Characterization of the angiotensin (AT1b) receptor promoter and its regulation by glucocorticoids

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Abstract

Angiotensin II acts through two pharmacologically distinct receptors known as AT1 and AT2. Duplication of the AT1 receptor in rodents into At1a and b subtypes allows tissue-specific expression of the AT1b in adrenal and pituitary tissue. Adrenal expression of this receptor is increased in the offspring of rat mothers exposed to a low-protein diet and this is associated with the undermethylation of its promoter. This phenomenon is blocked by the inhibition of maternal glucocorticoid synthesis by metyrapone. We have mapped the transcriptional start site of the promoter and demonstrated that a 1·2 kbp fragment upsteam of this site is effective in driving luciferase expression in mouse Y1 cells. A combination of bioinformatic analysis, electrophoretic mobility shift analysis (EMSA), and mutagenesis studies demonstrates: i) the presence of a putative TATA box and CAAT box; ii) the presence of three Sp1 response elements, capable of binding SP1; mutation of any pair of these sites effectively disables this promoter; iii) the presence of four potential glucocorticoid response elements which each bind glucocorticoid receptor in EMSA, although only two confer dexamethasone inhibition on the promoter; iv) the presence of two AP1 sites. Mutagenesis of the distal AP1 site greatly diminishes promoter function but this is also associated with the loss of dexamethasone inhibition. These studies will facilitate an understanding of the mechanisms by which fetal programming leads to long term alterations in gene expression and the development of adult disease.

Journal of Molecular Endocrinology (2009) **43,** 73–80

Introduction

The renin–angiotensin system plays a major part in the regulation of salt and water metabolism and consequently of blood pressure in mammals. Key components of this system are the receptors for angiotensin. Two G protein-coupled receptors (the AT1 and AT2 receptors) have been identified in mammals which have distinctive pharmacological and signal transducing characteristics (Clauser *et al.* 1996, Inagami 1999). However, the majority of the short-term salt and water regulatory functions are mediated through the AT1 receptor. In rodents, a duplication of the AT1 receptor gene has formed the AT1a and b receptor subtypes (encoded by *Agtr1a* and *Agtr1b* respectively). Although the ligand binding and signal transducing features of these two highly homologous receptors are indistinguishable, they do differ in their sites of expression, with the AT1b largely restricted to the adrenal cortex and pituitary (Kakar *et al.* 1992, Sandberg *et al.* 1992).

The physiological importance of the AT1b is not immediately obvious when studied in the mouse knockout models as compensatory increases in *Agtr1a* expression and/or the function can obscure its role. *Agtr1b*-knockout animals have impaired thirst-sensing and drinking, but are not hypotensive. However, in *Agtr1a*-knockout animals, studies with an AT1 antagonist further reduced the blood pressure, and an angiotensin II pressor effect mediated by the AT1b was observed. These aspects are reviewed by Audoly *et al.* (2000).

The selective advantage of having functionally the same receptor derived from distinct genes at these sites is not clear, but one probable benefit is the opportunity for each subtype to be driven and regulated by different promoters. The more widely active *Agtr1a* promoter has been characterized in some detail (Murasawa *et al.* 1993, 1995, Takeuchi *et al.* 1993, Bhat *et al.* 1994). However, little work has been reported on the rat *Agtr1b* promoter following its initial characterization (Guo & Inagami 1994).

The *Agtr1b* is of interest to us in view of our previously reported finding that a maternal low-protein diet results in an increased expression of the adrenal *Agtr1b* by 1 week of age in offspring (Bogdarina *et al.* 2007). It has previously been shown in this model of fetal programming that the adrenal shows increased mineralocorticoid responsiveness to Ang II (McMullen *et al.* 2004, McMullen & Langley-Evans 2005) and this increased expression of *Agtr1b* provides a potential mechanism for the hypertensive phenotype that

DOI: 10.1677/JME-09-0036
0952–5041/09/043–073
Online version via http://www.endocrinology-journals.org

develops after about 4 weeks of age in these animals (Langley-Evans 1997, 2000, Bertram & Hanson 2002). We showed that the putative *Agtr1b* promoter was undermethylated in this model, which may account for the increased gene expression (Bogdarina *et al.* 2007). No significant changes in the expression of *Agtr1a* or any other component of the renin–angiotensin system were found in any tissue studied at this early age.

