Mineralocorticoid Regulation of Transcription of Transfected Mouse Mammary Tumor Virus DNA in Cultured Kidney Cells

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Abstract. Renal cells contain two corticosteroid-binding entities defined on the basis of hormone-binding selectivity as type I (mineralocorticoid) and type II (glucocorticoid). The mineralocorticoid, aldosterone can bind to both type I and type II receptors. This poses problems in defining the characteristics of a true mineralocorticoid regulated expression of specific genes. We have used chimaeric constructs bearing the mouse mammary tumor virus (MMTV) promoter to study aldosterone action in the feline renal cell line CRFK. We have shown that in these cells aldosterone induces MMTV transcription through its own receptor (type I). This induction of MMTV transcription by aldosterone is a primary response to the hormone. We have shown that the DNA sequences that mediate the aldosterone response overlap the hormone response element (HRE) required for the glucocorticoid, progestin, and androgen induction of transcription at the MMTV long terminal repeat region. Thus the aldosterone regulation of MMTV long terminal repeat transcription is identical to the mode of action of the other steroid hormones at this promoter.

The steroid hormones that constitute the class corticosteroids are glucocorticoids and mineralocorticoids. Glucocorticoids regulate many developmental processes in higher eukaryotes and play an important role in cellular metabolism (for review see reference 7). Mineralocorticoids on the other hand promote sodium reabsorption across tight epithelia such as in the colon or in the distal nephron (22). Whilst the physiological function of glucocorticoids in the regulation of the expression of a number of genes have been intensively studied (for reviews see references 3 and 32), little information is available on the effect of mineralocorticoids on the expression of specific genes. One reason for this lack of information on mineralocorticoid action is the difficulty involved in distinguishing a true mineralocorticoid from a glucocorticoid response. Aldosterone, the available mineralocorticoid that can be used in transcriptional studies has a low but significant affinity for the glucocorticoid receptor (19). Similarly glucocorticosteroids also interact with the mineralocorticoid receptor (19).

One way of studying a true mineralocorticoid induction of specific gene transcription is to transfect the cloned mineralocorticoid receptor and a marker gene into cells that do not contain mineralocorticoid or glucocorticoid receptors. Such an approach has been used by Arriza et al. (1987) (1) to show that mineralocorticoids induce mouse mammary tumor virus long terminal repeat (MMTV LTR) transcription. Another approach is to use a mineralocorticoid antagonist in cells that contain corticosteroid receptors to determine which changes in gene expression could be attributed to the effect of the mineralocorticoid hormone.

In this report, we have investigated the effect of aldosterone on the transcription at the MMTV LTR promoter in the feline kidney cell line (CRFK) that contains both mineralocorticoid and glucocorticoid receptors. With the aid of the mineralocorticoid antagonist RU28318 (17 β-hydroxy-3-oxo, 7 α-propyl (17 α)-pregn 4-ene, 21 potassium carboxylate) (21), we have shown that aldosterone induces MMTV LTR transcription in these cells via the mineralocorticoid receptor. As in the case of the induction of MMTV LTR transcription by other classes of steroid hormones, sequences in the hormone response element (HRE) of the MMTV LTR play an important role in the aldosterone response. We have therefore identified the HRE as an important regulatory element for the mineralocorticoid induction of MMTV LTR transcription.

Materials and Methods

Hormones and Anti-Hormones

Non-radioactive dexamethasone and aldosterone were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2,4,6,7-3H]Dexamethasone 78.7 Ci/mmol and [1,2-1H]aldosterone 49 Ci/mmol were purchased from Amersham. The synthetic glucocorticoid RU28362 and the mineralocorticoid antagonist RU28318 were supplied by Roussel Uclaf (Romainville, France).

Construction of Plasmids

Plasmid pGR02 is an endogenous–exogenous hybrid provirus constructed from the plasmids pGR20 and pGR21 as previously described (23).
Plasmid pMMTVCAT is an MMTV LTR CAT fusion gene that contains MMTV LTR sequences (−631/+125) linked to the CAT gene as previously described (4).

