Human Mineralocorticoid Receptor Genomic Structure and Identification of Expressed Isoforms*

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Most of the known effects of aldosterone are mediated by the mineralocorticoid receptor, an intracellular receptor belonging to the steroid/thyroid hormone/retinoic acid receptor superfamily. We determined the genomic structure of the human MR (hMR) and identified 10 exons in the gene, including two exons (1 and 19) that encode different 5′-untranslated sequence. Expression of the two different hMR variants is under the control of two different promoters that contain no obvious TATA element, but multiple GC boxes. Our results indicate that hMR expression is regulated by alternative promoters perhaps in a tissue- or developmental-specific manner.

Molecular cloning of a cDNA encoding the human renal mineralocorticoid receptor (hMR)† (1) identified it as a member of the nuclear receptor superfamily, which includes the other steroid hormone receptors as well as the thyroid and retinoic acid receptors and a large group of orphan receptors (2, 3). Nuclear receptors are highly specialized ligand-dependent transcription factors which interact with specific cis-acting elements to enhance or repress target gene expression (4). They share the same structure-function organization, and their cDNAs are highly conserved particularly in those regions associated with DNA and hormone binding. The mineralocorticoid and glucocorticoid receptor are closely related, and together with the progesterone and androgen receptors they form a subfamily which is closely related both by sequence and functional criteria (3, 5).

The adrenal steroids aldosterone and cortisol both bind to the mineralocorticoid receptor (type I corticosteroid receptor); cortisol also binds to the glucocorticoid receptor (type II receptor). Specificity is conferred by the enzyme 11β-hydroxysteroid dehydrogenase, which converts cortisol to the less active compound cortisone, thus allowing aldosterone to bind to mineralocorticoid receptor (6, 7). The mineralocorticoid receptor is expressed in so called “classical” aldosterone target tissues, which are sodium transporting epithelia (kidney, colon, salivary, and sweat glands) and in a variety of non-epithelial target tissues, such as the central nervous system, mononuclear leukocytes, large blood vessels, and the heart (8). In the kidney, the mineralocorticoid receptor regulates the sodium, potassium, and hydrogen ion balance in the distal part of the nephron. In the central nervous system, the mineralocorticoid receptor is expressed in many areas of the forebrain with very high levels of expression reported in the rodent hippocampus. In some, but not all regions in the brain, the mineralocorticoid receptor responds to the diurnal fluctuations in cortisol levels and thus provides, together with the glucocorticoid receptor, a system capable of responding to normal and elevated cortisol concentrations, respectively (9).

Recent studies have reported the presence of multiple variants of the rat mineralocorticoid receptor mRNA, which encode for the same protein but diverge in their 5′-untranslated sequences (10). These mineralocorticoid receptor mRNAs are expressed in a tissue-dependent manner and are very likely to be under the control of different promoter sequences, allowing thus an independent regulation of each mRNA isoform.

The hMR gene has been localized to chromosome 4q31.1-4q31.2 (11, 12). We have characterized the structure of the hMR gene and determined the location of the hMR splice sites. Two different 5′-untranslated exons were identified which splice into a common translated region. Expression of these mRNA species is controlled by two distinct promoters.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Genomic Clones—A human placental cosmid library (Stratagene, La Jolla, CA), a human placental lambda phage library (13), two human leukocyte cosmid libraries, and a chromosome 4 enriched cosmid library were screened using three different EcoRI restriction fragments of the human mineralocorticoid receptor cDNA following standard colony hybridization techniques (14). Labeling was performed by random priming with nonamers and (α-32P) dCTP (Megaprime DNA labeling system, Amersham). Positively hybridizing colonies were picked and passaged until complete purification. DNA of positive clones was prepared following standard techniques (14). Cosmid and phage DNA was digested with different restriction enzymes and characterized by Southern blot analysis using specific (γ-32P) dATP-labeled oligonucleotides as probes (15, 16). Positive restriction fragments were subcloned and sequenced by the dideoxy chain termination method (17) using Sequenase (U. S. Biochemical Corp.). The sequence of the exon 19 intron P1 boundary and confirmation of the cDNA sequence was obtained by direct sequencing of the cosmid and by
subcloning into M13 for single-stranded sequencing using Taq polymerase (Promega Biotech) and formamide-containing polyacrylamide gels.

Inverse PCR—Inverse PCR was performed according to Ochman et al. (18). Briefly, human genomic DNA was digested with different restriction enzymes (HaeII, Rsal, MspI, and Sau3A). The purified digests were circularized and subcloned to 35 rounds of PCR amplification using exon-specific primer pairs. Oligonucleotide primers (20–23-mers) were designed, using the predicted exon-intron organization of the hMR (19, 20), to be located in the center of each exon to be amplified. PCR primers were as follows (numbering corresponds to the position of the 5'-nucleotide according to the published hMR cDNA sequence (1), S identifies primers in sense orientation and A in the antisense orientation): S180 and A83; S2190 and A2187; S2810 and A2803; S2969 and A2942. PCR products were fractionated on a 2% agarose gel, subcloned into Bluescript-KS, and sequenced.