Several theories for the development of fetal programming have been proposed (e.g. Simmons 2005, Fernandez-Twinn & Ozanne 2006, Lévy-Marchal & Czernichow 2006, Gluckman & Hanson 2007). One of the most widely accepted hypotheses is that maternal stress resulting from various causes leads to increased fetal exposure to maternal glucocorticoids and hence long-term alteration in gene expression or cell number in offspring (Langley-Evans 1997, Bertram & Hanson 2002). Since we have found that fetal programming leads to altered *Agtr1b* promoter methylation, we were particularly interested to investigate the possibility that glucocorticoids might alter DNA methylation of this gene. Administration of metyrapone, an inhibitor of corticosterone production, during the first 2 weeks of pregnancy in rats eating a low-protein diet is able to reverse the overexpression and to normalize the undermethylation of the *Agtr1b* gene (manuscript submitted). We have therefore set out to characterize this promoter in greater detail and in particular to investigate the mechanisms of any interaction with glucocorticoids which might in turn lead to alterations in methylation of this gene during development.

Methods

Cell culture, transfections, and luciferase assay

Mouse adrenocortical Y1 cells were maintained in high glucose DMEM/F10 medium (1:1) supplemented with 2·5% fetal bovine serum (FBS), 12% horse serum (HS), and penicillin/streptomycin at 37 °C and in 5% CO_2. For transfection, 200 ng of each plasmid were co-transfected with 20 ng of the pRL-CMV *Renilla* control vector (Promega, Southampton, UK) into cells in 12-well plates using calcium phosphate precipitation. Thirty-six hours later the cells were washed with PBS, lysed and the promoter activity was measured using the Dual Luciferase reporter assay protocol (Promega) with results normalized to *Renilla* luciferase activity. All experiments were performed three times, each time in triplicate. For dexamethasone stimulation experiments the cells were washed in PBS and cultured in fresh DMEM/F10 supplemented with dextran-coated charcoal-treated FBS and HS. Cells were stimulated with 10^{-7} M dexamethasone for 6 h.

DNA manipulations

5′-Rapid amplification of cDNA ends (RACE) was performed using GeneRacer kit (Invitrogen) according to the manufacturers instructions. Gel purified PCR products were cloned into the TOPO vector (Invitrogen) and sequenced.

Using rat adrenal genomic DNA as a template for PCR with primers (F-AGAGCTCCTTTCCATCTGTTT-GTTTCTG/R_ GATAGATCTTCCCAAGGTGGCAAG), a 1·3 kbp PCR product, containing the 5′-region of the *Agtr1b* promoter was cloned into SacI-BglII sites of pGL3-basic vector. This was subsequently digested with SacI and BamHI, the 1178 bp fragment was gel purified and recloned into SacI-BglII sites of pGL3. This plasmid, pGL3AT1b was used in further experiments for site-directed mutagenesis of three CpG sites. pGL3AT1b was used as a PCR template to create serial 5′ deletions of the promoter containing a 202 bp fragment or a 85 bp fragment of the *Agtr1b* promoter. Both fragments were cloned into SacI and BglII digested pGL3 basic vector. Mutated CpG sites in the proximal *Agtr1b* promoter were generated using the QuikChange XL site-directed mutagenesis protocol (Stratagene, Cedar Creek, TX, USA). Mutations were confirmed by sequencing. For oligonucleotide sequences for PCR and mutagenesis, see the Supplementary Table 1 in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol43/issue2/.

Electrophoretic mobility shift assays

Preparation of nuclear extracts – 10^6 HeLa cells grown in DMEM with 10% FBS were lysed with Dignam buffer A (10 mM HEPES, pH 7·9, 1·5 mM $MgCl_2$, 10 mM KCl, 0·5 mM dithiothreitol (DTT)) using 0·1% NP-40. After isolation of nuclei by centrifugation, the proteins were extracted at 4 °C for 60 min with Dignam buffer C (20 mM HEPES, pH 7·9, 25% glycerol, 0·42 M NaCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, 0·5 mM DTT) in the presence of protease inhibitors (0·5 mM phenylmethylsulfonylfluorid, 1 mM benzamidine, 30 mg/ml leupeptin, 5 mg/ml aprotinin, 5 mg/ml pepstatin). Nuclear extract was aliquoted and stored at −80 °C.