Plasmid pHc9 contains MMTV LTR sequences (−428/+125) linked to the CAT gene. This plasmid was constructed by substituting the HindIII/SstI MMTV LTR fragment (−631/+102) of pMMTVCAT with the HindIII/SstI MMTV LTR fragment (−428/+102) of ptkCAT5A (4).

Plasmid pHcwt contains MMTV LTR sequences (−237/+125) linked to the CAT gene. This plasmid was constructed by the removal of the BamHI/BstEII MMTV LTR fragment (−631/+110) of pMMTV CAT (4) with the BamHI/BstEII fragment (−237/+110) of ptkCAT5A (4).

Cell Culture and Transfection of Cells

A cloned population of feline kidney cells (designated 2C9) stably cotransfected with the construct pGRH02 and pSV2neo as described by (25) was cultured in DME supplemented with 3% FCS, stripped of hormone by charcoal treatment (CCS) (31). Transient transfection with these cells was carried out using the calcium phosphate method as described by (16). For transient transfection, the cells were cultured in DME supplemented with 10% FCS, and treated with "low" concentration (10⁻⁶ M) of the mineralocorticoid antagonist RU28318 for 24 h before and all through the transfection procedure. Cells treated with hormone were given 10⁻⁶ M aldosterone in addition to the 10⁻⁶ M RU28318. This concentration of aldosterone overcomes the effect of the antagonist. The cells were treated with the antagonist to reduce the basal level of mineralocorticoids in the culture medium.

CAT Assay

CAT assay with cellular extracts of transfected 2C9 cells was performed as previously described (4).

Hormone Receptor Measurements

Steroid hormone receptor levels in cytosol of 2C9 cells was determined in duplicates by the dextran–charcoal coated method (2). The cytosol was incubated with radioactive ligands for 24 h at 4°C. For aldosterone receptor measurements, saturating concentrations of [³H]aldosterone (10 nM) with RU28362 (1 µM) were used and the binding of the radioactive aldosterone was displaced by 1 µM unlabeled aldosterone. For glucocorticoid receptor measurements, saturating concentration of [³H]dexamethasone (10 nM) was used and binding to the receptor was displaced by 1 µM RU28362.

SI Nuclease Mapping

SI nuclease mapping experiments with RNA extracted from 2C9 cells were carried out as described by (29). For mapping the start of transcription at the MMTV LTR promoter a 2.1 kb Cia I/Hpa II fragment from plasmid p2.6 (10) was used. Transcripts initiated at the SV40 promoter of the cotransfected pSV2neo plasmid (25) were mapped using a 3.0-kb Eco RI/Bgl II fragment of the pSV2neo construct as probe.

Results

The Feline Kidney Cell Line CRFK Contains Both Mineralocorticoid and Glucocorticoid Receptors

We have used the feline (Felis catus) renal cell line CRFK (16) that had been co-transfected with an endogenous-exogenous hybrid MMTV proviral DNA (23) and the plasmid pSV2neo (25) to study the regulation of MMTV LTR transcription by mineralocorticoids. A cloned population of the transfected CRFK cells designated 2C9 (23) was chosen for these studies.

The mineralocorticoid receptor level in these 2C9 cells was measured as 14.9 ± 0.5 f moles/mg protein using the dextran–charcoal–coated method (2). In these measurements, a mixture of [³H]aldosterone and 100-fold excess of unlabeled glucocorticoid, RU28362 (11β, 17β-dihydroxy-6-methyl-17α-(1-propynyl)-androsta-1,4,6-trien-3-one) (20) was used as radioactive ligand. RU28362 prevents [³H]aldosterone from binding to the glucocorticoid receptor as aldosterone has some affinity for this receptor (19). RU28362 itself has a high affinity for the glucocorticoid receptor but virtually no affinity for the mineralocorticoid receptor (19). The combination of [³H]aldosterone and unlabeled RU28362 in the receptor determination protocol would ensure measurements of only mineralocorticoid receptor levels. To calculate the level of binding of the labeled aldosterone to the mineralocorticoid receptor, 100-fold excess unlabeled aldosterone was added in some experiments. The binding of [³H]aldosterone to its binding entity in the cytosolic extracts of the feline kidney cells was saturated by 10 nM. The level of binding was identical whether labeled aldosterone was used alone or in combination with RU28362. This indicates that under the conditions used aldosterone was only interacting with the mineralocorticoid receptor. The 2C9 cells also contain glucocorticoid receptor which was measured as 131.8 ± 20.4 fmol/mg protein using [³H]dexamethasone as radioactive ligand and RU28362 to displace the labeled dexamethasone specifically bound to the glucocorticoid receptor. No measurable levels of progesterone or androgen receptors were determined in these cells (data not shown).

