Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements

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Although the mineralocorticosteroid receptor (MR) belongs to the superfamily of hormone-dependent transcription factors, the molecular mechanism by which it regulates gene expression is poorly understood. Binding of the MR to target gene promoters has never been characterized, and specific mineralocorticosteroid response elements (MREs) remain to be identified. The human MR (hMR) was overexpressed in Sf21 insect cells using the baculovirus system. The high degree of similarity between the glucocorticosteroid receptor (GR) and the MR prompted us to examine the DNA-binding properties of the recombinant MR with glucocorticosteroid-regulated genes. Gel shift mobility assays demonstrated that the recombinant receptor interacted with oligonucleotides containing perfect and imperfect palindromic sequences of GRE. A monoclonal anti-hMR antibody (FD4) induced a supershift of protein–DNA complexes and identified the MR in Western blot analysis. *In vitro* DNAase I protection assays with the hormone-regulated murine mammary tumour virus promoter showed that recombinant hMR generated four footprints whose limits encompassed the GRE motifs. By means of these two complementary approaches, no difference between the interaction of free, agonist- or antagonist-bound MR and DNA was detected. We provide evidence that hMR functions as a sequence-specific DNA-binding protein.

INTRODUCTION

The mineralocorticosteroid receptor (MR) mediates aldosterone action in target cells. The MR is the largest member of the superfamily that includes the steroid/thyroid, vitamin D and retinoic acid receptors [1,2]. These ligand-dependent transcription factors modulate gene expression by binding to specific DNA sequences. The MR is closely related to the glucocorticosteroid receptor (GR), progesterone receptor (PR) and androgen receptor (AR), which constitute the GR subfamily [2]. Despite the extensive sequence similarity [3,4] and putative similar structural organization between GR and MR, the molecular mechanisms underlying MR function are still poorly understood, mainly because of the very low abundance of this receptor and the paucity of known aldosterone-regulated genes.

To facilitate the study of MR structure and function, new tools have been developed recently and shown to be particularly useful for precise molecular analyses. Using the baculovirus system, the human MR (hMR) when overexpressed at high concentrations behaves like the native protein in terms of hormone binding, hetero-oligomeric structure and immunological recognition [5,6]. However, the binding to DNA as a prerequisite of receptor transcriptional activity has yet to be established. Another major advantage of this expression system in insect cells is the absence of the GR, which is present in virtually every tissue and is known to share several common functional features with the MR, most notably similar hormonal binding and target recognition properties.

Very little is known about the initial steps of signal transduction mediated by the MR. Only a few studies have reported on the DNA-binding properties of the MR, and these have essentially examined its binding to DNA–cellulose [7,8] or measured the transcriptional activity of the MR through its interaction with a viral promoter [3,9,10]. Since the MR functions as a transcription factor, it should bind to specific sequences generally located upstream of regulated genes. These *cis*-acting DNA sequences, called hormone response elements (HREs), have been described for oestrogen, progesterone, glucocorticosteroid and androgen receptors ([11,12] and references therein), but no selective mineralocorticosteroid response element has ever been reported to date.

In the present paper, we describe the properties of the MR by characterizing the interaction of the recombinant protein, expressed in the baculovirus system, with DNA sequences using gel retardation and footprint DNAase I protection assays. As target genes we chose DNA fragments containing glucocorticosteroid response elements (GREs) present in two well-studied glucocorticosteroid-inducible promoters: that of tyrosine aminotransferase [13] and the long terminal repeat of the mouse mammary tumour virus (MMTV-LTR) [14,15]. We describe the characterization of the DNA-binding properties of the hMR and study whether the presence and/or the nature of the ligand (agonist or antagonist) modifies the receptor–DNA interaction. We provide the first evidence that the recombinant hMR binds specifically to GREs and protects four GRE-containing regions of the MMTV-LTR from digestion by DNAase I.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]Aldosterone (40–60 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from Amersham. [^{3}H]ZK91587 and unlabelled ZK91587 were from NEN (Les Ulis, France). Aldosterone and DNAase I (type IV

Abbreviations used: MR, mineralocorticosteroid receptor; hMR, human MR; GR, glucocorticosteroid receptor; PR, progesterone receptor; AR, androgen receptor; HRE, hormone response element; GRE, glucocorticosteroid response element; MMTV-LTR, long terminal repeat of the mouse mammary tumour virus; MRE, mineralocorticosteroid response element.