Electrophoretic mobility shift assays (EMSAs) were performed in a 20 µl binding reaction containing 10 µg of the nuclear extract. Double-stranded oligonucleotides were labeled using [γ-^{32}P] ATP (Perkin Elmer, Waltham, MA, USA) and T4 polynucleotide kinase (New England Biolabs, Hitchin, Hertfordshire, UK) and purified from 15% PAGE. The reaction mixtures were incubated on ice for 10 min, then ^{32}P-labeled probe was added and the incubation was continued for another 30 min at RT. In supershift experiments, the nuclear extract was preincubated on ice with anti-Sp1

antibody for 1 h before incubation with the ^{32}P-labeled probe. DNA–protein complexes were separated on 4–5% non-denaturing polyacrylamide gel in 0·5×TBE for 3 h at 200 V at 4 °C and subjected to autoradiography after drying. For competition experiments, 100-fold molar excess of unlabeled competitor was pre-incubated with the nuclear extract for 15 min before labeled probe was added.

Antibodies used for supershift studies were anti-*c-fos* (K-25): sc-253, and anti-*c-jun*: sc-44 from Santa Cruz (Wembley, Middlesex, UK), anti-GR (PA1-510A) rabbit polyclonal antibody from Affinity BioReagents (Cambridge, UK) and anti-SP1 rabbit polyclonal antibody (2873-24) – a gift from Prof Steve Jackson, Gurdon Institute, Cambridge, UK.

Results

Promoter mapping

The start site of transcription of the rat *Agtr1b* gene was identified using the 5′ RACE technique (Fig. 1a). Sequencing of cloned extended products suggested the existence of several potential transcription start sites although the major site was located at position 1437 (Gene Bank Accession number U01033) and 11/20 sequenced clones revealed a start site in this position. Examination of 1·6 kbp of genomic sequence upstream of this start site showed a sequence containing a probable TATA box (at −27 bp), a weak CAAT box at −59 bp and 7 CpG sites, of which three were located in the proximal promoter region. Analysis *in silico* using Transcription Element Search System (TESS, http://www.cbil.upenn.edu/tess) suggested the presence of three putative binding sites for Sp1 (5′ end at −43, −81 and −104 bp relative to the transcriptional start site). CpG sites 1 and 2 are located in one of the Sp1 binding sites (site 2 – Sp1-2) and CpG site 3 in Sp1-3 (Fig. 1b). Binding sites for several other transcription factors including AP1 (position −98 and −135 bp) were noted.

