Transcriptional regulation of the rat glucocorticoid receptor gene

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Declaration

I declare that this thesis and the work presented in it are entirely the result of my own independent investigation, except where stated in the text. This work has not been, and is not currently, submitted for any other degree.

James McCormick

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Publications from this thesis

Full Papers (appended to thesis)

McCormick, J.A., Lyons, V., Jacobson, M.D., Noble, J., Diorio, J., Nyirenda, M., Weaver, S., Ester, W., Yau, J.L.W., Meaney, M.J., Seckl, J.R. and Chapman. K.E. (2000) 5'-Heterogeneity of glucocorticoid receptor messenger ribonucleic acid is tissue-specific: differential regulation of variant transcripts by "early life" events. *Molecular Endocrinology*, **14**, 506-517.

Abstracts

McCormick, J.A., Lyons, V., Noble, J., Ester, W., Meaney, M.J., Seckl, J.R. and Chapman, K.E. (1998) Evidence for novel alternate tissue-specific promoters regulating rat GR gene transcription. *Journal of Endocrinology* **156** (suppl), P283.

McCormick, J.A., Lyons, V., Noble, J., Ester, W., Meaney, M.J., Seckl, J.R. and Chapman, K.E. (1998) Tissue-specific differences in rat glucocorticoid receptor gene transcriptional regulation. *Biochemical Society Transactions* **26**, S207.

McCormick, J.A., Lyons, V., Diorio, J., Francis, D., Weaver, S., Meaney, M.J., Seckl, J.R., Chapman, K.E. (1999) Programming of glucocorticoid receptor (GR) expression in hippocampus involves activation of a novel promoter. *Abstracts of the 81st annual meeting of the Endocrine Society*, 3-300.

List of abbreviations

5'-RACE PCR	5'-Rapid amplification of cDNA polymerase chain reaction
5-HT	5-hydroxytryptamine or serotonin
11β-HSD 1	11β-hydroxysteroid dehydrogenase 1
11β-HSD 2	11β-hydroxysteroid dehydrogenase 2
ACTH	Adrenocorticotrophic hormone
AP1 and AP2	Activating proteins 1 and 2
AVP	Arginine vasopressin
bp	Base pairs
BST	Lateral bed nucleus of the stria terminalis
CA	Cornus ammonis
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid binding globulin
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer-binding protein
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
cRNA	Complementary ribonucleic acid
CTP	Cholinephosphate cytidylyltransferase
DBD	DNA binding domain
Dex	Dexamethasone
DG	Dentate gyrus
DNA	Deoxyribonuleic acid
DNase	Deoxyribonuclease
ER	Oestrogen receptors
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptors
GRE	Glucocorticoid-responsive element
GTFs	General transcription factors
HNF	Hepatocyte nuclear factor

HPA axis	Hypothalamic-pituitary-adrenal axis
IGF	Insulin-like growth factor
IGFBP-1	Insulin-like growth factor binding protein-1
I-κB	Inhibitory κB
IL-1, IL-2, IL-6,	Interleukins 1, 2 and 6
Kb	Kilobases
MeCP	Methyl-CpG-binding protein
MMTV	Mouse mammalian tumour virus
MR	Mineralocorticoid receptors
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor κB
NGFI-A	Nerve growth factor-induced gene A
nGRE	Negative GRE
NIDDM	Non-insulin-dependent diabetes mellitus
NSE	Neuron-specific enolase
PBGD	Porphobilinogen deaminase gene
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PNMT	Phenylethanolamine-N-methyl transferase
POMC	Pro-opiomelanocortin
RNA	Ribonucleic acid
RNAP-II	RNA polymerase II
RNase	Ribonuclease
RPA	Ribonuclease protection assays
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEM	Standard error of the mean
sORF	Short open reading frame
TCR	T-cell receptor
TNFα	Tumour necrosis factor α
WT-1	Wilms' tumour 1

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Abstract

Glucocorticoids play key roles in cell differentiation, central nervous system function, intermediary metabolism and the immune response. During stress they act to restore homeostasis. Secretion of glucocorticoids by the adrenal glands is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Negative feedback effects at the hypothalamus and pituitary gland, and effects at other brain regions, especially the hippocampus, suppresses glucocorticoid secretion. Dysregulation of the HPA axis is associated with depression, Alzheimer's disease, obesity, type II diabetes, and altered immunocompetence. In rats, early life events can permanently increase (neonatal handling, via altered serotonergic neurotransmission) or decrease (*in utero* dexamethasone exposure) hippocampal glucocorticoid receptor (GR) levels, with life-long effects on HPA axis responsiveness. Levels of GR expression can also be permanently determined or "programmed" in peripheral tissues by perinatal manipulations. Hepatic GR is permanently increased by prenatal glucocorticoid exposure, altering glucose tolerance; this may be of relevance in the development of non-insulin-dependent diabetes mellitus in later life.

To begin to dissect the mechanisms of perinatal programming of GR, it is necessary to have an understanding of the transcriptional regulation of the GR gene. Regulation of the GR gene is likely to be complex since glucocorticoids have diverse functions in many tissues, and these functions vary during development. Previous work from this laboratory has identified 12 alternate untranslated exons 1 in GR mRNAs transcribed from the rat GR gene, which may reflect transcription regulated by alternative promoters. The majority of these alternate exons 1 lie within a CpG island. The aims of this thesis were to investigate tissue-specific regulation of the rat GR gene and how this relates to perinatal programming of GR levels.

RNase protection analysis was used to determine the relative tissue distributions of alternate exon 1-containing GR mRNAs. One alternate exon 1, exon 1_{10}, was found to be present in the majority (56-87%) of GR mRNAs in a variety of tissues,

including hippocampus, liver and thymus. Mapping of the 5' end of exon 1_{10} revealed substantial heterogeneity in transcription initiation points. Other alternate exons 1 exhibited tissue-specific distributions. For example, exon 1_1-containing GR mRNAs were restricted to thymus, while exon 1_7-containing GR mRNAs are hippocampus-specific.

