementary DNA: Structural and Functional Kinship with the Glucocorticoid Receptor

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Low-stringency hybridization with human glucocorticoid receptor (hGR) complementary DNA was used to isolate a new gene encoding a predicted 107-kilodalton polypeptide. Expression studies demonstrate its ability to bind aldosterone with high affinity and to activate gene transcription in response to aldosterone, thus establishing its identity as the human mineralocorticoid receptor (hMR). This molecule also shows high affinity for glucocorticoids and stimulates a glucocorticoid-responsive promoter. Together the hMR and hGR provide unexpected functional diversity in which hormone-binding properties, target gene interactions, and patterns of tissue-specific expression may be used in a combinatorial fashion to achieve complex physiologic control.

THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS INTEGRATES a variety of neuroendocrine inputs to regulate the synthesis and secretion of the adrenal corticosteroids. These steroid hormones exert effects on growth, development, and homeostasis by their interaction with intracellular receptor proteins that directly regulate the transcription of sets of target genes (*1, 2*). Two receptor systems have been defined for the corticosteroids; these are termed the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Early functional assays classified the corticosteroids as either glucocorticoid, by their effect in promoting glycogen deposition in the liver, or mineralocorticoid, by their effect in promoting sodium retention by the kidney. However, each steroid class is not restricted to interacting with only its cognate receptor, and glucocorticoids, in particular, can have substantial mineralocorticoid activity (*1–3*).

It is now evident that the MR has significant in vitro affinity for both glucocorticoids and mineralocorticoids (*3, 4*). Since the circulating levels of glucocorticoids are several orders of magnitude higher than those of aldosterone, the primary mineralocorticoid, glucocorticoid activation of the MR may be functionally significant. Whereas the secretory epithelia of tissues such as kidney and intestine regulate electrolyte and water balance in response to aldosterone, it is possible that additional mechanisms confer these tissues with sensitivity to mineralocorticoids (*5*). No clear functional role has emerged for the MR expressed in other tissues, but physiological responses in brain may result from glucocorticoid interactions with the MR (*5–7*).

Despite the availability of high-affinity radioactively labeled ligands, the MR has been refractory to purification, and its biochemical properties, in comparison to GR, remain poorly understood. Application of the techniques of molecular biology to the study of the MR would facilitate its biochemical characterization and, eventually, an understanding of the genes under its transcriptional control and the roles their products play in homeostasis.

Molecular cloning of the glucocorticoid (*8, 9*), estrogen (*10*), and progesterone (*11*) receptors has permitted the determination of their primary amino acid structures and prediction of functional domains common to this family of regulatory proteins. Experimental dissection of glucocorticoid (*12*) and estrogen (*13*) receptors has revealed a centrally located DNA-binding domain rich in cysteine, lysine, and arginine, and a carboxyl-terminal region where steroid hormones interact. Functional studies of GR suggest that hormone binding to the carboxyl terminus unmasks the DNA binding region to permit interactions of receptor with DNA and activation of transcription (*14, 15*). Comparison of the cysteine-rich DNA-binding regions of steroid and thyroid hormone receptors shows a high degree of relatedness between these molecules (*16*). The invariant cysteine residues have led to the hypothesis that coordination of Zn^{2+} metal atoms maintains a structural configuration for DNA binding analogous to that proposed for *Xenopus* 5S gene transcription factor IIIA (*17*). The steroid-binding regions of the steroid receptor family also show substantial conservation consistent with evolution of various receptor classes from a common ancestral precursor (*11, 16*).

We have used the structural similarity between steroid hormone receptors to isolate a gene product closely related to the human glucocorticoid receptor (hGR). Nonstringent hybridization with an hGR probe was used to isolate a human genomic DNA fragment

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highly related to the hGR cysteine-rich sequence. Using this DNA as a probe, we obtained complementary DNA's (cDNA's) that code for a molecule having a strong homology with the hGR from the cysteine-rich region to the carboxyl terminus. When expressed in cells, this molecule binds aldosterone with high affinity and activates aldosterone-responsive transcription of the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV). The overlap of the ligand and DNA sequence specificities of this human mineralocorticoid receptor with those of hGR suggest that the distinct roles traditionally assigned to these regulatory molecules should be reconsidered.

Isolation of hMR cDNA. For the identification of glucocorticoid receptor–related genes, human placenta DNA was digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and the fractions were hybridized with hGR1.2, an 1100-bp fragment of hGR cDNA containing sequences encoding the DNA-binding domain (*8, 15*). Southern blot analysis revealed several distinct bands specific to low-stringency hybridization conditions (compare Fig. 1, A and B). The 2.5-kilobase pair (kbp) Hind III fragment (bracketed by asterisks in Fig. 1B) was well resolved from other hybridizing bands and was judged suitable for direct genomic cloning. Hind III–digested DNA from human placenta was preparatively size-fractionated on an agarose gel, and the 2.5-kbp region was isolated for the construction of a genomic library. This λgt10 library was then screened under conditions of low-stringency hybridization with hGR1.2 as the probe. The insert from one positive genomic clone, λHGH, was nick-translated and used as a probe on a Southern blot under high-stringency hybridization conditions (Fig. 1C). The 2.5-kbp Hind III signal corresponded to that seen under nonstringent conditions, indicating that a portion of the desired genomic fragment had been isolated. Sequence analysis of the insert from λHGH revealed an exon of 140 base pairs (bp) flanked by intron sequences (Fig. 1D). Overall this exon has 68 percent nucleotide identity with the homologous hGR cDNA sequence, but a region conserving 85 nucleotides out of 104 probably confers its cross-hybridization properties. This highly conserved region corresponds to a portion of the hGR DNA-binding domain (*15*). The λHGH exon codes for 46 amino acids beginning with 16 nonconserved residues and followed by the first of the highly conserved cysteine residues characteristic of steroid hormone receptors (*8–11*). Of the next 30 residues, 28 are identical to hGR. These analyses demonstrated the isolation of a genomic fragment containing a sequence related to, but clearly distinct from, that found in the hGR cDNA sequence (*8*).