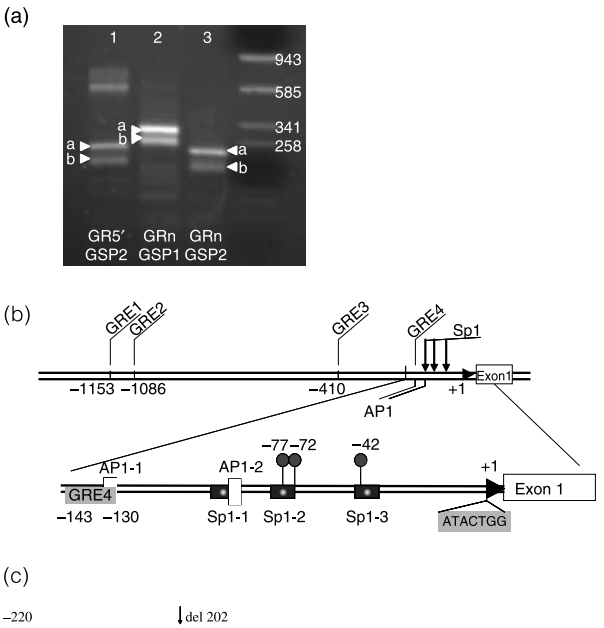

Figure 1 Mapping of the *Agtr1b* promoter. (a) Agarose gel showing the products of the 5′ RACE. Two major products (a and b) are found using independent reverse gene specific primers located at positions 639–654 (GSP1) and 537–559 (GSP2) of the Genebank genomic sequence S69961. Sequence analysis showed that the majority of clones derived from the 'a' band mapped a start site equivalent to position 1437 of the Genebank 5′ region and exon 1 sequence U01033. GR5′ is GeneRacer 5′ primer and GRn is GeneRacer nested primer from Invitrogen. (b) A diagrammatic representation of 1·3 kbp of promoter indicating the location of more distal GREs. Three CpGs sites located within the Sp1-2 and Sp1-3 are shown as closed circles. (c) DNA sequence of the putative proximal promoter showing the transcriptional start site (+1), the probable TATA, CAAT box (both underlined), GRE4, AP1-1 and -2 (gray boxes), and three SP1 sites. Vertical arrows labeled del 202 and del 85 represent the 5′ ends of the two deletion constructs of the promoter studied in luciferase assays.

Promoter function

To assess the function of this putative promoter, a 1·2 kb fragment was cloned into the pGL3 basic luciferase reporter vector. When transiently transfected into mouse Y1 adrenocortical cells significant luciferase activity was recorded. Deletion of this promoter to only the most proximal 202 bp showed that most promoter activity was retained in this fragment, although further deletion to 85 bp which removed both putative AP1 sites and the distal Sp1 site resulted in almost complete loss of activity (Fig. 2a). The three CpG sites of the proximal promoter (−77, −72, −42) were individually or simultaneously mutated changing C for T. Mutation in CpG1 reduced luciferase activity to about 40% of wild-type, mutation in CpG2 had little effect, and mutation in CpG3 reduced the activity to about 5% of wild-type. Combinations of any two CpG mutations or all three obliterated promoter activity (Fig. 2b). We had previously reported that *in vitro* methylation of these CpG sites resulted in complete loss of promoter activity (Bogdarina *et al.* 2007).

EMSA

All three of the proximal promoter CpG sites lie within the predicted Sp1 sites Sp1-2 or Sp1-3. Evidence for the interaction with Sp1 was sought by EMSA using

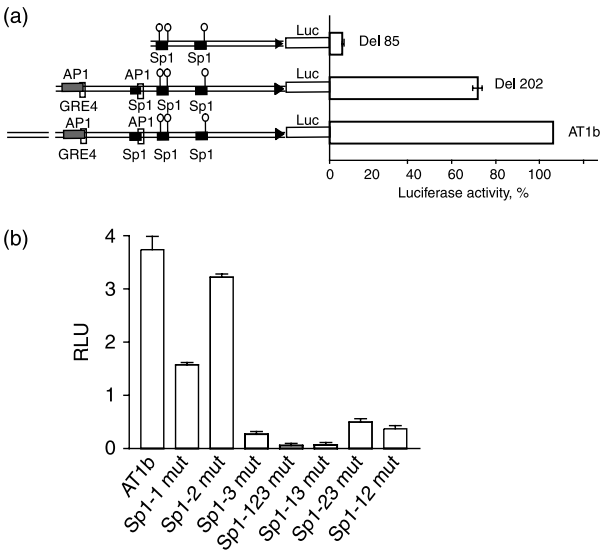

Figure 2 Function of the *Agtr1b* promoter in Y1 cells. (a) The full length, del 202, and del 85 promoter fragments were expressed in mouse Y1 cells and promoter activity monitored using the Dual Luciferase system. Loss of sequences upstream of Sp1-2 results in greatly reduced promoter function. (b) Functional analysis of the full length promoter and derivatives containing mutations of the Sp1 sites is shown in (a). Mut1 is a mutation in the Sp1-2 site at positions −77, mut2 is a mutation in the Sp1-2 site at −72, and mut3 is a mutation in the Sp1-3 site at −42. Mut 123 is a combination of all three mutations, and mut13, mut23, and mut12 mutations in the pairs of CpG sites so identified. All mutations represent a replacement of a C by T.