To address the mechanisms of perinatal programming of GR levels, RNase protection analysis was used to assess changes in the abundances of alternate GR mRNAs in the livers of adult rats exposed to dexamethasone *in utero*. A significant reduction (13%, $p<0.05$) in the proportion of exon 1_{10}-containing GR was detected, suggesting an increase in the proportion of a minor GR mRNA variant. Further experiments, however, did not identify a variant GR mRNA upregulated by this manipulation. 5'-Rapid amplification of cDNA ends PCR performed on primary hippocampal cultures revealed that the majority of GR mRNAs expressed by these cultures contain exon 1_{10} and strengthened the earlier finding that transcription initiation of this variant transcript exhibits considerable heterogeneity. RT-PCR performed on these cultures revealed that primary hippocampal cultures express GR mRNAs containing exon 1_7, which is specifically induced in the hippocampus by neonatal handling, suggesting that these cultures might provide a useful system to elucidate the mechanisms by which neonatal handling leads to permanently increased hippocampal GR.

To determine whether promoter activity is associated with alternate exons 1, a series of genomic constructs was generated and used in transient transfection assays. A construct spanning the entire CpG island had the highest activity of any construct in hepatoma, neuroblastoma and glioma cell lines. A construct designed to assess promoter activity associated with exon 1_7 alone had relatively higher activity in CNS-derived cell lines compared with hepatoma cells. In contrast, other constructs designed to determine promoter activity associated with individual exons 1 exhibited similar activity in all three cell lines. More detailed analysis identified a 134 base pair region conferring significant promoter activity which was substantially higher in neuroblastoma cells than hepatoma cells. The 134 base pair region is also able to act

as an orientation-independent enhancer on a heterologous promoter. Sequence analysis revealed the presence of a putative NGFI-A and an AP2 site in this region: NGFI-A and AP2 are induced in the hippocampus by neonatal handling. Thus, neonatal handling may increase hippocampal GR via a signal cascade culminating in induction of a promoter associated with exon 1_7 by NGFI-A or AP2. It is well established that glucocorticoids regulate expression of their own receptor. Transient transfection assays using glioma cells showed that 10^{-7}M dexamethasone was able to suppress promoter activity of the whole CpG island region by 37%, showing that GR autoregulation occurs at least partly at the level of the GR promoter.

These data show that regulation of the rat GR gene involves use of multiple tissue-specific promoters, which are used differentially during perinatal programming of GR levels. An understanding of the molecular mechanisms underlying programming of GR is of great importance in gaining an understanding of how tissue-specific regulation of GR occurs and how early life events influence adult disease.

Chapter 1: Introduction

1.1 Glucocorticoids

Glucocorticoids, secreted by the adrenal glands, act to restore homeostasis after stress, and play key roles in cell differentiation, central nervous system function, and intermediary metabolism. Gross alterations in circulating glucocorticoid levels results in disease. Primary adrenal insufficiency (Addison's disease), in which there is insufficient production of glucocorticoids, is characterised by weakness, fatigue, weight loss and gastrointestinal complaints (Orth D.N. et al. 1998). Cushing's syndrome is the result of excessive glucocorticoid secretion. Symptoms include central obesity, muscle atrophy, hypertension, diabetes, osteoporosis, depression and memory loss (Orth D.N. et al. 1998). Secretion of glucocorticoids is controlled by the hypothalamic-pituitary-adrenal (HPA) axis, with circulating glucocorticoids exerting important negative feedback effects to suppress their own production. Glucocorticoids act by binding to two types of cytoplasmic receptors, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), which on binding ligand translocate to the cell nucleus where they regulate gene expression by a variety of mechanisms. There are several ways in which glucocorticoid action can be modulated, e.g. by interconversion of hormone between active and inactive forms in different tissues by specific enzymes. Another important regulatory mechanism involves alterations in the levels of GR and MR themselves, which can have profound physiological effects. For example, permanent alterations in GR levels in the liver or in the hippocampal formation of the brain around birth lead to permanent physiological changes that can influence the development of pathology in later life. An understanding of how the GR gene is transcriptionally regulated is therefore crucial to understand not only how glucocorticoids act in general terms, but also how events in early life can "program" GR levels and affect the development of disease later in life.

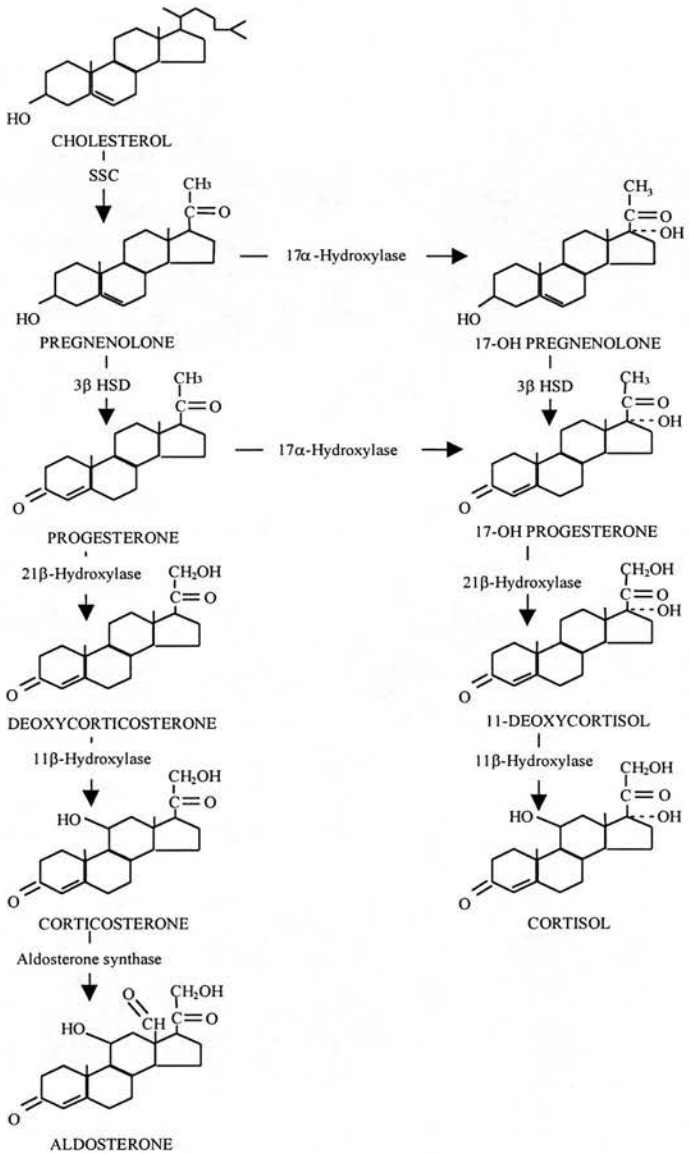


Figure 1.1: *Corticosteroid biosynthetic pathways in the adrenal cortex*

The names of the biosynthetic enzymes are shown above the arrows. SCC, side-chain cleavage enzyme; 3β HSD, 3β hydroxysteroid dehydrogenase.