The insert from λHGH was used as a probe to screen cDNA libraries for clones corresponding to this hGR-related gene. Mineralocorticoid receptor was considered a likely candidate to be encoded by such a gene. Since kidney is known to be a mineralocorticoid-responsive tissue, several human kidney cDNA libraries were screened. Eleven positive clones were isolated from these λgt10 libraries at a frequency of three to four per 10^6 recombinant phage. Two overlapping clones, λhk2 and λhk10, were subjected to nucleotide sequence analysis and together found to span 5823 nucleotides (Fig. 2). The exon-intron boundaries of λHGH were verified by sequencing these cDNA clones. The λhk10, encompassing nucleotides 1 to 3750, contains a large open reading frame predicting the entire primary amino acid sequence. The DNA insert from λhk2 extends from nucleotides 802 to 5823, but contains an internal 351-bp deletion from 2235 to 2586. Three additional clones were examined and determined to have the same structure as λhk10 in the deleted region. It is likely that the deletion in λhk2 represents either a cloning artifact or a rare messenger RNA (mRNA) splicing error (*18*). The sequence of the reported 3′-untranslated region downstream of nucleotide 3750 is derived from λhk2. The composite sequence of these two cDNA's is termed hMR (Fig. 2A). With the first in-frame ATG (position 223) downstream of an in-frame termination codon (position 136), hMR has a 5′-untranslated region of at least 216 nucleotides. The sequence surrounding this first ATG agrees with the consensus described by Kozak (*19*). This predicted initiator methionine codon begins an open reading frame encoding 984 amino acids. Following a termi-

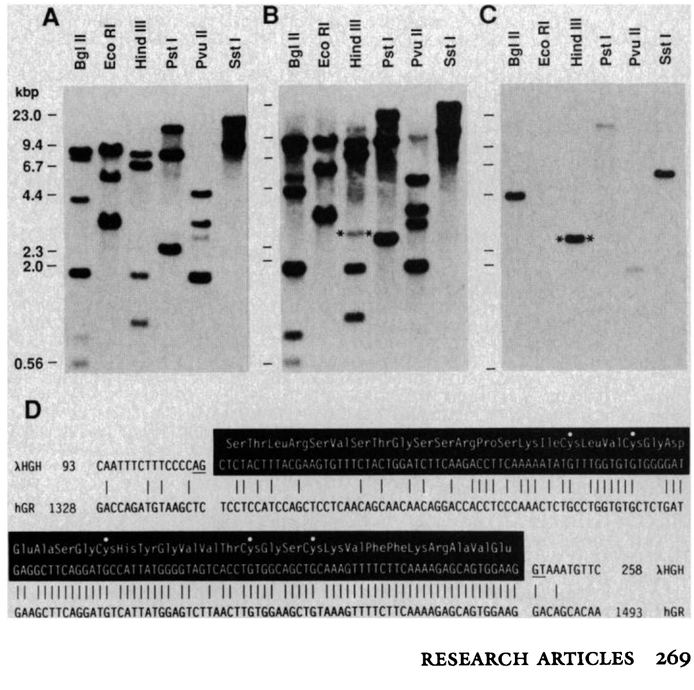
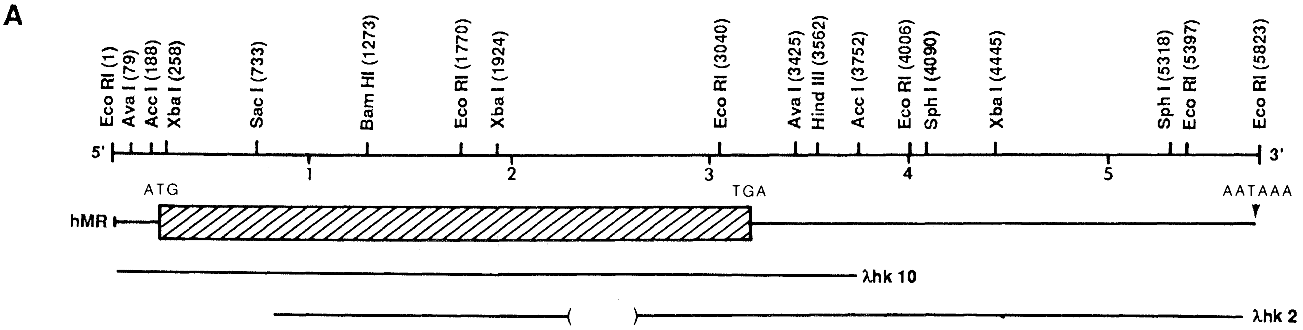