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from bovine pancreas) were purchased from Sigma (St. Louis, MO, U.S.A.). Poly(dI-dC) was from Pharmacia (Guyancourt, France).

Expression of recombinant hMR and preparation of cellular extracts

Sf21 insect cells were infected with the recombinant virus AcNPV-hMR, which contains the full-length cDNA of the hMR [5]. At 48 h post-infection, the cells were rinsed with cold PBS and homogenized in TEGW buffer [20 mM Tris/HCl, 1 mM EDTA, 10% (v/v) glycerol, 20 mM sodium tungstate, pH 7.4, at 20 °C]. The homogenates were centrifuged at 12000 *g* for 15 min at 4 °C in a microcentrifuge and the supernatants were utilized as cell extracts. In some experiments, infected cells were first rinsed in serum-free TNM-FH medium (Sigma) and incubated with 50 nM [^{3}H]aldosterone or [^{3}H]ZK91587 at 27 °C for 1 h. The cells were then rinsed with cold PBS and homogenized as described above; these are referred to as whole-cell extracts. The quantification of hMR contained in various cellular extracts was by binding assays as described in [5]. Protein concentration was determined by the Bradford technique [16].

Gel retardation assays

Synthetic oligonucleotides (see Table 1) purchased from the Institut Pasteur (Paris, France) were purified by gel electrophoresis (15% acrylamide, 7 M urea). The probes were prepared by annealing complementary strands in 50 mM Tris, pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$ and 1 mM dithiothreitol by heating at 85 °C for 4 min and cooling to room temperature over a 3 h period. After filling in the recessed ends of overlapping oligonucleotides, the probes were radiolabelled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase (Boehringer Mannheim) to a specific radioactivity of $(2–5) \times 10^7$ c.p.m./μg of DNA. Variable amounts of cellular extracts (crude, labelled with agonist or antagonists, or whole-cell extracts) were incubated with 0.1–1 ng ^{32}P-labelled oligonucleotides for 15 min at 25 °C in 20 μl of buffer containing 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol and 100 μg/ml BSA. All binding reactions contained 1 μg of poly(dI-dC) as a non-specific competitor. Unlabelled oligonucleotides were used as specific competitors and were added simultaneously to the binding assay. Murine monoclonal IgG1 antibodies (FD4 or MOPC31C) were added 15 min before the assays at 25 °C. A pre-electrophoresis (20 mA for 30 min) was performed and protein–DNA complexes were separated from free DNA by non-denaturing electrophoresis in a 4.5% acrylamide/bisacrylamide (39:1) gel for 1 h at 20 mA in 0.25 × TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA). Gels were dried and exposed to X-ray film at −70 °C.

Western blotting

After band shift mobility assays (see above), gels were electrotransferred onto a BA85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and, after blocking, incubated with a 1:500 dilution of FD4 overnight at 4 °C. The presence of hMR was visualized using the Vectastain ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA, U.S.A.).

DNAase I footprint experiments

The DNA probes were synthesized by the PCR using as a template the plasmid pFC31luc containing the MMTV-LTR promoter [17], and P1 or P2 oligonucleotide primer end-labelled with [γ-^{32}P]ATP by T4 kinase [18] and the opposite unlabelled primer. The sequences of the oligonucleotides P1 (−299 to −275) and P2 (+69 to +45) (numbers refer to the distance from the initiation of transcription) were GGGACAGTGGCTGGACTAATAGAAC and TAAGTGACGAGCGGAGACGGGATGG respectively. Thirty cycles of amplification with 0.25 unit of AmpliTaq (Perkin–Elmer Cetus) were carried out using an annealing temperature of 63 °C. The radioactive probes were purified on a 6% (w/v) polyacrylamide gel. The samples (20–50 μg of protein) were incubated with ^{32}P-labelled DNA probes for 15 min at room temperature in a total volume of 25 μl of buffer containing 20 mM Hepes, pH 7.9, 60 mM KCl, 0.8 mM $MgCl_2$, 0.5 mM phenylmethanesulphonyl fluoride, 1.25 mM dithiothreitol and 1 μg of poly(dI-dC). The DNA was digested with DNAaseI (1–4 units) for exactly 2 min at 0 °C. The reaction was stopped by the addition of 250 μl of 0.1% SDS, 5 mM EDTA and 300 mM NaCl containing 1 μg of dextran T40, followed by phenol/chloroform/3-methylbutan-1-ol (25:25:1, by vol.) extraction. The samples were ethanol-precipitated and analysed on a 6% sequencing gel.