1.1.1 Glucocorticoid biosynthesis

Glucocorticoids are synthesised in the zona fasiculata/reticularis of the adrenal cortex, though there is *in vitro* evidence of synthesis by other cell types e.g. thymic epithelial cells (Vacchio, M.S. et al. 1994) and possibly glial cells (reviewed in GarciaSegura, L. M. et al. 1996). Cholesterol is the precursor for all steroid hormones and is predominantly delivered to steroidogenic cells by low-density lipoprotein. Cholesterol can also be synthesised *de novo* from acetate or mobilised from intracellular cholesteryl ester pools. The biosynthetic pathway of glucocorticoid production is outlined in Figure 1.1. Firstly, the cholesterol side chain is removed by side-chain cleavage enzyme to form pregnenolone; this is the first step in the synthesis of all steroid hormones, and is rate-limiting. Subsequent reactions result in the production of corticosterone, the predominant glucocorticoid in the rat, or cortisol in humans. Glucocorticoids are not stored by the adrenals, but are released immediately into the circulation. The mineralocorticoid aldosterone is synthesised in the zona glomerulosa of the adrenal gland, which expresses aldosterone synthase.

1.1.2 Control of glucocorticoid production and secretion

1.1.2.1 Secretion from the adrenal cortex

Secretion of glucocorticoids by the adrenal cortex follows a circadian rhythm, regulated by the suprachiasmatic nucleus of the hypothalamus, with plasma glucocorticoid levels peaking prior to activity (i.e. in the morning in humans and in the evening in rats). Importantly, glucocorticoid secretion is also increased in response to "stress". Release of glucocorticoids is governed by the level of activity of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.2). The hypothalamus secretes corticotropin-releasing hormone (CRH), arginine vasopressin (AVP) and other peptides from its parvocellular paraventricular neurons in response to stimulatory inputs from a variety of brain regions. These peptides are released into the hypophyseal portal circulation and are delivered to the anterior pituitary, where

they increase production of pro-opiomelanocortin (POMC), the precursor for adrenocorticotrophic hormone (ACTH) (Orth, D. N et al. 1998). ACTH is the main stimulus for glucocorticoid synthesis at the adrenal cortex and binds to cell surface receptors coupled to adenylyl cyclase. Increasing cyclic adenosine monophosphate (cAMP) levels rapidly activate side-chain cleavage enzyme activity, and ACTH also increases the synthesis of steroidogenic enzymes over a period of days (Orth, D. N et al. 1998).

1.1.2.2 Feedback regulation at the hypothalamus and pituitary

Glucocorticoid secretion is autoregulated via a negative feedback loop. In the parvocellular neurons of the hypothalamus, glucocorticoid administration decreases and adrenalectomy increases CRH mRNA levels (Thompson, R. C. et al. 1987). GR may repress CRH expression by binding to a negative GRE (nGRE) in the CRH gene promoter (Malkoski, S. P. et al. 1997) (section *1.4.2.2*). In the posterior magnocellular division of the paraventricular nucleus, adrenalectomy increases AVP mRNA and this increase can be prevented by dexamethasone treatment (Davis, L. G. et al. 1986). In the pituitary, POMC synthesis and hence ACTH synthesis and secretion are reduced by glucocorticoids (Drouin, J. et al. 1990). Again, GR appears to repress POMC expression by binding to a nGRE (Drouin, J. et al. 1990). Mice expressing an antisense GR RNA ubiquitously have elevated ACTH and corticosterone levels, possibly due to hypothalamic and pituitary glucocorticoid hyposensitivity (Pepin, M. C. et al. 1992).

1.1.2.3 Central regulation of the HPA axis

The hippocampal region of the brain expresses high levels of both MR and GR (reviewed in deKloet, E. R. et al. 1998). The role of hippocampal GR in modulating HPA axis activity is a matter of controversy; *overall*, the hippocampus exerts an inhibitory influence (Jacobson, L. et al. 1991). The hippocampus does not influence HPA axis activity directly but projects excitatory neurons to the lateral bed nucleus of the stria terminalis (BST), which exerts an inhibitory effect on the hypothalamus

(reviewed in Raber, J. 1998). Maintenance of basal HPA activity appears to be mediated by hippocampal MR (high affinity glucocorticoid receptors). Intraventricular administration of a MR antagonist elevates basal plasma glucocorticoid levels at the morning nadir (Ratka, A. et al. 1989). Dexamethasone, a poor ligand for MR, but a high affinity ligand for GR, does not suppress adrenalectomy-induced elevations in ACTH levels when implanted in the dorsal hippocampus, whereas corticosterone (which binds to both GR and MR) implants do (Kovacs, K. J. et al. 1988). After stress, and during the circadian peak of glucocorticoid secretion, GR become progressively occupied. The GR antagonist RU38486 has no effect on nadir levels of plasma corticosterone (Ratka, A. et al. 1989) but decreases basal ACTH levels as glucocorticoid levels rise (vanHaarst, A. D. et al. 1997). Thus, hippocampal GR activation appears to disinhibit tonic maintenance of HPA axis activity by MR. These data correlate well with the opposing effects of GR and MR on neuronal excitability and hippocampal outflow (Joels, M. et al. 1995; Joels, M. et al. 1997; reviewed in deKloet, E. R. et al. 1998).

A large number of studies suggest that GR activation is involved in negative feedback regulation of the HPA axis (Figure 1.2). Reduced hippocampal GR levels are associated with glucocorticoid hypersecretion after stress (Sapolsky, R. M. et al. 1984a) and CRH inhibition is directly proportional to hippocampal GR occupancy (Sapolsky, R. M. et al. 1990). Hippocampal lesions increase CRH and AVP expression and inhibit suppression of the HPA axis following stress (Herman, J. P. et al. 1989a). Several manipulations that decrease hippocampal GR levels diminish the efficacy of feedback regulation, with elevated plasma glucocorticoid levels consistently associated with reduced hippocampal GR (Meaney, M. J. et al. 1989; Jacobson, L. et al. 1991; Henry, C. et al. 1994). These studies, however, are correlative, and it has been suggested that increased hippocampal GR indirectly improve HPA feedback regulation by altering behaviour, e.g. by strengthening the ability of an animal to "cope" with a stressful situation (Vallee, M. et al. 1997; Anisman, H. et al. 1998; reviewed in deKloet, E. R. et al. 1998) (section *1.2.6*).