Rapid Amplification of cDNA Ends (RACE)—Mapping of the hMR transcription initiation site by RACE (21) was performed using a commercial kit (5'-RACE Life Technologies, Inc.) following the instructions of the manufacturer. Total human kidney RNA was extracted by standard techniques (22) and submitted to reverse transcription using a reverse primer (23-mer) located at position 480 according to the published mineralocorticoid receptor cDNA sequence (1). The 3'-tailed cDNA was submitted to 30 rounds of PCR amplification using an oligo(dC) primer containing several cloning sites and a second nested reverse primer at position 311.

PCR included a denaturation step at 96°C for 30 s, an annealing step at 94°C for 5 min and followed by 10 min elongation. 0.05% of the initial PCR product was subsequently reamplified under the same conditions with a sense oligo(dC) primer containing several cloning sites and a second nested reverse primer at position 83. The PCR products were subcloned into Bluescript-KS and screened with an exon 1- or oligonucleotide (position 7); positive clones were sequenced by the dideoxy chain termination method.

Alternative 5' variants of the mineralocorticoid receptor mRNA were searched for by RACE using reverse primers located in exon 2 at position 311 and 280 for the first and second round of amplification, respectively. After subcloning PCR fragments, colonies which did not hybridize to an exon 1-specific oligonucleotide (position 7) probe were sequenced. Exon 1- or oligonucleotide probes were synthesized and used for direct sequencing of a cosmid containing exon 1β.

Nucleotide sequences were analyzed using the suite of programs provided by the Australian Genomic Information Service and by CTTI 2.

RESULTS

Positions of Cosmid Clones, Phage Clones, and Inverse PCR Products on the hMR Gene—Four cosmid clones and three phage clones were characterized after the screening for clones containing hMR gene sequences using the hMR cDNA as probe (Fig. 1). Cosmid clones cos25 and cos31 were mapped to the 3'-end of the gene using primer A3206 as a probe (primer position according to the published cDNA (1). 19b was characterized using oligonucleotide S1786 and A448 and mapped to the 5'-end of the mineralocorticoid receptor gene. Primer S2054, which maps to the DNA-binding domain, was used to characterize clone A14, and phage clone A131 was mapped to the hormone-binding domain of the receptor using oligonucleotides S2381 and A2683. Clones cos72 and cos953 were isolated using EcoRI fragments of the original hMR cDNA (1). Three different inverse PCRs were performed to specifically amplify putative exons not covered by the cosmid/cDNA clones as indicated (i1,i2,i3). The sequence of the exon for the published 5'-untranslated and its flanking sequence were determined from both cosmid clone cos72 and also using inverse PCR (ip1). Following the nomenclature used for the rat gene (10), this is designated as the 5'-untranslated. The cosmid cos72 also hybridized with both exon 2 and rat exon 1-β-specific probes. Exon 1-β 5'-untranslated was identified by RACE which enabled the sequencing of the flanking region and exon-intron junction from the cosmid. Using this strategy, we determined the exon-intron boundaries and flanking sequences of the mineralocorticoid receptor gene. As shown in Table I, the intron-exon boundaries have the canonical consensus sequence.

Exon-Intron Organization of the Gene—The hMR gene (Fig. 1A and Table I) consists of a total of 10 exons and spans over 60–90 kb, as deduced from Southern hybridization experiments (data not shown). Exons 1α and 1β are composed only of 5'-untranslated sequence. The N-terminal part of the receptor is encoded by exon 2, which contains 2 bp of 5'-untranslated region and 1757 bp of coding sequence. Two small exons, exons 3 and 4, encode each of the two zinc fingers of the DNA-binding domain of the receptor. The hormone-binding domain of the mineralocorticoid receptor is encoded by five exons, exons 5–9. Among the glucocorticoid/mineralocorticoid/androgen/proestrogen receptor subfamily of nuclear receptor genes (20, 23, 24) the length of the exons is identical for those encoding both the second zinc finger (exon 4 in the hMR) and the ligand-binding domain (6–8 in the hMR).