Fig. 1. Isolation of a genomic sequence related to the hGR gene. (**A**) High-stringency Southern analysis of human placenta DNA digested with the indicated restriction endonucleases. hGR cDNA (hGR1.2) was used as a probe. Sizes of lambda DNA fragment markers (in kilobase pairs) prepared by Hind III digestion are indicated next to the autoradiogram. (**B**) Low-stringency Southern analysis. The 2.5-kbp band bracketed by asterisks in the Hind III lane was the sequence targeted for direct genomic cloning. (**C**) Isolation of this genomic sequence in a clone designated λHGH is demonstrated by its use as a probe on a similar Southern blot. The λHGH genomic fragment contains the hybridizing internal Eco RI fragment isolated from this cloning. (**D**) The intron-exon structure of the λHGH genomic fragment and its homology with hGR. The hGR-related exon found within λHGH is boxed in black with its predicated amino acid sequence. Conserved cysteine residues are indicated with white dots. Portions of intron sequence with consensus splice donor and acceptor sites underlined are shown flanking the exon. Nucleotide homology with the hGR is shown underneath. Nucleotide numbers for hGR are from Hollenberg *et al.* (*8*). For Southern analysis, we digested DNA from human term placenta with restriction endonucleases, and products were separated on a 0.8 percent agarose gel. The DNA's were transferred to nitrocellulose paper and hybridized under either stringent or nonstringent conditions. Stringent hybridization was performed with 50 percent formamide, 5× SSPE (NaCl, NaH_2PO_4, EDTA, *p*H 7.4), 1× Denhardt's, 0.1 percent SDS, salmon sperm DNA at 100 μg/ml, and probe (10^6 cpm/ml) at 42°C. For nonstringent hybridization, 35 percent rather than 50 percent formamide was used. Washing conditions consisted of 0.1× SSC (standard saline citrate) with 0.1 percent SDS at 60°C for stringent analyses and 2× SSC with 0.1 percent SDS at 55°C for nonstringent filters. Washing conditions with the 338-bp insert from λHGH as probe were modified to 2× SSC with 0.1 percent SDS at 68°C. For isolation of λHGH, human placenta DNA (300 μg) was digested with Hind III and size-fractionated on a 1 percent low-melting agarose gel (Seaplaque, FMC). The gel was sliced in 0.5-cm strips, and the DNA was purified by phenol extraction and ethanol precipitation. DNA (2 μg) from the fraction corresponding in size to the band bracketed by asterisks in (B) was repaired with Klenow DNA polymerase for Eco RI linker addition. After digestion with Eco RI and removal of excess linkers on a Sepharose 4B column, this DNA was ligated to Eco RI–digested λgt10 DNA and packaged in vitro (λ arms and extracts from Vector Cloning Systems, San Diego, California). About 4 × 10^5 independent recombinants were screened under conditions identical to those used for the nonstringent Southern analysis to obtain λHGH.



B

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                                                                                                         10                            20
                                                                              MetGluThrLysGlyTyrHisSerLeuProGluGlyLeuAspMetGluArgArgTrpGlyGlnValSerGlnAlaVal
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               30                            40                            50                            60                            70
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               80                            90                            100                           110                           120
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      SerMetSerProAlaLysIleTyrGlnAsnValGluGlnLeuValLysPheTyrLysGlyAsnGlyHisArgProSerThrLeuSerCysValAsnThrProLeuArgSerPheMetSerAspSerGlySerSerValAsnGlyGlyVal
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               280                           290                           300                           310                           320
      SerHisCysSerValLysSerProValSerSerProAsnAsnValThrLeuArgSerSerValSerSerProAlaAsnIleAsnAsnSerArgCysSerValSerSerProSerAsnThrAsnAsnArgSerThrLeuSerSerProAla
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               330                           340                           350                           360                           370
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      ValProGlyPheAspGlyAsnCysGluGlySerGlyPheProValGlyIleLysGlnGluProAspAspGlySerTyrTyrProGluAlaSerIleProSerSerAlaIleValGlyValAsnSerGlyGlyGlnSerPheHisTyrArg
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               630                           640                           650                           660                           670
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               680                           690                           700                           710                           720
      LysLeuGlyLysLeuLysGlyIleHisGluGluGlnProGlnGlnGlnGlnProProProProProProProProGlnSerProGluGluGlyThrThrTyrIleAlaProAlaLysGluProSerValAsnThrAlaLeuValProGln
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               730                           740                           750                           760                           770
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               830                           840                           850                           860                           870
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               880                           890                           900                           910                           920
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               930                           940                           950                           960                           970
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      LysProLeuTyrPheHisArgLysEnd
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5701  ATATGTCTGTCTTTGCAAAGGACTGTAAGCAGATTACAATAAATCCTTTACTTTAAAAAAAAAAAAAAAAAAAAA
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nation codon (position 3175) is a 2.6-kb 3′-untranslated region with a typical polyadenylation signal (AATAAA) found 17 nucleotides upstream of a 70-nucleotide poly(A) (polyadenylated) tract. Long 3′-untranslated regions are a characteristic feature of steroid hormone receptor mRNA's (*8–11*).

The DNA- and hormone-binding regions. The protein encoded by hMR cDNA has the structural properties of a steroid hormone receptor closely related to hGR. Comparison of the predicted amino acid sequence of hMR with that of hGR demonstrated high degrees of homology with both the hGR DNA binding and steroid binding domains. The hMR gene encodes a protein of 984 amino acids with a predicted molecular size of 107 kD, significantly larger than the 777 residues of hGR. This size discrepancy is primarily due to the large amino terminus, which bears no homology to hGR. Considerable heterogeneity of size and sequence for this region exists between the receptors for glucocorticoid, estrogen, and progesterone (*8–11*). Amino acid homology begins in the centrally located DNA region with 94 percent amino acid identity in 68 residues (Fig. 3). Separating the DNA-binding domain and the carboxyl-terminal steroid-binding domain is a region with relatively low sequence conservation found between other steroid hormone receptors. It has been speculated that the region may serve as a molecular hinge between the two domains (*12, 13*). Comparison with hGR shows this region of hMR to contain an additional 24 amino acids including a sequence of 4 glutamines followed by 8 prolines encoded by repetitive nucleotide elements. The significance of this unusual sequence in terms of origin and function is unclear, but structure-breaking prolines are consistent with a hinge region. A comparison of the carboxyl-terminal 250 amino acids of hMR with hGR shows 57 percent amino acid identity as well as a number of conservative amino acid substitutions. Some of these substitutions may preserve hydrophobic regions necessary for steroid hormone interaction.

Expression and hormone binding. We have previously used transfection of the monkey kidney cell line CV1 and its derivative (that is, SV40 T antigen–transformed) cell line COS-1 (referred to as COS) to study glucocorticoid receptor function (*12*). High levels of polypeptide expression from transfected hMR were essential to facilitate steroid-binding experiments in transfected cells. Since plasmids containing the SV40 origin of replication can replicate to high copy numbers in COS cells, an expression vector for hMR coding sequences similar to pRShGRα, used previously in hGR studies, was constructed (*12*). The plasmid, pRShMR, contains the hMR coding sequence, under the control of the promoter from Rous sarcoma virus, and the SV40 origin of replication (Fig. 4A).