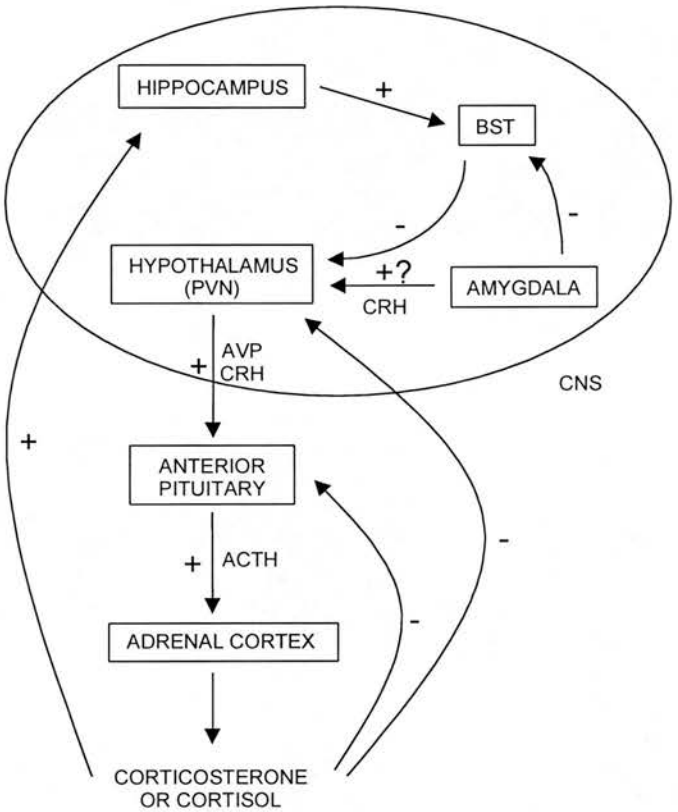


Figure 1.2: *The hypothalamic-pituitary-adrenal axis*

Secretion of CRH and AVP from the paraventricular nucleus (PVN) of the hypothalamus into the pituitary portal circulation stimulates ACTH release from the anterior pituitary, leading to subsequent glucocorticoid secretion from the adrenal cortex. Glucocorticoids exert negative feedback effects at the level of the pituitary and hypothalamus. At the circadian peak of glucocorticoid secretion, and during the stress response, negative feedback effects may also indirectly via the hippocampus. The amygdala exerts positive effects on HPA axis activity, either by direct actions of CRH on the paraventricular nucleus or by suppressing activity of the bed nucleus of the stria terminalis (BST) which sends inhibitory inputs to the hypothalamus. The hippocampus suppresses HPA axis activity by activating BST outflow.

The amygdala also plays a role in central HPA axis regulation, though in contrast to the hippocampus, the effect is stimulatory (reviewed in Raber, J. 1998). The amygdala plays a key role in anxiety and fear-related memory (section *1.2.6*) and these processes are relevant in stress; thus during stress, the amygdala increases HPA axis activity, and fear memory is strengthened (reviewed in Raber, J. 1998). The amygdala inhibits outflow from the BST and may also directly project excitatory neurons onto the hypothalamus (reviewed in Raber, J. 1998). Indeed, lesions of the BST attenuate corticosterone secretion induced by conditioned fear (Gray, T. S. et al. 1993).

1.1.3 The mechanism of GR action

In common with all members of the steroid hormone family, glucocorticoids exert their actions primarily by binding to intracellular receptors. In tissues expressing little or no 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (section *1.1.4.1*), e.g. the hippocampus (Robson, A. C. et al. 1998), MR are essentially occupied by glucocorticoids and activated under basal conditions. GR, in contrast, is a lower-affinity site for glucocorticoids and is only ~10% occupied by basal glucocorticoid levels but becomes progressively occupied (75-100% during the diurnal peak or stress response (deKloet, E. R. 1991). Tissue levels of MR may therefore affect GR-mediated effects of glucocorticoids. This may be of particular importance in feedback regulation of HPA axis activity by the hippocampus (section *1.1.2.3*).

The steroid hormone receptors are a family of structurally related proteins that include the glucocorticoid receptor, (GR), mineralocorticoid receptor (MR), oestrogen receptors (ER) forms α and β (encoded by distinct genes), and progesterone receptors forms A and B (encoded by a single gene); all form a subgroup, with the androgen receptor, of the nuclear receptor (reviewed in Beato, M. 1989; reviewed in Beato, M. et al. 1996). All members of the steroid receptor family function via a similar mechanism, as ligand-dependent transcription factors that interact with specific DNA sequences to either increase or decrease their rate of transcription (section *1.4.2*). Structurally, these receptors consist of a variable

amino-terminal transactivation domain, a central and highly conserved DNA binding domain (DBD), and a moderately conserved carboxy-terminal domain responsible for ligand-binding, dimerisation and transactivation (Mangelsdorf, D. J. et al. 1995; Evans, S. J. et al. 1998).

As discussed above, glucocorticoids act by binding intracellular receptors. There is, however, some evidence suggesting the existence of distinct membrane-associated glucocorticoid receptors (Harrison, R. W. et al. 1979; Orchinik, M. et al. 1991; Evans, S. J. et al. 1998) that signal via G proteins (Iwasaki, Y. et al. 1997). These putative receptors may mediate the rapid, nongenomic effects of glucocorticoids that have been observed, particularly in neuronal tissues (Iwasaki, Y. et al. 1997; Evans, S. J. et al. 1998). It is generally accepted that the highly lipophilic glucocorticoids enter the cytoplasm of cells by free diffusion across the lipid bilayer, though there may be an active transport system involved (Orth, D. N et al. 1998). In the absence of ligand, GR is located in the cytoplasm in an inactive state, as a result of its association with the regulatory heat shock protein complex which includes hsp 90 and hsp 56 (Pratt, W. B. 1993; Smith, D. F. et al. 1993). On ligand binding, GR is activated by a change in conformation, dissociation from the hsp complex, and hyperphosphorylation (Picard, D. et al. 1987; Bodwell, J. E. et al. 1991; Sanchez, E. R. et al. 1994). Activated GR translocates to the cell nucleus and binds to specific DNA sequences as a homodimer (reviewed in Beato, M. 1989; reviewed in Beato, M. et al. 1996). The DNA binding domain of GR consists of two zinc ions co-ordinated with eight cysteine residues to form zinc finger motifs (Luisi, B. F. et al. 1991). The third and fourth cysteines of the first zinc finger form the first turn of an α-helix. GR subunits interact with the major grooves of the DNA via this α-helix; the other finger is involved in protein-protein interactions between the dimer subunits (Hard, T. et al. 1990; Pina, B. et al. 1990; Archer, T. K. et al. 1991). The DNA sequence to which GR homodimers bind (glucocorticoid-responsive element, GRE) is related to the consensus sequence 5'-GGTACAnnnTGTTCT-3' (reviewed in Beato, M. 1989). Once bound to a GRE, GR can activate or repress transcription of the target gene though repression by GR can also occur via mechanisms not involving binding of GR to a GRE (section *1.4.2.2*).