Aldosterone Induces the Transcription of Transfected Chimaeric MMTV LTR Proviral DNA in the Feline Kidney Cell Line CRFK

The 2C9 cells were cultured in medium supplemented with FCS that had previously been stripped of hormone (CCS) according to the method of (31). These cells were then treated for 16 h with different concentrations of aldosterone and RNAs were extracted and analyzed by the SI nuclease mapping procedure.

SI nuclease mapping studies with RNAs from cells cultured in medium supplemented with untreated serum showed a high level of MMTV LTR transcription even in the absence of added aldosterone. Although medium supplemented with 10% CCS reduced this level of MMTV LTR transcription, an even lower basal level was obtained when the cells were cultured in medium containing 3% CCS. Alternatively this basal activity of the MMTV LTR promoter could be lowered by treating the cells with the mineralocorticoid inhibitor RU28318. This indicates that FCS contains mineralocorticoid agonists that could induce transcription at the MMTV LTR promoter even in the absence of added aldosterone. Similar data have already been obtained by Arriza et al. (1). In the SI nuclease mapping experiments with RNA from 2C9 cells cultured for a week in medium supplemented with 3% CCS, we observed that the correct start of transcription was used at the 3' MMTV LTR promoter. The amount of RNA initiated at this site was increased dose dependently by aldosterone (Fig. 1 B, lanes 1–5). As slight differences may exist in the amounts of RNA used for these studies, we used as an internal control the amount of transcript initiated at the hormone insensitive SV40 promoter (Fig. 1 C, lanes 1–5) to standardize the calculation of the relative intensity of the SI nuclease signals. We achieved this by scanning the bands corresponding to the correctly initiated MMTV LTR and SV40 transcripts (see arrows in Fig. 1, B and C) and expressing them as a ratio of the MMTV LTR band over the SV40
band. We then presented these values in a graph against the concentration of aldosterone used in the experiment (Fig. 1D). From this graph, we calculated the concentration of aldosterone required for half maximal induction of MMTV LTR transcription. This value (7 nM) is close to the dissociation constant of aldosterone for the mineralocorticoid receptor measured in brain and pituitary cells of the rat (15) and in the 2C9 cells (K_d = 3 nM) (results not shown). Thus the induction of MMTV LTR transcription by aldosterone occurs most likely through the mineralocorticoid receptor.

**Hormone Specificity**

To investigate whether the aldosterone response is indeed mediated by the mineralocorticoid receptor, the effect of the mineralocorticoid antagonist, RU28318 on the aldosterone-induced MMTV LTR transcription was studied. As a control, the effect of RU28318 on glucocorticoid induced MMTV LTR transcription was also studied. The induction of MMTV LTR transcription by 10^-7 M aldosterone was dose dependently inhibited by RU28318 (Fig. 2). RU28318 (with an antagonist/agonist molar ratio of 100) inhibits 65% of the aldosterone induced MMTV LTR transcription. Transcription at the MMTV LTR promoter induced by 10^-7 M dexamethasone was not affected by any of the concentrations of RU28318 tested (10^-8 to 10^-5 M) (Fig. 2).