RESULTS

The hMR has been expressed in Sf21 insect cells infected by the recombinant baculovirus AcNPV-hMR [5], which contains the full-length coding sequence of hMR [3]. At 48 h after infection the level of hMR expression was measured in cytosolic fractions by an aldosterone-binding assay as previously described [5], and it reached a value of approx. 2 pmol/mg of protein. Prior to use in subsequent experiments, the receptor was partially purified by ion-exchange chromatography resulting in an enrichment factor of about 10.

A gel shift assay was used to characterize the interaction of recombinant hMR with the DNA-containing GREs shown in Table 1. The first oligonucleotide (TAT), a DNA sequence derived from the promoter of the rat tyrosine aminotransferase gene, contains the motif TGTACATGTTCT that confers positive regulation by glucocorticosteroids [13,19]. The second oligonucleotide (MST) is derived from the previous sequence by mutation of two base pairs (A-T, T-A) in the motif. Studies on the identification of protein contact sites have previously shown that these bases are critical for the GR–DNA interaction [20,21]. The third oligonucleotide (VIT), derived from the natural sequence of the oestrogen response element in the *Xenopus* vitellogenin A2 gene (−555 to −222) was mutated to obtain a perfect GRE palindromic motif [22]. One of the known aldosterone target genes is Na^+,K^+-ATPase [23]. Elements of the rat Na^+,K^+-ATPase α1 gene promoter have been described recently [24] and we chose an oligonucleotide within this promoter sequence (fourth oligonucleotide; ANA) as a potential mineralo-

Table 1 Sequence of the oligonucleotides used

The specific motifs are underlined and in bold type. These oligonucleotides have been designed according to [13,19–22,24,25].

Oligonucleotide	Sequence
TAT	5′-GGATC**TGTACA**GGA**TGTTCT**AGATCCA-3′
MST	5′-GGATC**TGTTCA**GGA**TGATCT**AGATCCA-3′
VIT	5′-GATCCAAAGTC**AGAACA**CAG**TGTTCT**GATCAAAGA-3′
ANA	5′-GGAGCCGGT**GTCA**GGTT**TGCTC**CGGTAA-3′
GRE4	5′-AGTTTTG**GTTACA**AAC**TGTTCT**TAAA-3′

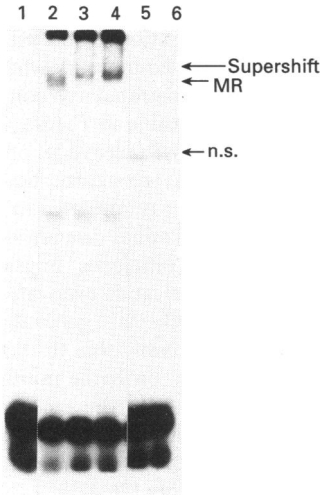

Figure 1 Specific binding of the recombinant hMR to HREs

Gel shift assay with the TAT oligonucleotide. [^{32}P]TAT probe (lane 1) (~ 0.5 ng) was incubated with 20 μg of cell extract of Sf21-infected cells containing ~ 40 fmol of expressed hMR (lanes 2–4) or 20 μg of protein of cellular extracts from uninfected Sf21 cells (lanes 5 and 6). A 1 μl (~ 3 μg) (lane 3) or 2 μl (lanes 4 and 6) sample of purified FD4, a monoclonal anti-MR antibody, was added to the binding assays. MR indicates the position of the specifically bound hMR–TAT complexes. n.s. represents non-specific binding.

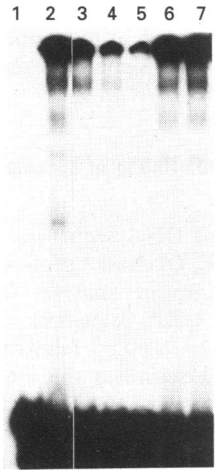

Figure 2 Sequence specificity of the DNA binding by expressed hMRs

[^{32}P]TAT probe (~ 0.6 ng) (lane 1) was incubated with recombinant hMR (10 μg of protein of cellular extracts) alone (lane 2) or in the presence of 50, 100 or 200 ng of unlabelled TAT (lanes 3–5), 100 ng of MST (lane 6) or 100 ng of ANA (lane 7).

corticosteroid response element (MRE). The last oligonucleotide used in our work (GRE4) corresponds to the upstream GRE (−185 to −171) of the mouse mammary tumour virus LTR [14,25] and contains an imperfect palindromic motif.