1.1.4 Modulation of glucocorticoid actions

1.1.4.1 General mechanisms

There are several main mechanisms by which the actions of glucocorticoids can be modulated. Firstly, the amount of glucocorticoid available to bind GR can vary depending on levels of synthesis. As described in section *1.1.2.1*, plasma glucocorticoid concentrations fluctuate diurnally, with a peak in the morning in humans (evening in rodents), and under conditions of stress. Binding to corticosteroid binding globulin (CBG) further modifies availability. *In vivo,* about 90% of circulating glucocorticoids are bound to CBG; the majority of the remainder is bound to albumin (Dunn, J. F. et al. 1981). There are two conflicting hypotheses with regard to the role of CBG and steroid-binding plasma proteins in general. The free hormone hypothesis states that the intracellular concentration of a hormone and therefore its biological activity are proportional to the concentration of free hormone in plasma, and not to the protein-bound hormone concentration (reviewed in Mendel, C. M. 1989). In contrast, the free hormone transport hypothesis suggests that a hormone enters tissues exclusively after dissociation from CBG (reviewed in Mendel, C. M. 1989). The principal function of CBG may be to ensure uniform ligand distribution among the cells of target tissues by acting as a glucocorticoid delivery system (Pardridge, W. M. 1987). Specific, high activity receptors for CBG are present on target cell membranes (Singer, C. J. et al. 1988; Rosner, W. 1990). CBG shares a high degree of homology with members of the serine protease inhibitor superfamily (Hammond, G. L. et al. 1987). Cleavage of CBG at the plasma membrane by a serine protease (which acts as the CBG receptor) results in release of bound glucocorticoid (Pemberton, P. A. et al. 1988; Hammond, G. L. et al. 1990), possibly resulting in local delivery of a larger amount of glucocorticoid than is possible by a free-hormone mechanism.

Metabolism of glucocorticoids is another important means by which their bioavailability is altered. The 11β-hydroxysteroid dehydrogenase isozymes (11β-HSDs) convert cortisol (corticosterone in the rat) to inactive cortisone (11-

dehydrocorticosterone in rats) (11β-HSD2) or catalyse the reverse reaction to generate active glucocorticoids (11β-HSD1) (reviewed in Chapman, K. E. et al. 1997). Since MR has high affinity for glucocorticoids, 11β-HSD2 "protects" MR from glucocorticoids in aldosterone-target tissues such as kidney (Funder, J. W. et al. 1988; Edwards, C. R. W. et al. 1988; Agarwal, A. K. et al. 1989). It also protects the developing foetus from potentially harmful levels of maternal glucocorticoids by forming a "barrier" in the placenta (Stewart, P. M. et al. 1995; Benediktsson, R. et al. 1997). 11β-HSD1 reactivates inactive glucocorticoids in glucocorticoid-target tissues (Lakshmi, V et al. 1988). This may be important in regulating the actions of glucocorticoids in the hippocampus (Rajan, V et al. 1996). Glucocorticoids can also be inactivated by a variety of reduction, oxidation and hydroxylation reactions prior to urinary excretion (Orth D.N. et al. 1998).

The activity of GR may be modulated directly in the nucleus by interactions with other transcription factors (discussed in detail in section *1.4.2.2*). The two components of the transcription factor AP1, Jun and Fos have been shown to antagonize the action of GR *in vivo* (Konig, H. et al. 1992) and *in vitro* (Kerppola, T. K. et al. 1993) by a mechanism likely to involve direct protein-protein interaction (Pfahl, M. 1993). It has been suggested that MR and GR may form heterodimers capable of binding GREs (Trapp, T. et al. 1994). MR and GR homodimers can affect transcription from different GRE-containing genes to different degrees, so heterodimer formation may exert subtle effects on gene expression in tissues in which MR and GR are colocalized (Rupprecht, R. et al. 1993). Furthermore, a splice variant of human GR has been described, GRβ, that differs from GRα at its C-terminus and is hence unable to bind ligand (Hollenberg, S. M. et al. 1985). GRβ is also widely expressed but is localized to the cell nucleus in the absence of ligand and is unable to activate transcription of glucocorticoid-responsive reporter genes (Hollenberg, S. M. et al. 1985; Giguere, V et al. 1986). It may therefore antagonize GR effects by acting as a dominant negative regulator of transactivation (Bamberger, C. M. et al. 1995; Oakley, R. H. et al. 1996; Oakley, R. H. et al. 1997; Oakley, R. H. et al. 1999). However, the relevance of GRβ *in vivo* has been questioned (Hecht, K.

et al. 1997; De Lange, P. et al. 1999), and due to the lack of a homologous splice site, GRβ is absent from mice (Otto, C. et al. 1997).

1.1.4.2 Regulation of GR expression levels

The level of cellular GR expression is closely correlated with the magnitude of glucocorticoid sensitivity *in vitro* (Vanderbilt, J. N. et al. 1987). *In vivo*, mice expressing antisense GR RNA ubiquitously (with a subsequent reduction in GR protein levels to around 30-50% of wild type levels) show signs of glucocorticoid resistance (Pepin, M. C. et al. 1992). Similarly, humans expressing GR from only one allele have receptor levels 50% of normal and are glucocorticoid resistant (Karl, M. et al. 1993). Thus, effective regulation of GR expression is critical.