Ligand specificity of the hMR protein was determined by preparing cytosol extracts from COS cells transfected with pRShMR. Two days after transfection, cells were harvested, and hormone binding was measured by a dextran-treated charcoal assay. Mock-transfected control extracts had no specific binding activity for [^{3}H]aldosterone, whereas extracts from pRShMR-transfected cells bound significant amounts of [^{3}H]aldosterone with high affinity. A dissociation constant (K_D) of 1.3 n*M* for the binding of [^{3}H]aldosterone was determined by Scatchard analysis (Fig. 4B). This value is in good agreement with those reported for aldosterone binding to mineralocorticoid receptor (*2, 20*). Competition experiments were then performed to examine the ability of different unlabeled steroids to compete with 5 n*M* [^{3}H]aldosterone for binding when present at 1-, 10-, or 100-fold molar excess (Fig. 4, C and D). This provided a measure of the relative affinity of each of these steroids for hMR. The results of these experiments show that aldosterone, corticosterone, deoxycorticosterone, and hydrocortisone (cortisol) all have very similar affinities for hMR. Dexamethasone, progesterone, and spironolactone demonstrated weaker binding affinity while estradiol competed very poorly for binding to hMR. Overall, this hierarchy of affinities indicated that hMR encoded the human mineralocorticoid receptor (*2, 20*).

Transcriptional activation. Steroid hormone action is characterized by hormone-dependent modulation of target gene transcription. An assay for transcriptional regulation by transfected hGR in CV1 cells (*12*) was adapted to hMR (Fig. 5). The expression plasmid used for steroid-binding assays, pRShMR, was cotransfected with a reporter plasmid called GMCAT, which contains the MMTV LTR linked to the bacterial gene for chloramphenicol acetyltransferase (CAT). Thus CAT activity provides an enzymatic assay for the transcriptional activity of the MMTV promoter. The MMTV promoter contains several glucocorticoid response elements (GRE's), enhancer-like DNA sequences that confer glucocorticoid responsiveness via interaction with the GR (*21*). It was possible that hMR, because of the near identity of its DNA-binding domain to that of hGR, might also recognize the MMTV LTR. When CV1 cells were cotransfected with pRShMR and GMCAT, we observed full CAT activity. This activity was independent of added aldosterone suggesting that, in contrast to transfected hGR, sufficient hormone was present in serum (fetal calf serum, 5 percent) to fully activate hMR (Fig. 5B). In the presence of charcoal-treated serum (*22*) CAT activity became responsive to the addition of exogenous aldosterone (Fig. 5C), indicating that hMR cDNA encodes a functional steroid hormone receptor. While the hMR was also activated by the glucocorticoid agonist dexamethasone, the hGR did not respond to even supraphysiological concentrations (10 n*M*) of aldosterone.

Tissue-specific expression. We examined the expression of MR mRNA homologous to hMR cDNA in rat tissues by Northern blot hybridization (*23*). Classical mineralocorticoid target tissues such as kidney (*24*) and gut (*25*), as well as tissues such as brain, pituitary, and heart, contained mRNA homologous to hMR (Fig. 6). Aldosterone-sensitive cells in kidney are primarily restricted to the distal and cortical collecting tubules (*2*), and therefore a modest level of expression in this tissue was not unexpected. High levels of MR (type I corticosteroid-binding sites) have been reported in rat brain, particularly in the hippocampal formation (*4, 6*). In comparing dissected hippocampal RNA with RNA prepared from total brain, we found a striking enrichment of message in the hippocampus. While aldosterone binding has been reported for pituitary (*26*), cultured aortic cells (*27*), and spleen (*28*), no such activity has been reported in muscle. Liver expresses GR, but has no detectable high-affinity aldosterone-binding activity (*29*), and as would be expected no hybridization to liver RNA was observed. Reprobing of the same Northern blot with an analogous portion of hGR cDNA demon-

Fig. 2. Nucleotide sequence and primary amino acid structure of human mineralocorticoid receptor. (**A**) Composite structure of hMR aligned with a line diagram of some restriction endonuclease cleavage sites (Eco RI sites shown at nucleotides 1 and 5823 and derived from linkers). The composite was assembled from two overlapping λgt10 clones, λhk10 and λhk2. Parentheses in the line diagram of λhk2 indicate a 351-bp deletion. The hatched box indicates predicted coding sequence with initiator and termination codons indicated. (**B**) Complete nucleotide sequence of hMR and its predicted primary amino acid sequence. Underlined are a 5′ in-frame termination codon upstream of the predicted initiator methionine and four potential polyadenylation sites (AATAAA). Human kidney λgt10 libraries (*18*) were screened with the insert from λHGH under the same conditions described for Southern analysis under high-stringency conditions with this probe. Overlapping deletions of each cDNA were obtained (*36*) by the Cyclone rapid deletion subcloning method (International Biotechnologies). Deletion clones were sequenced by the dideoxy procedure (*37*), and any gaps or ambiguities were resolved by the chemical cleavage method (*38*). DNA sequences were compiled and analyzed by the programs of Devereux *et al.* (*39*) and Staden (*40*).

```
                ┌──DNA──►
hMR  548   SSFPPVNTLVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRSVSTGSS
                               |  |            |||    |
hGR  374   NLTSLGTLNFPGRTVFSNGYSSPS........MRPDVSSPPSSSSTATTG
                ┌──DNA──►
hMR  598   RPSKICLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQHNYLCAGRNDCII
            | | |||| |||||||||| |||||||||||||||||||||||||||||
hGR  416   PPPKLCLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCII
                         ◄──DNA──┐
hMR  648   DKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPP
           ||||||||||||  ||||||||| ||| ||  |          |
hGR  466   DKIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATTGVSQ......
                                               ┌── Steroid ──►
hMR  698   PPPPQSPEEGTTYIAPAKEPSVNTALVPQLSTISRALTPSPVMVLENIEP
                             | | |             |||  |  || |||
hGR  510   ..................ETSENPGNKTIVPATLPQLTPTLVSLLEVIEP

hMR  748   EIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLED
           |  ||||||| ||       ||| | | | |  |||||  ||| || | |
hGR  542   EVLYAGYDSSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLDD

hMR  798   QITLIQYSWMCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYE
           | || ||||| |  ||| ||||       | |||||  ||  |    ||
hGR  592   QMTLLQYSWMFLMAFALGWRSYRQSSANLLCFAPDLIINEQRMTLPCMYD

hMR  844   LCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMRT
            |  |   |    |||   |||  || |||||  ||||||||  | | |
hGR  642   QCKHMLYVSSELHRLQVSYEEYLCMKTLLLLSSVPKDGLKSQELFDEIRM

hMR  898   NYIKELRKMVTKCPNNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFR
            ||||| |   |   || | |||||||||||||||  |  ||  || ||
hGR  692   TYIKELGKAIVKREGNSSQNWQRFYQLTKLLDSMHEVVENLLNYCFQTF.
                                 ◄── Steroid ──┐
hMR  948   ESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK  984
                  ||| || |||  | ||   || | | || |
hGR  741   LDKTMSIEFPEMLAEIITNQIPKYSNGNIKKLLFHQK  777
```