Overexpressed hMR binds specifically to a variety of GREs

As compared with the [^{32}P]TAT probe alone (Figure 1, lane 1), the recombinant hMR contained in the cytosol fractions of Sf21 insect cells infected with the baculovirus AcNPV-hMR is able to form a complex with the [^{32}P]TAT probe, as demonstrated by the appearance of a major retarded band (lane 2). Increasing amounts of hMR led to an enhancement of the retarded band signal without modification of the degree of migration (results not shown). To establish unambiguously that the DNA–protein complexes contained hMR, we used the monoclonal antipeptide anti-hMR antibody FD4 (M. Lombès, N. Binart, F. Delahaye, E. E. Baulieu and M.-E. Oblin, unpublished work). Addition of purified FD4 resulted in a supershift of the DNA–protein complexes (Figure 1, lanes 3 and 4). To control the specificity of the supershift induced by FD4, we used an unrelated IgG1 antibody (MOPC31C) which did not modify the mobility of the complexes (results not shown). Cytosolic extracts from uninfected Sf21 insect cells alone (lane 5) or in the presence of FD4 (lane 6) did not give any specific signal, further demonstrating that the observed bands corresponded to [^{32}P]TAT–hMR complexes.

Noticeable amounts of radioactive probe were visualized in the loading wells only where the samples contained recombinant hMR (Figure 1, lanes 2–4), suggesting an aggregation of DNA–protein complexes unable to penetrate the non-denaturing gel. This phenomenon was not affected even when the reticulation of the gel was decreased (from 39:1 to 79:1 acrylamide/bisacryamide), or by increasing the ionic strength (KCl) by treatment with a non-ionic detergent (Nonidet P40), or by modifying the Mg^{2+} content of the incubation buffer. It appeared that when the electrophoresis was performed at 4 °C (as in Figure 4), migration of the DNA–protein complexes was facilitated, consistent with the involvement of hydrophobic bonds in this aggregation process.

As shown in Figure 2, increasing concentrations of non-radioactive TAT oligonucleotide (50–200 ng) were able to compete with the major retarded band (lanes 3–5) corresponding to [^{32}P]TAT–hMR complexes (lane 2). It is noteworthy that radioactive material present in the wells of the gel disappeared in the presence of unlabelled TAT. In contrast, the oligonucleotide MST, containing the mutated GRE sequence of TAT (lane 6), and the oligonucleotide ANA (lane 7), possessing a putative steroid response element, could not compete with the [^{32}P]TAT–hMR complexes either at the level of the retarded band or at the top of the gel, indicating that the aggregates that could barely penetrate into the gel may indeed correspond to specific TAT–hMR complexes. Moreover, no complexes were formed between hMR and an oestrogen responsive element of the vitellogenin A2 gene promoter (results not shown). Altogether, these results indicated that recombinant hMR binds specifically to an oligonucleotide presenting a GRE sequence motif.

We next checked whether the recombinant hMR was also able to interact with a perfect palindromic GRE sequence by comparing the gel patterns obtained with [^{32}P]TAT and [^{32}P]VIT oligonucleotides (Figure 3). The retarded band of [^{32}P]TAT–hMR (lane 2) was supershifted by addition of antibody FD4 (lane 3) and disappeared in the presence of an excess of unlabelled TAT (lane 4). The 35-mer oligonucleotide [^{32}P]VIT alone migrated more slowly (lane 5) than the 27-mer [^{32}P]TAT. Incubation of [^{32}P]VIT with hMR resulted in a retarded band migrating at the same position as the [^{32}P]TAT–hMR complexes (lane 6). Moreover, addition of FD4 led to an upwards shift arising from the formation of antibody–hMR–DNA ternary complexes with the same migration as that of TAT complexes (compare lanes 3 and 7). A 10- or 100-fold excess of unlabelled VIT decreased the labelling of [^{32}P]VIT–hMR complexes (lanes 8 and 9). A non-specific band was observed with cytosol proteins from uninfected insect cells (lane 10), confirming that the formation of protein–DNA complexes was dependent on the presence of expressed hMR in the cytosolic extracts of Sf21 insect cells infected with the recombinant baculovirus AcPNV-hMR.