The mechanisms by which levels of GR are regulated are poorly understood and most studies addressing this issue have focussed on autoregulation of GR gene expression. Hippocampal GR are downregulated under conditions of glucocorticoid excess, including chronic stress, high-dose glucocorticoid treatment, and age-related glucocorticoid hypersecretion (Sapolsky, R. M. et al. 1984b; Sapolsky, R. M. et al. 1985; Herman, J. P. et al. 1989b; Makino, S. et al. 1995; Kitraki, E. et al. 1999). In contrast, adrenalectomy upregulates GR at the protein and mRNA level (Herman, J. P. et al. 1989b; Reul, J. M. H. M. et al. 1989; Herman, J. P. S. 1998). Thus, glucocorticoids appear to modulate their own effects in the hippocampus by regulating their own receptor. Downregulation of GR mRNA levels follows high-dose glucocorticoid treatment in kidney, spleen and adrenal gland (Kalinyak, J. E. et al. 1987), and in kidney adrenalectomy leads to upregulation (Kalinyak, J. E. et al. 1987).

In vivo studies of GR expression in rat liver (Dong, Y. et al. 1988), and *in vitro* studies using rat hepatoma (Dong, Y. et al. 1988), rat pancreatic tumour (Rosewicz, S. et al. 1988), rat colonic adenocarcinoma (Meyer, T. et al. 1997) and human lymphoma (Rosewicz, S. et al. 1988) cell lines indicate that autoregulation is likely to occur chiefly at the level of transcription in most cell types. A comparison of the

effect of dexamethasone on the half-lives of GR mRNA and protein suggests an element of post-translational control (Dong, Y. et al. 1988). In the human GR gene a region –250 to –750 relative to the transcription start of the exon 1 in the published human GR cDNA is implicated in autoregulation, and an unidentified protein binds to this region (Leclerc, S. et al. 1991). A mechanism for autoregulation of the human GR gene has been proposed in which ligand-bound monomeric GR interacts with the Jun component of the AP1 transcription factor, reducing induction of GR expression by AP1 binding to sites in the GR promoter (Vig, E. et al. 1994).

Other regulators of GR expression include MR (in the hippocampus) (Herman, J. P. S. 1998; Chao, H. M. et al. 1998), 5-hydroxytryptamine (5-HT or serotonin) (Seckl, J. R. et al. 1990; Mitchell, J. B. et al. 1990a; Mitchell, J. B. et al. 1992; Yau, J. L. W. et al. 1994), and cAMP-mediated pathways in human bronchial epithelial cells and in HeLa cells (Korn, S. H. et al. 1998; Penuelas, I et al. 1998).

In vivo, GR are expressed ubiquitously (Ballard, P. L. et al. 1974), though GR levels vary widely between tissues. For example, testis expresses low levels of GR (Kalinyak, J. E. et al. 1987; Whorwood, C. B. et al. 1992) whereas lung expresses high levels (Kalinyak, J. E. et al. 1987; Whorwood, C. B. et al. 1992). Levels of GR vary within tissues (e.g. Herman, J. P. et al. 1989b), during development (Cole, T. J. et al. 1995a) and in response to environmental manipulations (Olsson, T. et al. 1994 and section *1.3*). Since GR is expressed ubiquitously, it is a member of the family of genes referred to as "housekeeping" genes (reviewed in Dynan, W.S. 1986). However, the mechanisms of transcriptional regulation of the GR gene are poorly understood (section *1.4.4*), particularly with regard to how varying degrees (up or down) of GR expression are superimposed onto a basal level of GR expression (e.g. by programming (section *1.3*)).

1.2 Physiological actions of glucocorticoids

1.2.1 The Stress Response

The actions of glucocorticoids during stress are probably their most important during adult life. Acute physical (e.g. infection) or emotional stress activates HPA axis activity thereby increasing glucocorticoid secretion. The effects of glucocorticoids on metabolism enhance energy availability. Furthermore, during illness glucocorticoids protect the body from over-reactivity by suppressing the immune response. The stress response is terminated by the negative feedback actions of glucocorticoids on HPA axis activity (section *1.1.2*); effective feedback control is critical to prevent the development of pathologies associated with prolonged exposure to even slightly raised glucocorticoid levels.

1.2.2 Transgenic models

Glucocorticoids have effects on virtually all tissues due to the ubiquitous expression of GR (section *1.1.4.2*). In recent years, elucidation of the role of glucocorticoid action has been helped greatly by the development of several mutant mouse models. The various models will be outlined in this section, and in later sections, they will be referred to, where relevant, with regard to GR function in specific tissues.

The earliest mouse model derived expresses an antisense GR RNA (Pepin, M. C. et al. 1992). The original intention of this model was to restrict expression of the antisense GR RNA to brain by placing the transgene under the control of a human neurofilament gene promoter element, but in practice the transgene was also expressed in liver and pituitary (Pepin, M. C. et al. 1992), and in thymus (section *1.2.4*), and possibly ubiquitously (though no other tissues were examined). In the hypothalamus and frontal cortex, endogenous GR mRNA was reduced by 50-70%; in liver the reduction was 30-55% (Pepin, M. C. et al. 1992). The reduction in GR mRNA was reflected by decreased GR protein levels (Pepin, M. C. et al. 1992). These mice exhibit a general disturbance of HPA regulation, including decreased

glucocorticoid feedback efficiency (Pepin, M. C. et al. 1992; Stec, I et al. 1994; Barden, N. et al. 1997; Karanth, S. et al. 1997), enhanced CRH- and stress-induced increases in plasma ACTH but not corticosterone (Montkowski, A. et al. 1995; Barden, N. et al. 1997; Karanth, S. et al. 1997) and adrenal hyporesponsiveness to ACTH (Barden, N. et al. 1997). Pepin et al. showed an increase in basal plasma corticosterone levels in these transgenic mice (Pepin, M. C. et al. 1992), though later analysis showed no difference (Barden, N. et al. 1997), suggesting the levels originally reported were not basal.

To determine the actions of glucocorticoids in T-cell development, a transgene expressed predominantly in immature thymocytes has been developed (King, L. B. et al. 1995). In this case, antisense GR is targeted with the *lck* promoter, which is active mainly in immature thymocytes, but not in mature peripheral T-cells (Garvin, A. M. et al. 1990). Expression of this transgene appears to be thymus-specific, resulting in a 2-fold reduction in GR mRNA and protein, and there are no changes in plasma corticosterone levels (King, L. B. et al. 1995).