HeLa cell nuclear extracts and oligonucleotides corresponding to Sp1-1, 2 or 3. All probes bind a complex of a similar mobility to that which binds a consensus Sp1 site (Fig. 3a – arrowed). A mutated Sp1-2 or Sp1-3 oligonucleotide was unable to bind a complex (Fig. 3a). One hundred-fold molar excess of the consensus Sp1 sequence effectively competes for complex binding on both these Sp1 probes (Fig. 3b). The mutant consensus sequence or mutant Sp1-2 or Sp1-3 sequence was unable to compete (Fig. 3b). Similar results are found using Y1 cell nuclear extracts (data not shown). Further evidence that this factor was Sp1 was sought using supershift with Sp1 antibodies. As can be seen in Fig. 3c, complexes formed with both probes and the consensus Sp1 probe show supershift. We had previously shown that the influence of each of these CpG sites on the promoter function was significantly reduced when methylated *in vitro* (Bogdarina *et al.* 2007). We therefore investigated whether Sp1 binding was influenced by methylation using specifically methylated probes in EMSA. In all cases when each of the CpG sites were methylated on both strands a complex migrating as for the unmethylated probe was seen and was supershifted by Sp1 antibody. Therefore, alternative mechanisms such as methyl-DNA binding proteins binding to these methylated CpG sites are more likely to explain the inhibition of gene expression associated with methylation.

Glucocorticoid regulation

Using Y1 cells transfected with the full length *Agtr1b*-luciferase vector pGL3AT1b, it was possible to demonstrate a potent suppressive effect of 6 h treatment with dexamethasone on luciferase activity. Dose response studies indicated an IC_{50} of $4{\cdot}4 \times 10^{-10}$ M (Fig. 4a).

To investigate whether the glucocorticoid effect was likely to be mediated through the putative GREs their ability to bind glucocorticoid receptor was investigated by EMSA. This confirmed that all four GREs bound a complex with similar mobility to that formed with a consensus GRE, and that a 100-fold excess of a consensus GRE competed these away. Addition of dexamethasone (10^{-7} M) to the culture medium for 6 h did not influence the intensity or size of complex formation (Fig. 4b). However, site-directed mutagenesis of GRE1 and 3, but not GRE2 and 4 in the context of the full length promoter led to loss of significant glucocorticoid responsiveness (Fig. 4c),

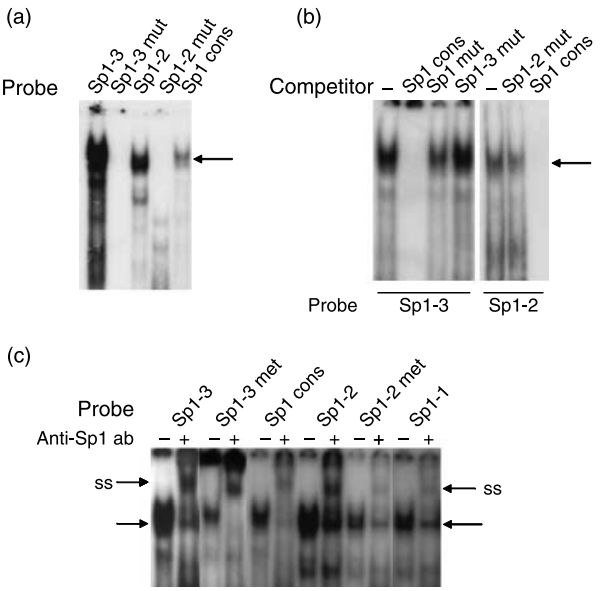

Figure 3 Sp1 binding to proximal promoter elements. (a) EMSA using a consensus Sp1 or Sp1-2 or Sp1-3 as probes. All probes bind a similar sized complex (arrowed) although mutated Sp1-2 or Sp1-3 fail to bind. (b) Sp1-2 and Sp1-3 probes are competed by a consensus Sp1 sequence, but not by mutant sequences. (c) The consensus Sp1 and Sp1-1, -2, and -3 probes are supershifted by antibody to Sp1 (arrow labeled ss). DNA methylation of Sp1-2 or Sp1-3 (labeled SP1-2met and Sp1-3met) does not reduce complex formation (unlabeled arrow) or supershifted band after pre-incubation with antibody.

suggesting that the glucocorticoid effect was mediated in part through these two sites (GRE1 and 3) acting as a negative GRE.