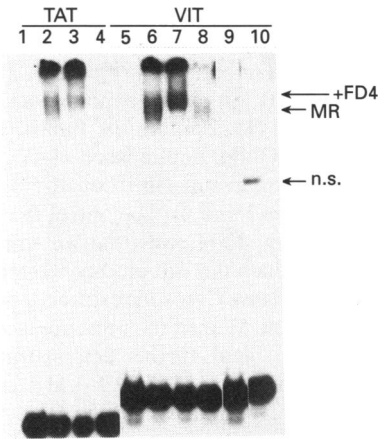

Figure 3 hMR binding to imperfect and perfect palindromic GREs

[^{32}P]TAT probe (~ 0.6 ng) (lane 1) was incubated with expressed hMR (10 μg of protein of cellular extracts) alone (lane 2) or in the presence of 2 μl of purified FD4 (lane 3) or with a 200-fold excess of unlabelled TAT (lane 4). [^{32}P]VIT probe (lane 5) was incubated with the same amount of recombinant hMR alone (lane 6), in the presence of 2 μl of purified FD4 (lane 7) or with a 10- or 100-fold excess of unlabelled VIT (lanes 8 and 9). A non-specific band (n.s.) was detected after incubation of 10 μg of cytosolic protein from uninfected cells incubated with [^{32}P]VIT probe (lane 10).

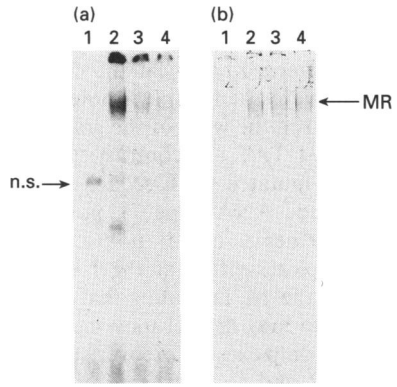

Figure 4 Immunological identification of GRE4–hMR complexes

(**a**) Autoradiogram of a gel shift mobility assay. [^{32}P]GRE4 probe was incubated with 20 μg of cytosolic protein from uninfected cells (lane 1) or with the same amount of protein containing the recombinant hMR alone (lane 2) or in the presence of a 100- or 200-fold excess of unlabelled GRE4 (lanes 3 and 4) respectively. n.s., non-specific binding. (**b**) Western blotting. An identical gel shift mobility assay was performed in parallel and the gel was transferred to a nitrocellulose membrane. The presence of immunoreactive hMR was detected by a Western blot with purified FD4 antibody (dilution 1:500).

Oligonucleotides that contain a GRE sequence motif specifically interact with the hMR. Figure 4(a) shows that, as expected, the GRE4 oligonucleotide was capable of forming specific complexes with the recombinant hMR (lane 2) compared with the non-specific signal observed with uninfected cell extracts (lane 1). The major retarded band disappeared in the presence of increasing amounts of unlabelled GRE4 (lanes 3 and 4). To identify hMR in the GRE4–protein complexes, we performed a Western blot after transfer of the gel shift assay containing the same samples (Figure 4b). Signals revealed by FD4 antibody (lanes 2–4) fit perfectly with the labelling of the major retarded band (autoradiogram; Figure 4a, lanes 2–4). No detectable immunoreactive protein was seen in uninfected cell extracts (lane 1). In some infected cell preparations, we observed in gel shift assays minor bands with increased mobility whose specificity for the oligonucleotide was demonstrated by competition experiments. Such a minor band is visible in Figure 4(a) (lane 2) and may correspond to a proteolytic cleavage product of hMR. However, FD4 was unable to recognize this protein band, suggesting that this protein either is unrelated to hMR or has lost the epitope located in the N-terminal domain of the receptor.

The specific DNA–hMR complexes migrate very slowly through the non-denaturing gel and, even after complete migration of free oligonucleotide, they are generally recovered very close to the top of the gel, probably due to the high molecular mass of the receptor and/or its probable multimeric structure. Moreover, the signals produced by these complexes are often broad and thick, preventing clear identification of distinct bands which have already been reported as monomeric or dimeric entities for other steroid receptors [26,27].