Taken together, these results confirm published data that RU28318 interacts weakly but virtually only with the mineralocorticoid receptor (21). Our results also demonstrate that the aldosterone induction of MMTV LTR transcription in the

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**Figure 1.** Aldosterone induces transcription at the MMTV LTR promoter of a transfected chimaeric proviral DNA in feline kidney cells. (A) 2C9 cells are a cloned population of feline kidney cells co-transfected with a chimaeric endogenous-exogenous MMTV LTR construct pGR102 (23) and pSV2neo (25). Transcripts derived from the 3' LTR of the pGR102 construct were mapped using an S1 nuclease mapping procedure with a 2.l-kb C1a I/Hpa II fragment from plasmid p 2.6 (10) labeled at the 5' end of the Hpa II site. Correctly initiated transcripts protect a 106 nucleotide fragment of this probe. Transcripts originating at the correct SV40 promoter of the co-transfected pSV2neo construct protect 392 nucleotides of a 3.0-kb Eco RI/Bgl II probe labeled at the 5' end of the Bgl II site. The open bar of pGR102 represents the endogenous 5' LTR and gag sequences and the striped bar represents the 3' exogenous LTR and env, sequences (23). The broken lines in the pSV2neo construct represent pBR322 sequences. The probes and RNA transcripts have been indicated. (B and C) 30 μg RNAs derived from 2C9 cells cultured 1 wk in DME supplemented with 3% hormone-stripped FCS (CCS) and treated with the indicated concentrations of aldosterone (Ald) were used in the S1 nuclease mapping procedure. The 2.1-kb C1a I/Hpa II fragment of p 2.6 (10) and the 3.0-kb Eco RI/Bgl II fragment of pSV2neo (23) were used as probes for the S1 nuclease mapping experiments. The arrows in B and C show the correct MMTV LTR start of transcription and the correct start of transcription at the SV40 promoter. M represents labeled Hae III fragments of pBR322 used as marker. (D) The bands in the S1 nuclease autoradiographs corresponding to the 106 and 392 nucleotide fragments were scanned and plotted as a ratio of the intensity of the 106nt fragment over the 392nt fragment against the concentration of aldosterone.
feline kidney cells is mediated by the mineralocorticoid receptor.

**The Induction of MMTV LTR Transcription by Aldosterone Requires No De Novo Protein Synthesis**

Cycloheximide, the protein synthesis inhibitor, inhibits the aldosterone-dependent sodium transport in renal A6 cells (22). This suggests that this action of aldosterone requires new protein synthesis. We have studied the effect of cycloheximide on the aldosterone-induced MMTV LTR transcription in our feline renal cells (2C9) using the S1 nuclease mapping procedure to determine whether this drug inhibits this response too.

The induction of MMTV LTR transcription by aldosterone in the absence of cycloheximide was rapid; occurring within 30 min after hormone addition and reaching a maximum by 3 h (Fig. 3, lanes 1–6). The same kinetic of induction of MMTV LTR transcription by aldosterone was determined in 2C9 cells treated with cycloheximide (Fig. 3, lanes 7–12). These experiments indicate that de novo protein synthesis is not required for the aldosterone induction of MMTV LTR transcription. As in the case of the induction of MMTV LTR transcription by glucocorticoids (8), progesterin or androgen (5), the stimulation of MMTV LTR transcription by aldosterone represents a genuine primary response to this hormone.

**Sequence Overlapping the HRE Mediate the Mineralocorticoid Induction of MMTV LTR Transcription**

The HRE located between nucleotides -202 and -59 5' upstream of the start of transcription at the MMTV LTR promoter, mediates the glucocorticoid (48), progesterin and androgen (5) induction of LTR transcription. To determine whether the HRE also plays a role in the aldosterone induction of MMTV LTR transcription, we transiently transfected different MMTV LTR chimaeric constructs into the 2C9 feline kidney cells. The transfected constructs were MMTV LTR 5'-deletion fragments linked to the bacterial chloramphenicol acetyl transferase (CAT) gene in such a way that the MMTV LTR promoter drives the transcription of the CAT gene (Fig. 4 A).