Fig. 3. Amino acid homology of mineralocorticoid receptor with glucocorticoid receptor. The primary amino acid sequence of hMR has been aligned with that of hGR for maximum homology by introducing gaps as indicated by dots. Numbers were taken from Fig. 2 for hMR and from Hollenberg *et al.* (*8*) for hGR. No significant homology was found upstream of the region shown. Vertical lines indicate identical amino acid residues. Arrows show putative boundaries of the DNA-binding (DNA) and steroid-binding (Steroid) domains. The amino-terminal border of the DNA-binding domain was arbitrarily defined by the first conserved cysteine residue while the carboxyl-terminal limit was chosen on the basis of mutagenesis studies which indicated sequences necessary for DNA-binding and transcriptional activation (*15*). Several conserved basic residues that follow the DNA binding domain may also be important for these functions. The limits of the steroid-binding domain, while defined by the region of amino acid homology, are also consistent with mutational analysis. Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

strated hybridization to mRNA species of different sizes, and indicated that the MR and GR do show differential patterns of tissue-specific expression.

Chromosome mapping. To determine the chromosomal location of the mineralocorticoid receptor gene, we tested hMR against a panel of rodent-human somatic cell hybrids retaining different combinations of human chromosomes (*30*). The DNA fragments specific for the mineralocorticoid receptor gene segregated concordantly with human chromosome 4 in 15 hybrid cell lines. Discordant segregation was observed for all other human chromosomes, including chromosome 5, site of the glucocorticoid receptor gene (*8, 31*). To confirm the assignment to chromosome 4, we tested a restricted set of microcell hybrids, each of which carry one to three human chromosomes (*32*), for the hMR gene by Southern analysis (Fig. 7). Six Eco RI fragments detected by the coding portion of λhk2 co-segregate with chromosome 4 in this hybrid panel. In particular, the hMR gene is present in HDm-1132B, a cell line that carries chromosome 4 as its only human chromosome.

Implications for adrenal corticosteroid physiology. Human mineralocorticoid receptor cDNA encodes a polypeptide that is highly homologous to the human glucocorticoid receptor. In the DNA-binding domain, hMR maintains approximately 94 percent amino acid identity to hGR while the steroid-binding domain localized in the carboxyl terminus has 57 percent identity. The recently reported sequence (*11*) of the rabbit progesterone receptor (rPR) also has a high degree of relatedness to hMR. Comparison of the amino acid identity in hGR and rPR structural domains with that of hMR (Fig. 8) demonstrates the remarkable similarity of these functionally distinct regulatory proteins. The homology of hMR with rPR is almost identical to the hGR-hMR comparison, with 90