To examine whether the hMR–DNA interaction was dependent on the presence and/or the nature of the ligand, we tested the binding of the expressed hMR to GRE under various conditions. In one set of experiments we were unable to observe any difference either in the intensity of the signals or in the mobility of the retarded complexes whether using unbound hMR or hMR incubated with mineralocorticosteroid agonists (aldosterone, cortisol) or antagonist ZK91587. Similarly, there was no major difference in the apparent receptor–DNA affinity or the mobility of the retarded DNA–hMR complexes whether or not we used *in vivo* aldosterone- or ZK91587-labelled receptors (results not shown). However, as mentioned above, the smeared aspect of the specific band signals prevented the detection of subtle mobility differences.

Mapping of DNAase I footprinting of specific hMR-binding regions in the MMTV-LTR

To precisely localize the DNA sequences covered by the hMR after its binding to GRE, DNAase I protection experiments were carried out. These footprint analyses were performed with fragments containing GRE sequences of the MMTV-LTR extending from −299 to +69 bp relative to the transcription initiation site [15]. We examined the interaction between the recombinant hMR expressed in Sf21 cells and the 368 bp fragment amplified by the PCR using either ^{32}P-labelled P1 or P2 oligonucleotide and the corresponding unlabelled primer in order to delineate the exact boundaries of protected regions in the sense and the antisense strand of the DNA. This fragment contains four separated regions of high-affinity steroid receptor binding sites [14] responsible for the hormonal induction, corresponding to GRE4 (−185 to −171), GRE3 (−115 to −110), GRE2 (−99 to −94) and GRE1 (−84 to −79). A nuclear factor 1 (NF1) binding site has been also identified [28]. The MMTV-LTR fragments were incubated with hMR preparations, subjected to digestion with various concentrations of DNAase I and displayed on denaturing polyacrylamide gels.

As shown in Figure 5, four footprints on the sense strand were detected only in the presence of infected cell extracts containing recombinant hMR (lane 2) and not with the same amount of protein from uninfected cell extracts (lane 1). The most clearly protected region corresponds to the positions −186 to −165 bp (GRE4) from the transcription start site, adjacent to three nearly contiguous footprints at positions −110, −94 and −81 (GRE3, 2 and 1 respectively) whose exact limits could not be clearly determined because of the stacked bands. These protected sequences encompass the GREs already described for this viral

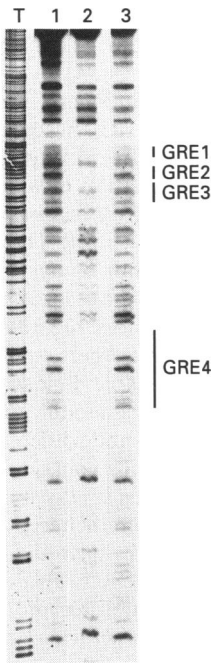

Figure 5 DNAase I footprint analysis of hMR binding to the MMTV-LTR fragment

A 5′-end-labelled sense strand MMTV fragment (−299 to +69) was incubated with 10 μg of protein (whole cellular extracts prepared from Sf21 cells incubated with 50 nM aldosterone) and then digested by 4 units of DNAase I as described in the Materials and methods section. Lane 1, uninfected Sf21 cells; lane 2, AcNPV-hMR-infected Sf21 cells; lane 3, AcNPV-hMR-infected Sf21 cellular extracts + 200 ng of GRE4 competitor oligonucleotide. Positions of footprints were identified from parallel dideoxy A, G, C and T reactions. A representative dideoxy T sequencing ladder is shown on the left of the Figure. Vertical lines indicate the positions of protected regions and are quoted as GRE sequences.

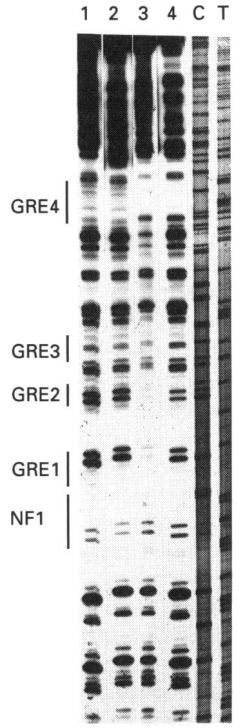

Figure 6 DNAase I footprinting of hMR binding sites on the antisense strand of the MMTV promoter