For technical reasons, we were unable to transfec 2C9 cells cultured for a week in medium supplemented with 3% CCS. To decrease the high basal activity of the MMTV LTR promoter in the absence of aldosterone, we cultured the 2C9 cells in medium containing 10% FCS and treated them 24 h before transfection and all through the transfection period and thereafter with 10^{-6} M RU28318. Cells treated with hormone received 10^{-6} M aldosterone in addition to the mineralocorticoid inhibitor. This concentration of aldosterone overcomes the inhibitory effect of the rather weak mineralocorticoid antagonist RU28318.

Different levels of CAT activity were measured in the absence of hormone in the 2C9 cells transfected with the different MMTV LTR-CAT constructs (Fig. 4 B). These differences in CAT activity could be attributed to the presence of activator or repressor elements that we have identified in the 5' upstream region of the MMTV LTR (results not shown). Regardless of what these elements are and how they function, all the constructs with the exception of pGREOOCAT when transfected showed an aldosterone-induced CAT activity of 3–7-fold (Fig. 4 B). As cells transfected with the pHC-wt construct showed an aldosterone induction of CAT activ-
Aldosterone induction of MMTV LTR transcription requires sequences that overlap the HRE on the MMTV LTR. (A) The indicated MMTV LTR 5' deletions fragments were linked to the CAT gene in such a way that the MMTV LTR drives the transcription of the CAT gene. The HRE (hatched box) contains the hormone receptor-binding sites (24, 28). These constructs were transiently transfected into 2C9 cells and CAT activity was measured in the presence and absence of aldosterone, dexamethasone and RU28318. The induction factors were derived from the averages of four independent transfection experiments with at least two different plasmid DNA preparations. (B) CAT activity of the various MMTV LTR 5' deletion constructs in cells treated with or without aldosterone. Ch stands for chloramphenicol and AcCh stands for the acetylated form of chloramphenicol. CAT activity determination and the calculation of induction factors were all as previously described (8). 200-μg protein from cellular extracts of the transfected cells were used for the CAT assay. (C) The construct pH'Cwt was transiently transfected into the 2C9 feline kidney cells and CAT activity was measured after the cells had been treated with aldosterone and/or RU28318. CAT activity induced by 10⁻⁶ M aldosterone of the transfected pH'Cwt is inhibited by 10⁻⁴ M of the mineralocorticoid antagonist RU28318 (Fig. 4 C). This anti-hormone had no effect on the dexamethasone-induced CAT activity (Fig. 4 D). This indicates that aldosterone at the concentration used in the transient transfection experiments mediated a true mineralocorticoid response.

Taken together, the results we have presented identify nucleotides −237 to −73 that overlap the HRE as the sequence necessary for the mineralocorticoid induction of MMTV LTR transcription.

Discussion

Little progress has been achieved in general on studies of the effect of mineralocorticoids on gene expression for a number of reasons. Firstly, so few cells are targets for mineralocorticoid action that an extensive search for the appropriate cells and marker genes has to be pursued. Secondly, even among the target cells for mineralocorticoids, the determination of the levels of mineralocorticoid receptor remains a problem as no true mineralocorticoid ligands are available. The ever popular mineralocorticoid, aldosterone, has a high affinity for its own receptor and some affinity for the glucocorticoid receptor (19). A possibility of blocking off these glucocorticoid-binding sites by glucocorticosteroids has met with little success. Most glucocorticoids including the potent mineralocorticoid antagonist RU28362 have been developed that has a high affinity for the glucocorticoid receptor and virtually no affinity for the mineralocorticoid receptor (2). We have used this "pure" glucocorticoid together with labeled aldosterone to show that in cytosolic extracts of a cloned population of cat kidney cells (2C9 cells) aldosterone binds exclusively to the mineralocorticoid receptor.

We have also demonstrated that the mineralocorticoid, aldosterone induces the transcription of transfected MMTV LTR chimaeric constructs in these 2C9 cells through its own receptor. These studies were made possible by the availability of a mineralocorticoid antagonist RU28318 (21) that reacts specifically with the mineralocorticoid receptor and inhibits a true mineralocorticoid response. The induction of MMTV LTR transcription by aldosterone follows a similar mode of action as the other steroid hormones that induce the transcription of this DNA (5, 8). The induction is rapid and requires no new protein synthesis. Above all, the DNA sequences on the LTR that mediate the aldosterone response overlap the HRE at the MMTV LTR promoter required for the response to other steroids (5, 18).