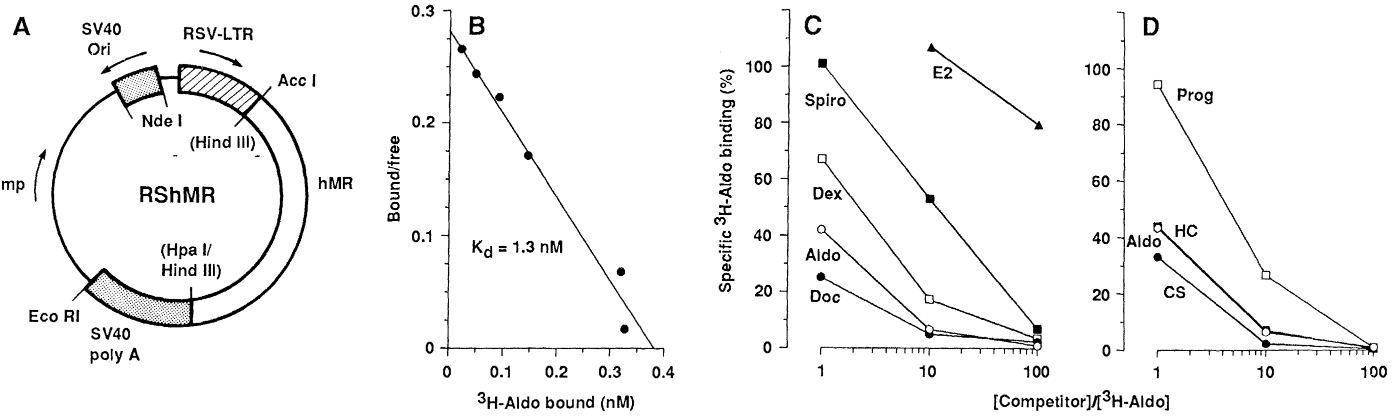

Fig. 4. Steroid-binding properties of expressed hMR. (**A**) Structure of pRShMR, the hMR expression plasmid (*41*). (**B**) Scatchard analysis of tritiated aldosterone binding in extracts prepared from pRShMR-transfected COS cells. Each point was assayed in triplicate with 100 μg of extract protein in a 200-μl incubation at 0°C for 2.5 hours. The nonspecific binding determined with a 500-fold excess of unlabeled aldosterone was approximately 20 percent of total counts. No specific binding was seen in mock-transfected cells. (**C** and **D**) Competition of unlabeled steroids for binding with 5 n*M* [^{3}H]aldosterone in transfected COS cell extracts. The results of two independent trials representative of these competition experiments are shown. Cold competitor was present in 1-, 10-, or 100-fold molar excess. The value for 100 percent binding was determined by subtracting the number of counts per minute bound in the presence of 1000-fold excess of unlabeled aldosterone from the counts bound in the absence of competitor. Abbreviations: Aldo, aldosterone, Doc, deoxycorticosterone; Dex, dexamethasone; Spiro, spironolactone; E2, 17β-estradiol; CS, corticosterone; HC, hydrocortisone; and Prog, progesterone. Subconfluent COS cells were transfected by the DEAE-dextran method (*42*) with 10 μg of pRShMR per dish. Cells were maintained for 2 days in DMEM (Dulbecco's modification of Eagle's minimum essential medium) with 5 percent charcoal-treated fetal calf serum, then harvested [in 40 m*M* tris-HCl (*p*H 7.5), 150 m*M* NaCl, and 1 m*M* EDTA] and lysed by Dounce homogenization in hypotonic buffer containing 10 m*M* tris-HCl (*p*H 7.8), 10 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Na_2MoO_4, 5 m*M* dithiothreitol, antipain (5 μg/ml), leupeptin (5 μg/ml), and 500 μ*M* phenylmethylsulfonyl fluoride. After centrifugation at 15,000*g* for 10 minutes, extracts were adjusted to 100 m*M* NaCl and 5 percent glycerol before binding. Labeling reactions with [^{3}H]aldosterone (specific activity 78 Ci/mmol, Amersham) were incubated for 2.5 hours at 0°C in a total volume of 200 μl, and then for 10 minutes with 20 μl of 50 percent dextran-coated charcoal (10:1 activated charcoal:dextran). After centrifugation at 15,000*g* for 2 minutes at 4°C, tritium in supernatant was quantified by liquid scintillation spectrophotometry.

percent of the amino acids shared in the DNA-binding domain and 56 percent in the steroid-binding region. In contrast, a comparison of the same regions of hMR with human estrogen receptor (*10*) indicates 56 percent identity in the DNA binding domain and 21 percent sequence identity in the steroid-binding carboxyl terminus. The degree of structural homology shared by hMR, hGR, and rPR, and the structural relatedness of their ligands, suggests that they may comprise a subfamily of steroid hormone receptors.

Expression of the hMR polypeptide in COS cells by transient transfection permitted the evaluation of its steroid-binding potential. The results of these analyses indicated that hMR cDNA encodes a human mineralocorticoid receptor. Scatchard analysis demonstrated that extracts from cells transfected with pRShMR bound [^{3}H]aldosterone with a K_D of 1.3 n*M*, while reported K_D values for aldosterone binding to MR range from 0.5 to 3 n*M* (*2*). This is the single most important criterion in defining this gene product as the human mineralocorticoid receptor. Steroid-binding competition studies have further supported this identification of hMR. The mineralocorticoid deoxycorticosterone and the glucocorticoids corticosterone and cortisol compete as effectively as aldosterone itself, whereas the synthetic glucocorticoid dexamethasone and progesterone have lower affinities for the hMR.

The extensive amino acid sequence identity in the presumptive steroid-binding domains of hMR, hGR, and rPR is compatible with the similar ligand-binding properties of these receptors. The mineralocorticoid, glucocorticoid, and progesterone receptors exhibit a limited ability to discriminate between the similar 21-carbon atom structures of the mineralocorticoids, glucocorticoids, and progestins. This lack of specificity is particularly relevant to the MR and GR. For example, the MR binds glucocorticoids with an affinity equal to that for aldosterone. Indeed, it may be that only in tissues such as kidney, where additional mechanisms confer selective response to aldosterone, does the MR function as a classical mineralocorticoid receptor (*3, 5*). The MR also binds progesterone with a high affinity, but one lower than its affinity for corticosteroids. There is some indication that progesterone may act as a partial agonist or antagonist of mineralocorticoid action (*33*), and it is not