The 5′-end-labelled MMTV fragment was incubated in binding reactions with 10 μg of cell extract protein from either uninfected cells (lane 1) or AcNPV-hMR-infected cells in the absence (lane 3) or presence (lane 4) of 20 ng of TAT competitor oligonucleotide. Buffer was used as control (lane 2). All reactions were digested by 2 units of DNAase I. The C and T lanes given on the right of the figure correspond to the sequencing reactions. Vertical lines indicate the positions of different motifs contained in the MMTV promoter.

promoter [14,15,25,29]. To ensure that the recombinant hMR specifically binds to these particular sequences, we performed a competition assay using the oligonucleotide GRE4 extending from −193 to −167 bp. As seen in lane 3, a large excess of GRE4 completely prevented the appearance of the distal footprint and, to a lesser extent, of the three proximal target sequences.

In order to map the specific receptor-binding regions on the antisense strand of the MMTV-LTR, we analysed the pattern of DNAase I-generated fragments using uninfected and infected cellular extracts (Figure 6). Interestingly, in the presence of recombinant hMR (lane 3), there was evidence for three major protected sequences at positions −185 to −163 bp (GRE4), −102 to −87 bp (GRE2) and −84 to −72 bp (GRE1). Protection within the GRE3 sequence was less complete than that observed within the sense strand (Figure 5). Moreover, an excess of the oligonucleotide TAT (lane 4), which is closely related to the GRE consensus (see Table 1), was able to partially extinguish the protecting effect of hMR within the footprints GRE1, GRE2 and GRE4. Due to the lower amount of oligonucleotide used in this experiment, the competition by TAT was less pronounced than that of GRE4 (see Figure 5), consistent with a lower affinity of hMR for this TAT sequence.

As in the case of gel shift mobility assays, we examined whether the presence and/or the nature to the bound ligand modified the hMR-generated footprinting patterns. For this purpose we directly incubated the infected Sf21 cells with a mineralocorticosteroid agonist or antagonist before the preparation of cellular extracts and assessed in these conditions the receptor binding. Aldosterone- and ZK91587-labelled hMR complexes bind to the same sequence regions of the MMTV promoter without apparent differences in the intensity and limits of the protected sites (results not shown). Similarly, we found that the steroid-free or aldosterone-bound hMRs were equally potent in interacting with the GRE sequences of the MMTV, as analysed by *in vitro* footprintings.

DISCUSSION

In this work we provide evidence that recombinant hMR expressed in the baculovirus system is able to specifically interact with HREs. This was done by the means of gel shift mobility assays and *in vitro* footprinting, two complementary technical approaches which allow detailed analyses of protein–DNA interaction.

The baculovirus expression system is now widely utilized to produce large quantities of eukaryotic proteins [30], in particular biologically active steroid receptors [31–35]. With respect to the MR, since authentic receptors are poorly represented in aldosterone target cells, it is of particular interest to produce a plentiful supply of recombinant receptors. In addition, in all experimental models studied up to now, the cross-occupancy by glucocorticosteroids and mineralocorticosteroids of their cognate receptors prevents the clear distinction of their respective con-

tributions to the biological effects. Furthermore, the high degree of sequence identity in the DNA-binding domain (94%) and the identical P box between two closely related members of the same subfamily, the hGR and the hMR [2,3], suggests that the structures of these domains are almost identical. The response elements to which these receptors bind are likely to be similar in sequence and organization [12]. The availability of substantial amounts of hMR together with the lack of GRs makes the baculovirus system an interesting and suitable model for studying receptor–target gene interactions. It has previously been shown that the LTR of the MMTV, a glucocorticosteroid-inducible promoter, may also direct aldosterone-dependent gene regulation through the MR [3,9,10]. Transcriptional activity measurements suggested that the HRE included in this viral promoter confers the aldosterone responsiveness.

The present results of the gel shift mobility assays demonstrate, for the first time, a specific interaction of the hMR with a DNA fragment. Several lines of evidence indicated the specificity of this interaction for the recombinant hMR, since AcNPV-hMR-infected cells display retarded protein–DNA complexes which are supershifted by the addition of the monoclonal anti-hMR antibody FD4 but are absent from uninfected cells. Western blot analyses confirm that the hMR is included in these protein–DNA complexes.