Transcription Initiation Site of hMRα and 5'-Flanking Region (P1)—Two different hMR 5'-mRNA ends were identified, designated as hMRα and hMRβ. The 5'-untranslated sequence containing exon 1α was obtained both from the cosmid clone cos72 and by inverse PCR (Fig. 1). A 2.1-kb HindIII fragment of cos72 shown to hybridize with a rat exon 1α probe yielded sequence extending from intron A (900 bp) through the 5'-untranslated and into the flanking region (P1:950 bp). The inverse PCR fragment obtained was ~1.2 kb in size and similarly extends from intron A to include ~750 bp of flanking sequence. Fig. 2 shows the hMRα 5'-untranslated and flanking sequences.
sequences obtained from these clones. No consensus TATA box sequence is present in the putative promoter sequence, and no CCAAT motif is found within the first 100 bp upstream of the transcription initiation site, although two are found at positions −172 and −225 (these sequences could be found by chance every 1025 bp). The region is very rich in GC boxes, and computer-assisted analysis revealed seven consensus sequences for Sp1-binding sites are boxed, and those for transcription factors Ap-1 and PEA3 are underlined; consensus sequences for AP-2 are indicated by a line above the sequence. The exonic sequence is indicated in bold letters. Numbering is relative to the transcription initiation site of hMRα.

**Table 1**

| Exon | Size | 5'-Donor site | 3'-Acceptor site | Letter | Size |
|------|------|---------------|-----------------|--------|------|
| 1b   | 216  | GAAAG (220)   | gtagct   | tggtag (221) | GATGG |
| 1a   | 256  | AGGAGC (220)  | gtagct   | tggtag (221) | GATGG |
| 2a   | 1759 | ATCATAG (179) | gtagct   | tggtag (221) | GATGG |
| 3    | 140  | TGGAGA (2119) | gtagct   | tggtag (221) | GATGG |
| 4    | 117  | TAGGAG (2236) | gtagct   | tggtag (221) | GATGG |
| 5    | 351  | TTCCAG (2587) | gtagct   | tggtag (221) | GATGG |
| 6    | 145  | TAAAG (2732)  | gtagct   | tggtag (221) | GATGG |
| 7    | 131  | GCCAAG (2863) | gtagct   | tggtag (221) | GATGG |
| 8    | 158  | CATGAC (3021) | gtagct   | tggtag (221) | GATGG |
| 9    | 2733 | Exon ends at  | TACTTT   (5754) |

* ND, not determined.

**Fig. 2. DNA sequence of the region upstream of exon 1a (P1).** The transcription initiation site is indicated by an arrow. Consensus sequences for Sp1-binding sites are boxed, and those for transcription factors Ap-1 and PEA3 are underlined; consensus sequences for AP-2 are indicated by a line above the sequence. The exonic sequence is indicated in bold letters. Numbering is relative to the transcription initiation site of hMRα.

In the present study we have determined the genomic organization of the hMR gene. The two different mRNA 5'-ends isolated by RACE suggested the existence of alternative promoters in the hMR gene, as has been reported for the mouse glucocorticoid receptor (34) and suggested for the rat mineralocorticoid receptor (10, 32). The expression of the two mRNAs is not mutually exclusive, since the two transcripts were both found in the kidney. Alternative transcription initiation from exon 1a or 1b generates two different hMR transcripts, hMRα and hMRβ, with the same translation product. A third mRNA isoform was found in rat hippocampus (33). The longest transcripts obtained by subsequent RACE using exon 1b-specific reverse primers at position 124 and 103 of the cloned cDNA enabled us to determine a putative transcription initiation site of hMRβ (underlined in Fig. 3). Sequencing of cosmids cos72 with 5'-untranslated-specific primers enabled the identification of both the exon1b-intron P1 junction and the region flanking the 5'-untranslated (P2). It is possible, given the very GC-rich nature of the region which rendered sequencing difficult, that the RACE products are prematurely terminated in the 5'-untranslated. Sequence homology with the rat exon 1b cDNA is observed over 216 bp to the putative transcription initiation site of hMR 1b, after which the sequence similarity decreases. Additional experiments are required to precise whether alternative transcription start sites exist in the human and the rat MRβ promoter. Computer-assisted analysis of P2 revealed two binding sites for transcription factor Sp1 (−62, −150) and one consensus sequence for binding of transcription factor AP-2 (−26).

**Discussion**

In the present study we have determined the genomic organization of the hMR gene. The two different mRNA 5'-ends isolated by RACE suggested the existence of alternative promoters in the hMR gene, as has been reported for the mouse glucocorticoid receptor (34) and suggested for the rat mineralocorticoid receptor (10, 32). The expression of the two mRNAs is not mutually exclusive, since the two transcripts were both found in the kidney. Alternative transcription initiation from exon 1a or 1b generates two different hMR transcripts, hMRα and hMRβ, with the same translation product. A third mRNA isoform was found in rat hippocampus (33). The longest transcripts obtained by subsequent RACE using exon 1b-specific reverse primers at position 124 and 103 of the cloned cDNA enabled us to determine a putative transcription initiation site of hMRβ (underlined in Fig. 3). Sequencing of cosmids cos72 with 5'-untranslated-specific primers enabled the identification of both the exon1b-intron P1 junction and the region flanking the 5'-untranslated (P2). It is possible, given the very GC-rich nature of the region which rendered sequencing difficult, that the RACE products are prematurely terminated in the 5'-untranslated. Sequence homology with the rat exon 1b cDNA is observed over 216 bp to the putative transcription initiation site of hMR 1b, after which the sequence similarity decreases. Additional experiments are required to precise whether alternative transcription start sites exist in the human and the rat MRβ promoter. Computer-assisted analysis of P2 revealed two binding sites for transcription factor Sp1 (−62, −150) and one consensus sequence for binding of transcription factor AP-2 (−26).
particular tissues.