An alternative mechanism for glucocorticoid inhibition of gene expression is that it may be mediated through an AP1 site. Two consensus AP1 sites are present in the proximal promoter, one of which (AP1-2) overlaps Sp1-1 site, whilst the other (AP1-1) overlaps GRE4. EMSA demonstrated that the putative AP1-2 site formed a complex that had similar mobility to that of a consensus AP1 site. Mutation of this site prevented formation of this complex which was also competed by a consensus AP1 oligonucleotide (Fig. 5a). The AP1-1 site also formed a similar complex which was competed by a AP1 consensus oligonucleotide and by GRE4, but not by GRE1, GRE2, GRE3 or mutant consensus AP1 (Fig. 5b). Supershift experiments with anti-*c-fos*, anti-*c-jun*, and anti-GR suggested AP1 and GR could interact with the AP1-1 sequence (Fig. 5d).

Dexamethasone responsiveness was not affected by mutagenesis of AP1-1. Mutagenesis of AP1-2 resulted in greatly reduced activity of the promoter and this was associated with loss of dexamethasone inhibition (Fig. 6).

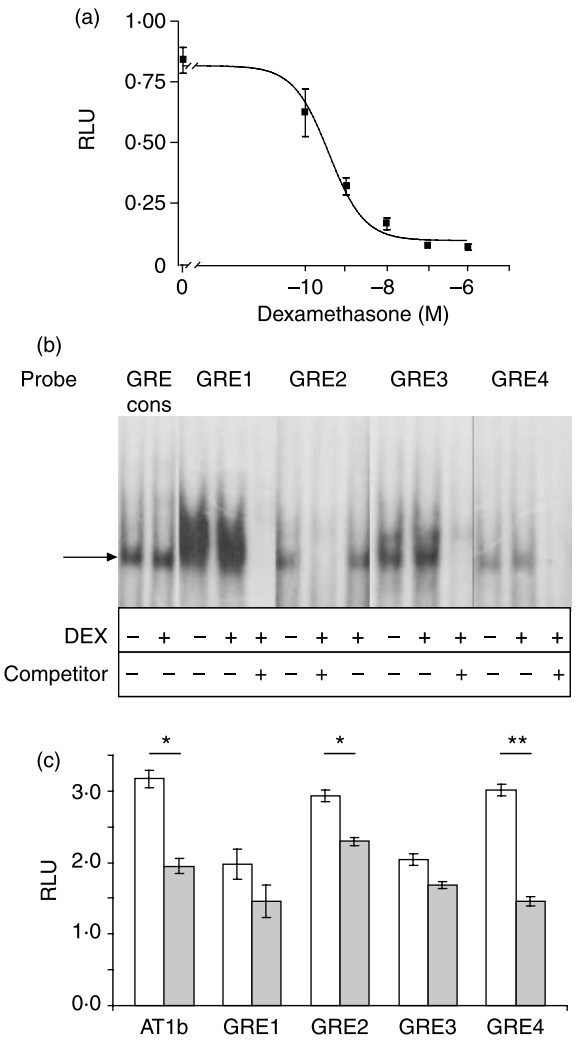

Figure 4 Glucocorticoid responsiveness of the proximal promoter. (a) The full length promoter-luciferase reporter shows a dose dependent inhibition by dexamethasone in Y1 cells. (b) EMSA with a consensus GRE, or GRE1–4 from the *Agtr1b* shows a similar complex formation (arrowed) which is not significantly altered by the pre-treatment of cells with dexamethasone. All of the *Agtr1b* GRE probes show successful competition by a consensus GRE. (c) The *Agtr1b* promoter reporter still shows adequate function in the presence of mutations of each of the GREs, and glucocorticoid inhibition is retained with the GRE2 and GRE4 mutants, but is lost when GRE1 and 3 are mutated. RLU, relative light units. $^{*}P<0{\cdot}05$; $^{**}P<0{\cdot}001$.