An early attempt to create mouse completely lacking GR by inserting a neomycin cassette into exon 2 was unsuccessful (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b), since mRNA encoding an amino-terminal truncated protein containing the DNA-binding domain persists (Tronche, F. et al. 1998). The majority of mice homozygous for this hypomorphic allele ($GR^{hypo/hypo}$) die soon after birth, due to atalectasis of the lungs, indicating that absence of GR is incompatible with survival, but 5-10% survive to adulthood (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b). All $GR^{hypo/hypo}$ express low levels of an N-terminus truncated form of GR; those surviving may be expressing a level of this form of GR just above the threshold needed for survival as a result of subtle differences in conditions experienced *in utero* (e.g. receiving more nutrients by being the first pup in contact with the maternal circulation). Indeed, the number of surviving mice has been reported to be up to 20% (Finotto, S. et al. 1999). In surviving mice, glucocorticoid feedback on the HPA axis is impaired, as reflected by increased plasma levels of ACTH and corticosterone (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b). Furthermore, in the

hypothalamus, there is little change in AVP levels but a massive increase in CRH levels suggesting CRH, not AVP, is the major target for glucocorticoid feedback at the hypothalamus (Kretz, O. et al. 1999).

A complete GR null mouse ($GR^{null/null}$) has been generated recently by deleting exon 3 (encoding zinc finger 1) of the mouse GR gene (Finotto, S. et al. 1999). The $GR^{null/null}$ genotype leads to neonatal lethality in 100% of mice. In common with the $GR^{hypo/hypo}$ mouse, $GR^{null/null}$ mice have impaired induction of gluconeogenic enzymes and glucocorticoid-induced T-cell apoptosis is abolished (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b; Tronche, F. et al. 1998). Due to the lethality of this transgene soon after birth, however, this mouse will be most useful for determining the actions of glucocorticoids during gestation.

Using a Cre transgene under the control of the nestin promoter, a mouse with a brain-specific disruption of the GR gene (GR is reduced by 90%) has recently been generated (Tronche, F. et al. 1999). In the hypothalamus of this mouse, CRH is increased dramatically but AVP is not in comparison to wild type mice, in agreement with data from the $GR^{hypo/hypo}$ mouse (Kretz, O. et al. 1999). Basal plasma corticosterone levels are raised suggesting glucocorticoid feedback at the pituitary is insufficient to counter the increased POMC production caused by raised CRH levels, and central MR cannot compensate for the lack of GR in the brain to suppress the HPA axis (Tronche, F. et al. 1999).

A "knock-in" mutant expresses GR with a point mutation in its DNA binding domain/dimerisation interface (Reichardt, H. M. et al. 1998). *In vitro* studies showed that this mutant form of GR, GR^{dim}, no longer binds co-operatively to GREs (Heck, S. et al. 1994). Furthermore, in the presence of glucocorticoids, GR^{dim} shows a reduced ability to induce a mouse mammalian tumour virus (MMTV)-based reporter (Heck, S. et al. 1994) and completely fails to induce the tyrosine amino transferase (TAT) gene (Reichardt, H. M. et al. 1998). However, repression of AP1 dependent gene activation, which relies on direct protein-protein interactions between GR and not DNA binding by GR (section *1.4.2.2*), is unaffected. Mice generated with this

mutation share the transactivational and repressive properties of GR^{dim} *in vitro* (Heck, S. et al. 1994; Reichardt, H. M. et al. 1998). Due to impaired feedback control by GR, plasma levels of corticosterone levels are raised under basal conditions, and in the pituitary, levels of ACTH and its precursor POMC are increased (Reichardt, H. M. et al. 1998). It should be noted, however, that dimerisation of ligand-occupied GR could possibly occur via protein-protein interactions between the ligand binding domains of GR monomers, so $GR^{dim/dim}$ mutant GRs may not truly be unable to dimerize. Furthermore, *in vitro*, activity of an MMTV-based reporter is not completely ablated, so genes with high affinity GREs may still be activated at a low level in the mouse knock-in due to non-co-operative binding of GR monomers at GRE half-sites.

1.2.3 Intermediary metabolism

Glucocorticoids regulate practically all metabolic processes. After food, glucocorticoids and insulin act together to increase energy stores by effects on glucogenic and glycolytic pathways. Hepatic glycogen stores are increased by activation of glycogen synthase and inactivation of glycogen phosphorylase, the glycogen-mobilizing enzyme (Orth, D. N et al. 1998). Absolute glycogen synthase levels are unchanged by glucocorticoids, but activation occurs via dephosphorylation, either by glucocorticoid–induced activation of a phosphatase or indirectly by inactivation of glycogen phosphorylase (Orth, D. N et al. 1998). During starvation, glucocorticoids inhibit glucose uptake and utilisation in peripheral tissues and increase gluconeogenesis in the liver, partly by increasing substrate availability by stimulation of the release of glucogenic amino acids from peripheral tissues, such as skeletal muscle (Orth, D. N et al. 1998). In addition, glucocorticoids directly induce the expression of key hepatic gluconeogenic enzymes, notably glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (Nyirenda, M. J. et al. 1998; Orth, D. N et al. 1998). When administered exogenous glucocorticoids, $GR^{hypo/hypo}$ mice exhibit impaired induction of hepatic gluconeogenic enzymes (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b), as do $GR^{dim/dim}$ mice (Reichardt, H. M. et al. 1998). Furthermore, $GR^{dim/dim}$ mice show no induction of

hepatic tyrosine amino transferase (Reichardt, H. M. et al. 1998), which diverts tyrosine into the gluconeogenic pathway. Finally, glucocorticoids acutely activate lipolysis in adipose tissue, thus enhancing free-fatty acid release (Orth, D. N et al. 1998). At birth, glucocorticoids in combination with other hormones, especially glucagon, activate key hepatic gluconeogenic enzymes e.g. glucose-6-phosphatase and PEPCK (Liggins, G. C. 1976; Imai, E. et al. 1993). Exposure to glucocorticoids *in utero* also has an important effect on the development of the liver, as well as hippocampus (section *1.3*).

1.2.4 Immune System

Glucocorticoids are powerful immunosuppressors: their actions on the immune system have led to therapeutic applications e.g. in the treatment of asthma (reviewed in Barnes, P. J. 1995) and rheumatoid arthritis (Kirkham, B. W. et al. 1991). In this section I shall focus on the effects of glucocorticoids on the thymus (the site of T-cell generation and selection) and T-cell development.