It is interesting to compare the genomic structure of the hMR, hGR, hAR, and hPR (20, 23, 24), all belonging to the same steroid receptor subfamily (3, 5). Although in these genes the same exon-intron organization is used to assemble functional domains of the receptor protein (eight exons), no structural conservation is found for 5'-untranslated exons and regulatory regions. hGR has a unique 5'-untranslated exon, whereas only eight exons compose the hAR gene. The expression of both is driven by a unique promoter, whereas the hPR gene (24, 35) contains two different hormone-inducible promoters (Fig. 4).

The DNA surrounding the transcription initiation site of hMR (5'-CCCTCC-3'TCT-3') resembles the initiator sequence from the terminal deoxynucleotidyltransferase gene 5'-CCCTCA-3'TTCT-3', which appears to be important in TATA-less promoters, and which has been shown to interact in a position-dependent manner with Sp-1 binding sites to direct high levels of transcription (36, 37). This sequence also shares homology with the androgen receptor transcription initiation site I (TISI) surrounding sequence (CCCTC-12C-13GAGA), and with the sequence surrounding the putative transcription initiation site of the rat MR (CTTC-1TGCGC). As with the promoters directing expression of other steroid receptors (20, 24, 34, 35, 38, 39), including the rat mineralocorticoid receptor (32), both P1 and P2 do not contain any TATA-less exons or regulatory regions. hGR has a unique 5'-untranslated exon, whereas only eight exons compose the hAR gene. The expression of both is driven by a unique promoter, whereas the hPR gene (24, 35) contains two different hormone-inducible promoters (Fig. 4).

The MR gene is thus regulated by alternative promoters (40, 41). This mechanism allows more flexibility in the control of expression and generally, alternative promoters are associated with tissue- and/or developmental-specific gene expression. In the rat, MRα is the predominant form expressed in the kidney, whereas in the hippocampus MRβ and MRγ are expressed in equal proportions (10). In addition, alternative transcription initiation can affect both the stability of the transcripts and the efficiency of mRNA translation (42). Indeed, mineralocorticoid receptor expression seems to be regulated in a tissue-specific manner by the level of its ligand, although conflicting data are reported in the literature. Whereas there is good evidence for hormonal regulation of mineralocorticoid receptor in the hippocampus (10, 43, 44), for the kidney both regulation (45) and lack of regulation by corticosteroids have been reported (44). For the rat distal colon mineralocorticoid receptor, protein and mRNA levels are neither up-regulated after adrenalectomy nor down-regulated in response to a mineralocorticoid receptor agonist (46).

Although mineralocorticoid receptor is known to bind specific consensus sequences, such as the GREs contained in the MMTV promoter (1), no specific mineralocorticoid-responsive element has yet been identified. P1 contains a sequence resembling a GRE in an inverted orientation, which has been shown to confer hormone responsiveness to exogenous promoters in an orientation-independent manner (47). It is worth noting that a significant and selective increase in MRα mRNA levels has been reported in rat hippocampus after adrenalectomy, which was reversed by exogenous corticosterone administration, whereas MRβ mRNA levels did not change. Thus, in the rat, MRα may be the hormonally regulated mRNA variant, suggesting that the putative hormone responsive sequence identified in P1 might be of functional significance.

In conclusion, the determination of the genomic structure of the hMR will allow the study of its transcriptional regulation and of the tissue-specific and developmental expression of distinct hMR mRNA species. It will also facilitate the search for mutations in the human mineralocorticoid receptor gene which may be responsible for disorders of salt and water balance, such as mineralocorticoid resistance or hypertension.

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REFERENCES

1. Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) Science 237, 268–275
2. Evans, R. M. (1988) Science 240, 889–895
3. Fuller, P. J. (1991) FASEB J. 5, 3092–3099
4. Beato, M. (1989) Cell 56, 335–344
5. Laudet, V., Hänni, C., Coll, J., Calzaferr, F., and Stéhelin, D. (1992) EMBO J. 11, 1003–1013
6. Edwards, C. R., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., De Kloet, E. R., and Monder, C. (1988) Lancet 2, 586–589
7. Funder, J. W., Pearce, P. T., Smith, R., and Smith, A. I. (1988) Science 242,