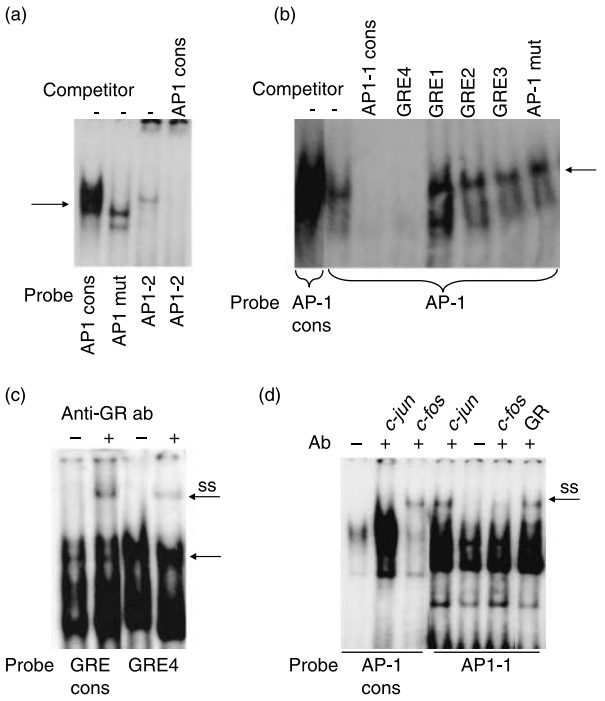

Figure 5 Characterization of AP1 sites. (a) EMSA demonstrating that complexes formed on a consensus AP1 sequence and AP1-2 migrate as a complex with similar mobility, and that a consensus oligonucleotide effectively competes with AP1-2 for binding. Note that the mutant AP1 sequence also reveals a non-specific doublet complex of greater mobility than that formed with AP1. (b) EMSA demonstrating that complexes formed on AP1-1 and a consensus AP1 oligonucleotide migrate with similar mobility, but that AP1-1 binding is competed by a consensus oligonucleotide or by the GRE4 oligonucleotide, but not by a mutant consensus AP1 fragment or the other GRE oligonucleotides. (c) The GRE4 probe binds a complex that is supershifted by glucocorticoid receptor antibody (arrow labeled ss), similar to that observed with the consensus GR probe. (d) The AP1-1 probe binds a complex that is supershifted with anti-*c-jun* and anti-GR antibodies, and to a lesser extent with anti-*c-fos* antibody.

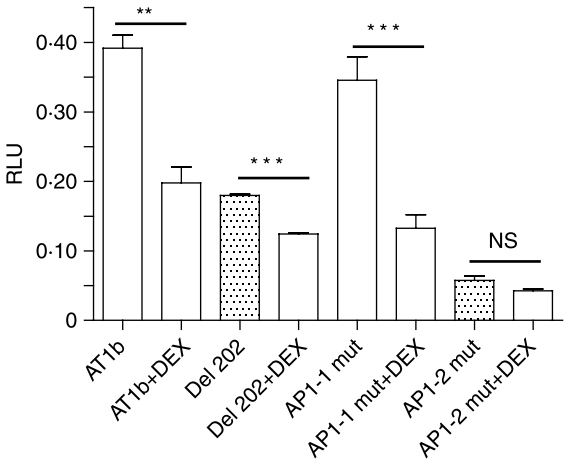

Figure 6 Role of AP1 sites in dexamethasone responsiveness. Dexamethasone (10^{-6} M) inhibited the activity of constructs containing the full length promoter and the del 202 construct with and without a mutated AP1-1 site. Mutation of the AP1-2 site resulted in greatly reduced promoter activity and no evidence of additional dexamethasone suppression of this construct was observed. Statistical comparison was made using Two-way Anova with a Bonnferroni correction. $^{**}P<0{\cdot}001$; $^{***}P<0{\cdot}0001$; NS, not significant ($P>0{\cdot}05$).

Discussion

As outlined in the Introduction, our interest in the *Agtr1b* promoter is driven by our observations on its expression in fetal programming models of hypertension. One candidate mechanism for this phenomenon is that stress-induced maternal glucocorticoid excess may provide a common pathway linking various maneuvers that ultimately lead to similar cardiovascular and metabolic endpoints in the adult. Consequently, we were particularly interested to observe the effects, if any, of glucocorticoids on expression of the *Agtr1b* gene. Previously, Chansel *et al.* (1996) reported that dexamethasone reduced *Agtr1b* mRNA expression in rat mesangial cells, but no effect of dexamethasone was observed in vascular smooth muscle cells (Guo *et al.* 1995). We hypothesized that glucocorticoid targeting of the *Agtr1b* gene may also direct reduced methylation of the gene, and if so, it would be of great interest to identify the mechanism of this effect.