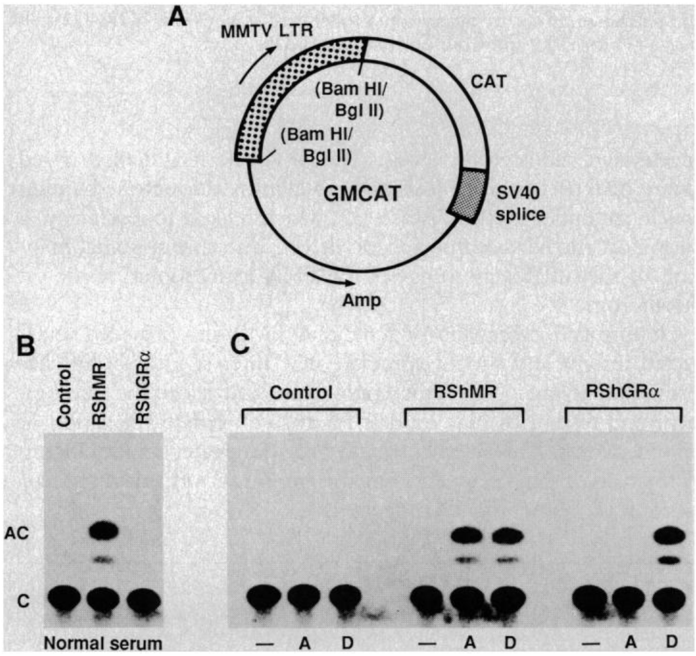

Fig. 5. Transcriptional activation of MMTV LTR by hMR and hGR expression plasmids in transfected CVI cells. (**A**) Structure of GMCAT. This plasmid was cotransfected with the steroid receptors as a reporter gene for hormone-dependent transcriptional activation (*12*). (**B**) Differential CAT enzyme activity found after hMR or hGR transfection with normal serum. Transfected cells were maintained in DMEM with 5 percent fetal calf serum. Serum was treated with charcoal to eliminate free steroids in subsequent experiments so that the effects of exogenous steroids could be determined. (**C**) Differential induction of CAT activity by aldosterone or dexamethasone in cells transfected with hMR or hGR. CV1 cells were cotransfected with 10 μg of either pRSVgal (control), pRShMR, or pRShGRα and 10 μg of the reporter GMCAT and cultured in the absence (−) or presence of 10 n*M* aldosterone (A) or 10 n*M* dexamethasone (D). AC, 3-acetylchloramphenicol; C, chloramphenicol. Two days after transfection by calcium phosphate coprecipitation (*43*), extracts were prepared for CAT assay (*44*). The assays were incubated for 6 hours with 50 μg of protein extract.

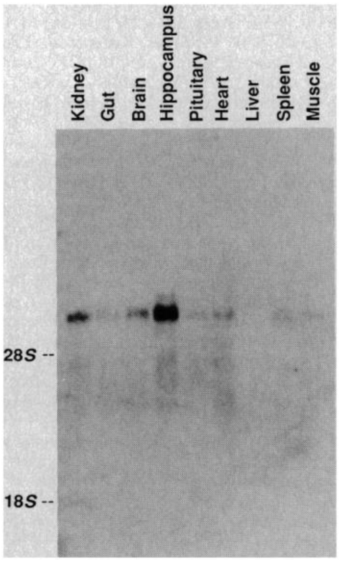

Fig. 6. Northern analysis of mineralocorticoid receptor mRNA's in rat tissues. The 1270-bp Eco RI fragment (1770 to 3040) from λhk10 was used as a probe for the expression of homologous mRNA's in rat. Ten micrograms of poly(A)$^+$ mRNA was used in all lanes. Migration of ribosomal RNA's (28*S* and 18*S*) are indicated for size markers. After hybridization under stringent conditions, the filter was washed twice for 30 minutes each time in 2× SSC with 0.1 percent SDS at 68°C.

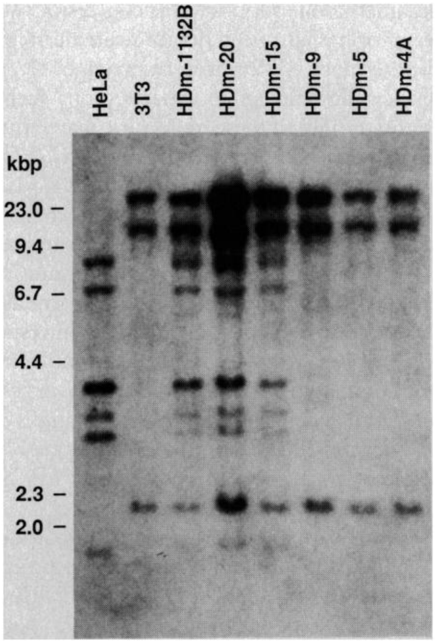

Fig. 7. Chromosomal localization of hMR gene by Southern analysis of microcell hybrids. The construction and characterization of these hybrids has been previously described (*32*). The human chromosome content of each is as follows: HDm-4A (chromosome 20), HDm-5 (chromosome 14 and an unspecified E group chromosome), HDm-9 (chromosomes 20, 14, and 21), HDm-15 (chromosomes 21, 11, and 4), HDm-20 (chromosomes 7 and 4), and HDm-1132B (chromosome 4 only). Human (HeLa) and mouse (3T3) control DNA samples are also shown. Genomic DNA from microcell lines (10 μg) was digested with Eco RI and subjected to electrophoresis through a 1.0 percent agarose gel, transferred to a nylon membrane (Nytran, Schleicher & Schuell), and hybridized with an hMR cDNA probe under high-stringency conditions (Fig. 1). Radioactive probe was synthesized by the Klenow fragment of *Escherichia coli* DNA polymerase from two randomly primed (*45*) hMR cDNA templates (the 1000- and 800-bp Eco RI fragments of λhk2). The sizes of Hind III–digested lambda DNA fragments are indicated next to the autoradiogram.

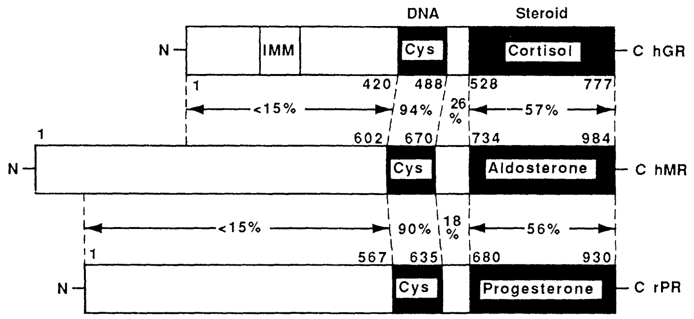

Fig. 8. Schematic amino acid comparisons of the hGR, hMR, and rPR structures. Primary amino acid sequences have been aligned schematically with the percentage amino acid identity indicated for each region of homology in the intervals between dotted lines. The amino acid position of each domain boundary is shown for each receptor. N and C represent the amino and carboxyl termini, respectively. Cys corresponds to the cysteine-rich region encoding the putative DNA-binding domain while Steroid (cortisol, aldosterone, or progesterone) designates the steroid-binding domain. The immunogenic region (IMM) of the hGR is also indicated. Amino acid residue numbers are taken from Hollenberg *et al.* (*8*) for hGR, Loosfelt *et al.* (*11*) for rPR, and from our data for hMR.

clear whether glucocorticoids act as full agonists in binding to the mineralocorticoid receptor. Similarly, the GR binds glucocorticoids with a K_D between 20 to 40 n*M* and it binds aldosterone with a K_D between 25 to 65 n*M* (*2*). Therefore, the important distinction between the hormone-binding properties of MR and GR may not be one of ligand specificity, but rather of a high-affinity versus a lower affinity receptor for the corticosteroids.