Using a series of oligonucleotides, we showed that the MR binds to specific DNA sequence motifs corresponding to the GRE of the tyrosine aminotransferase (TAT). These sequences contain two half-sites composed of hexanucleotides TGTACA and TGTTCT separated by a strict spacing of three bases. These GRE half-sites, which are in an inverted orientation, seem to form the contact points, since double mutations such as found in MST abolish MR binding. Our results indicate that the hMR is able to recognize GREs, but do not allow a clear definition of a specific MRE. Such regulatory elements have been evoked in the promoter of the α_1 subunit of the Na^+,K^+-ATPase, an aldosterone-regulated protein [24]. However, the hMR does not bind to ANA, suggesting that this sequence is not involved in the transcriptional regulation. The binding efficiency of steroid receptors for oestrogen response elements and GREs is dependent not only on the sequence but also on the relative orientation and spacing of the repeated motifs of response element [36–38]. All of these parameters need to be determined in order to establish a better definition of the MRE and to identify such response elements in other aldosterone-regulated gene promoters. Upon DNA binding, steroid receptors form homodimers in a co-operative manner [22,33,37]; such a mechanism remains to be established for MR.

Since recombinant hMR bound to diverse GREs, we used footprint experiments to investigate the precise location and defined sequences of the hMR-protected regions in the MMTV-LTR. Four footprints were detected on the two opposite DNA strands of the proximal promoter (Figures 5 and 6) that correspond to GRE1, 2, 3 and 4, suggesting that the receptor surrounds the DNA helix at its site of binding. Competition experiments in which DNAase I-digested fragments reappeared in the presence of an excess of TAT and GRE4 oligonucleotides provided additional evidence for a specific binding of the hMR to GRE sequences. We have shown that the hMR binds to the same regions of MMTV as the GR. It is possible that the intimate molecular interaction of the hMR with DNA differs from that of the GR, as already described for the GR and the PR within the HRE of MMTV; most notably, the PR generates a longer footprint and contacts three additional guanine residues [39]. In any case, since the MR binds to the same DNA sequences as the GR, differential gene regulation or distinct receptor interactions with other transcriptional factors may explain the selective discrimination between gluco- and mineralo-corticoid effects.

Using two different techniques, we showed that the recombinant hMR binds to GREs whether complexed with an agonist or an antagonist, or free. In agreement with our data, previous studies using methylation or *in vitro* footprint experiments did not demonstrate any effect of ligand binding on the interaction of PR [40] or the GR [41] with specific DNA sequences. In contrast, gel shift assays give divergent results. Some authors have reported that target sequences could form complexes with steroid receptors regardless of the agonist or antagonist bound [31,42] while others emphasize the importance of the agonist to determine stable dimeric receptor–DNA complexes [22]. Moreover, structural modifications of receptors have been proposed to account for subtle differences in the mobility of agonist and antagonist–receptor complexes [26,42,43]. Overall, one can question the role played *in vitro* by the ligand in ensuring an adequate interaction between the receptor and DNA. Indeed, recent crystallographic analyses have indicated that the DNA-binding domain of the GR alone is also able to bind its target element [44]. Similarly, truncated receptors lacking the steroid-binding domain have been shown to be transcriptionally active [45], confirming that an appropriate receptor–DNA interaction could occur even in the absence of ligand. According to the classical mechanism of steroid action, hormone binding induces dissociation of the receptor from an inactive complex containing the 90 kDa heat shock protein (hsp90), allowing subsequent receptor dimerization, high-affinity DNA binding and transcriptional activation. MR is an hetero-oligomer including hsp90 in aldosterone target cells [46] as well as in insect cells [5]. However, we cannot exclude the possibility that part of the recombinant receptor is still capable of interacting with DNA even if it does not bind ligand.

The kinetic aspects of ligand–receptor–DNA interactions probably play an important role in modulating receptor activity. For instance, it has been proposed that the rapid dissociation of anti-mineralocorticoids from the MR is involved in their antagonistic activity [47,48]. As suggested earlier, anti-hormones may function by establishing unstable receptor–DNA complexes which are transcriptionally non-productive [49]. The binding of hormone has been shown to accelerate the kinetics of GR and PR binding to target DNA [50], giving another example of the importance of kinetic parameters which should therefore be taken into consideration more often.

In conclusion, DNA-binding studies using baculovirus-expressed recombinant hMRs have shown that the receptor is functional, although they have not allowed definite conclusions to be drawn regarding the role of the ligand in directing receptor–DNA binding. The availability of large amounts of the purified MR should aid in the identification of high-affinity MREs that confer aldosterone responsiveness to known or as yet unidentified genes.

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