In order to achieve this it was necessary to characterize this promoter and its function in the adrenocortical cells. Previous studies (Guo & Inagami 1994) had identified a putative promoter region and suggested that it may contain glucocorticoid response elements, though these were not found to be functional in vascular smooth muscle cells, which do not express this receptor endogenously. In this work we have characterized the 5′ end of the gene using the RACE technique and used this information to re-define the promoter as a probable TATA-box, CAAT-box, and Sp1 containing promoter. We have shown that Sp1 binds to three consensus Sp1 sites and this is required for a normal expression of the gene. Mutation of CpG sites 1 and 3 significantly reduces gene expression whereas mutation of CpG site 2 has little effect. However, methylation of these CpG sites does not reduce Sp1 binding, as is the case for most, but not all, Sp1 binding sites (Zhu *et al.* 2003, Liedtke *et al.* 2005).

The 1·2 kbp of promoter studied is clearly capable of signaling a negative glucocorticoid effect in Y1 cells with an IC_{50} characteristic of a glucocorticoid receptor mediated effect. By sequence comparison there are four potential consensus GREs in this promoter, two of which correspond to those identified previously (Guo *et al.* 1995). Two of these, GRE1 and 3 appear to mediate in part the negative glucocorticoid effect. However, significant glucocorticoid inhibition was also seen with the proximal 202 bp of promoter which only includes GRE4. This GRE does not seem to influence luciferase expression directly, but closely overlies one of the two AP1 sites in this region. As there are several examples of negative glucocorticoid effects being mediated via protein–protein interaction between GR and AP1 (Yang-Yen *et al.* 1990, Heck *et al.* 1994, Wargnier *et al.* 1998, Tuckermann *et al.* 1999) this alternative was investigated. Mutagenesis of AP1-1 had a relatively small effect on gene expression and the glucocorticoid repression was retained. Mutagenesis of AP1-2 led to substantial loss of expression of the gene, which was accompanied by loss of glucocorticoid inhibition raising the possibility that this is an additional means of glucocorticoid inhibition.

Although the acute negative glucocorticoid regulation observed in these promoter studies might appear to contradict the long-term enhancement of function observed in programmed animals, we believe the latter effect is mediated by reduced methylation. Conceivably the interaction of this promoter with the GR may influence the deposition of methylated sites during the critical period of development at which this occurs.

A further observation of interest is that the Sp1-3 sequence in addition to consensus sites for GAGA factor and *zeste* in exon 1 form a potential *polycomb* response element (PRE). PREs are of particular importance in early development where, in mammalian cells, they may

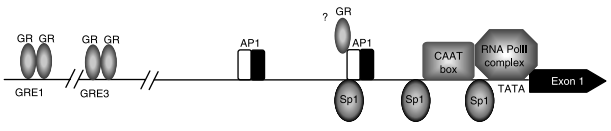

Figure 7 A diagrammatic representation of the key elements of the rat *Agtr1b* promoter (not drawn to scale). The interaction between GR and the proximal AP1 site (AP1-2) is speculative.

bind several proteins that can influence the chromatin structure in the surrounding vicinity as a result of histone methylation (Simon & Tamkun 2002, Lomberk & Urrutia 2005, Schwartz & Pirrotta 2007, Vasanthi & Mishra 2008). Susceptibility of a DNA region to methylation in the course of development may depend on the chromatin structure and thus be influenced by PRE activity. To what extent maternal glucocorticoids can influence PRE occupancy is not known.

In summary, we report the characterization of the *Agtr1b* promoter in Y1 cells, and glucocorticoid inhibition of expression has been identified acting in part through upstream promoter GREs as well as other incompletely clear mechanisms. Our findings are summarized in Figure 7. How this relates to the function of this promoter in the perinatal rat adrenal and its pattern of developmental methylation is not clear.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by a grant from the British Heart Foundation.

Acknowledgements

We are grateful to Prof Steve Jackson (Gurdon Institute, Cambridge) for the gift of Sp1 antibody.

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Received in final form 22 April 2009
Accepted 1 May 2009
Made available online as an Accepted Preprint 1 May 2009