As concentrations of 10⁻⁶ M aldosterone had to be used in the transient transfection experiments for an appreciable induction of CAT activity to be observed, we wondered whether the results of the CAT assay reflected a true mineralocorticoid response. We therefore transfected the construct pH'Cwt into the 2C9 cells and treated the cells with either 10⁻⁶ M aldosterone or 10⁻⁸ M dexamethasone. Both aldosterone and dexamethasone induced CAT activity in the transfected cells (Fig. 4, C and D). However the CAT activity induced by aldosterone as well as the basal level of CAT activity was drastically reduced by 0.1 mM of the mineralocorticoid antagonist RU28318 (Fig. 4 C). This anti-hormone had no effect on the dexamethasone-induced CAT activity (Fig. 4 D). This indicates that aldosterone at the concentration used in the transient transfection experiments mediated a true mineralocorticoid response.

Figure 4. Aldosterone induction of MMTV LTR transcription requires sequences that overlap the HRE on the MMTV LTR. As concentrations of 10⁻⁶ M aldosterone had to be used in the transient transfection experiments for an appreciable induction of CAT activity to be observed, we wondered whether the results of the CAT assay reflected a true mineralocorticoid response. We therefore transfected the construct pH'Cwt into the 2C9 cells and treated the cells with either 10⁻⁶ M aldosterone or 10⁻⁸ M dexamethasone. Both aldosterone and dexamethasone induced CAT activity in the transfected cells (Fig. 4, C and D). However the CAT activity induced by aldosterone as well as the basal level of CAT activity was drastically reduced by 0.1 mM of the mineralocorticoid antagonist RU28318 (Fig. 4 C). This anti-hormone had no effect on the dexamethasone-induced CAT activity (Fig. 4 D). This indicates that aldosterone at the concentration used in the transient transfection experiments mediated a true mineralocorticoid response.

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The HRE of the MMTV LTR contains four receptor-binding sites with the hexanucleotide core 5'→TGTTCT→3'.
These sequences are bound by both the glucocorticoid and progesterone receptors (24, 28). Sequence analyses of the cDNAs for these two receptors show that their DNA binding domains have a high degree of sequence homology (85% amino acid sequence homology) (II, 12). This might explain why both receptors bind to identical sequences on the HRE. Our finding that aldosterone requires sequences on the HRE for the induction of MMTV LTR transcription makes it very likely that the aldosterone receptor also binds to sequences on the HRE and possesses a DNA binding domain highly homologous to identical domains in the glucocorticoid and progesterone receptors. Indeed recent sequencing of the cloned human mineralocorticoid receptor has shown that the DNA-binding domain of this receptor shares a high degree of relatedness to the DNA binding domains of the glucocorticoid and progesterone receptors (94 and 90% amino acid sequence identity) (I).

In the mouse, the transcription of MMTV proviral sequences occurs predominantly in mammary gland cells (26) where these sequences have been implicated in the development of mammary carcinomas (9). Mammary cells are usually not targets for mineralocorticoid action, although the presence of mineralocorticoid receptors in some neoplastic aldosterone induction of MMTV LTR transcription that we have demonstrated here would be an important process in murine mammary tumor cells. These cells may be an important process as MMTV expression in mammary cells is correlated with the development of renal adenocarcinomas (30).

Recently exogenous MMTV proviruses have been identified in renal cells of the mouse (30). Although the LTR of these proviruses differ from the mammary cell MMTV LTR in the U3 region, the HREs of both LTRs are intact. The HRE in the renal cell MMTV LTR mediates glucocorticoid response (30). From our results, we would predict that aldosterone would also stimulate the transcription of this MMTV LTR in the renal cells through the same HRE. The aldosterone induction of MMTV LTR transcription in renal cells may be an important process as MMTV expression in these cells has been correlated with the development of renal adenocarcinomas (30).

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