The function of the hMR in vivo is complicated by the serum cortisol-binding protein, transcortin. This protein sequesters cortisol and, because of its differential distribution, transcortin could influence local glucocorticoid concentration. High levels of transcortin in kidney would reduce available cortisol from plasma to favor aldosterone sensitivity, whereas low levels of transcortin in the brain would suggest that, in the central nervous system, glucocorticoids may be the predominant hMR ligand. Thus, the preferred physiologic ligand for hMR apparently varies depending on the site of receptor expression (*3*). This model and others (*5*) have been proposed to explain the responsiveness of some tissues to aldosterone despite much higher levels of competing glucocorticoids.

The degree of homology between hMR and hGR in the DNA-binding domain (only four amino acid residues differ in this conserved 68-residue region) suggests that these receptors may recognize similar regulatory elements. The activation of the MMTV LTR by the transfected hMR in response to both aldosterone and dexamethasone supports this conclusion, although the progesterone receptor has also been demonstrated to regulate this promoter (*21*). Furthermore, differences between hMR and hGR in the DNA-binding domain, or in other regions such as the highly divergent amino termini of these molecules, may influence target gene specificity in ways not revealed in this assay. However, we have utilized transcriptional regulation of the MMTV LTR by hMR and hGR to examine their activation by mineralocorticoids and glucocorticoids. While the hMR response was approximately equivalent with either 10 n*M* aldosterone or dexamethasone, hGR was activated by dexamethasone but was insensitive to aldosterone in this assay. Transcriptional activation by hMR in response to exogenous cortisol was also observed. These data indicate that in transfected cells both mineralocorticoids and glucocorticoids can activate hMR-mediated gene transcription. On the basis of this functional property, we conclude that the hMR is highly responsive to adrenal corticosteroids and therefore may function as a glucocorticoid receptor.

In addition to elucidating the pharmacologic and physiologic function of the mineralocorticoid receptor in coordinating response to corticosteroids, the isolation of hMR cDNA should allow an investigation of the role of hMR in a number of disease states, among them hypertension and pseudohypoaldosteronism (PHA). An association of mineralocorticoids with hypertension has been recognized for several decades, and it may be that hMR-mediated sodium retention and increased blood volume are, in part, responsible for some forms of hypertension (*34*). PHA is an autosomal recessive disorder characterized by lack of responsiveness to normal or elevated aldosterone levels. Recent work has demonstrated diminished or complete loss of high-affinity aldosterone-binding sites in patients with this disease (*35*) which is likely to result from a mineralocorticoid receptor genetic defect. The chromosomal mapping of the hMR gene suggests the PHA locus should reside on chromosome 4.

Cloning and expression of functional hMR has provided unexpected insight and should stimulate new interest into the mechanisms underlying physiologic complexity, and allow the development and testing of new models for the coordinate regulation of gene networks. This in turn should provide greater understanding of the role of gene regulation in the evolution, organization, and function of the neuroendocrine system.

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41. Construction of pRShMR. The 3.75-kb insert from λhk10 was ligated into the Eco RI site of pGEM4 (Promega) oriented with the mp18 polylinker adjacent to the 5′ end of hMR coding sequence. Digestion of this plasmid (phk10) with Hind III generated at Hind III fragment spanning the polylinker site to the hMR site at position 3562; this fragment was isolated and the ends were repaired with the Klenow fragment of DNA polymerase I. Plasmid pRSVCAT [C. M. Gorman, G. T. Merlino, M. C. Willingham, I. Pastan, B. H. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982)] was digested with Hind III and Hpa I, and the Hind III–Hpa I fragment containing pBR322 sequences, the RSV LTR, and the SV40 polyadenylation site was also repaired. Ligation of the hMR fragment to the fragment from pRSVCAT yielded a vector which, in the correct orientation, has hMR coding sequence driven by the RSV promoter. Sites bracketed in Fig. 4A were lost in this cloning step. To improve translational efficiency several upstream initiation and termination codons in the 5′-untranslated region were deleted by digesting the vector with Acc I to remove an ~200-bp sequence from the mp18 polylinker to position 188 in the hMR 5′-UT region. Finally, an Nde I–linkered SV40 origin of replication was introduced into the Nde I site (*12*) to generate pRShMR.
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46. We thank M. Thomas, D. Gruol, B. Sefton, G. Wahl, M. G. Rosenfeld, D. Fanestil, and J. Funder for advice and discussion; V. Giguère and S. Hollenberg for assistance in developing the plasmids and steroid receptor function assays adapted to this study, G. Bell (Chiron Corp.) for providing human kidney cDNA libraries, N. Drascopoli for providing hybrid DNA's, and M. ter Horst for artwork. Supported by predoctoral training grant (J.L.A.) to the Department of Biology, University of California, San Diego, by a predoctoral grant from the NIH Medical Scientist Training Program (T.M.G), by a grant from the Hereditary Disease Foundation (B.L.H.), and by grants from the NIH and the Howard Hughes Medical Institute.

8 April 1987; accepted 9 June 1987