All lymphocytes express functional GR (Plaut, M. 1987), though levels differ considerably depending on cell maturity and location (Miller, A. H. et al. 1998). Immature thymic T-cells express high levels of GR whereas mature splenic cells express relatively low levels (Miller, A. H. et al. 1998); this might be related to the role of GR in T-cell selection (see below). Adrenalectomy leads to thymic hypertrophy, suggesting that basal levels of glucocorticoids are important in maintaining a normal thymus.

Glucocorticoids are involved in thymic development during gestation. In rats, the thymus primordium is initially colonized by T-cell precursors from foetal liver on days 13-14 of gestation (Vicente, A. et al. 1998). Subsequently, $CD4^+CD8^+$ cells appear (day 18), followed by mature cells in the thymus on day 20 and in the periphery on day 21 (Vicente, A. et al. 1998) (the role of glucocorticoids in T-cell selection is discussed below). Adrenalectomy of pregnant rats accelerates all stages of this pattern of development by one or two days in the developing foetus, and

corticosterone replacement reverses this effect (Sacedon, R. et al. 1999a). Mice expressing antisense GR RNA ubiquitously (Pepin, M. C. et al. 1992) exhibit similar advancement in the arrival of precursors into the thymus and their intrathymic proliferation (Morale, M. C. et al. 1995). These data differ slightly to those obtained using mice expressing antisense GR RNA targeted specifically to developing T-cells which showed no change in precursor arrival (King, L. B. et al. 1995). These differences could be due to the more drastic changes in whole body physiology induced by a lack of maternal glucocorticoids or ubiquitous transgene expression. Another consideration is expression of steroidogenic enzymes and secretion of glucocorticoids even in foetal and early life by a subset of thymic epithelial cells (Vacchio, M. S. et al. 1994). Local secretion of glucocorticoids may account for the high level of T-cell apoptosis observed after maternal adrenalectomy (Sacedon, R. et al. 1999a), or alternatively prevention of antagonism of T-cell receptor (TCR)-induced apoptosis by GR may be responsible (see below).

A key effect of glucocorticoids in the thymus is the induction of apoptosis in immature thymocytes. Physiological glucocorticoid levels achieved during stress are sufficient to induce apoptosis (Gruber, J. et al. 1994). In contrast, mature T cells are resistant to glucocorticoid-induced apoptosis (Miller, A. H. et al. 1998). This difference in glucocorticoid sensitivity may be due to the difference in GR levels between mature and immature T-cells (Miller, A. H. et al. 1998). The apoptotic effect is important in the selection of T-cells bearing TCRs that recognise self-antigens with low-to-moderate avidity, rather than those with near-nil avidity (which would be useless immunologically), or high avidity (which would pose autoimmune problems). The mechanism of glucocorticoid action appears to involve an interaction between GR- and TCR- signalling pathways (King, L. B. et al. 1995; reviewed in Ashwell, J. D. et al. 1996; Tolosa, E. et al. 1998). Glucocorticoids or TCR-ligation independently cause apoptotic death of T-cell hybridomas and immature thymocytes, but simultaneous stimulation results in survival (Zacharchuk, C. M. et al. 1990; Iwata, M. et al. 1991). Inhibition of local glucocorticoid synthesis *in vitro* by metyrapone increases thymocyte apoptosis in response to TCR activation (Vacchio, M. S. et al. 1994). Furthermore, inhibition of local glucocorticoid

synthesis in mice expressing a TCR transgene increases apoptosis, with increased death of the $CD4^+CD8^+$ subset of cells that would normally have undergone positive selection (Vacchio, M. S. et al. 1997). MRL-lpr/lpr mice are spontaneously autoimmune due to selection of a subset of $CD4^-CD8^-$ cells bearing a specific TCR (TCR Vβ) (Theofilopoulos, A. N. et al. 1985). Mice bearing the T-cell-specific GR transgene were backcrossed onto the MRL-lpr/lpr for six generations and then intercrossed (Tolosa, E. et al. 1998). Phenotypically, the resulting mice exhibit a reduction in numbers of TCR Vβ cells and reduced autoimmunity (Tolosa, E. et al. 1998). Thus, in the absence of a normal response to glucocorticoids, the binding of TCRs that normally cause positive selection results in apoptosis. These data suggest that the balance of GR and TCR activation is critical in determining whether developing T-cells survive or die. The precise mechanism of glucocorticoid action during apoptosis is unknown, but regulation of Bcl-2, an important component of the apoptotic pathway, may be important (reviewed in Ashwell, J. D. et al. 1996).

Recently, an alternative mechanism of T-cell selection has been proposed. Using the mouse model ubiquitously expressing antisense GR RNA, the reduction in $CD4^+CD8^+$ cells during gestation was proposed to be due to a partial blockade of differentiation at an intermediate stage of selection rather than apoptosis in response to glucocorticoids (Sacedon, R. et al. 1999b). Furthermore, $GR^{hypo/hypo}$ and $GR^{dim/dim}$ mice exhibit a lack of T-cell apoptosis in response to dexamethasone (Tronche, F. et al. 1998). However, the reduction in thymic GR specifically in $GR^{hypo/hypo}$ and $GR^{dim/dim}$ mice may have been insufficient to exclude a role for apoptosis in T-cell selection in wild type animals.

In the foetal thymus of transgenic mice ubiquitously expressing antisense GR RNA there are large alterations in the thymic stroma, including disappearance of the laminin network under some blood vessels which in the adult results in the appearance of large areas devoid of epithelial cells (Sacedon, R. et al. 1999b). Such epithelium-free areas have previously been identified in thymus, but in the transgenic mice they are much larger (Sacedon, R. et al. 1999b). Thus, glucocorticoids appear to be responsible for maintenance of the thymic epithelium. Indeed, thymic

epithelial cells are responsive to glucocorticoids (Stojanovic, S. et al. 1995) and *in vitro* express levels of GR ten times higher than thymocytes (Dardenne, M. et al. 1986).

The functions of various immune cells alter in response to glucocorticoids. In lymphocytes, an important effect is the inhibition of nuclear factor kappa B (NF-κB) (Mukaida, N. et al. 1994; Ray, A. et al. 1994; Scheinman, R. I. et al. 1995a), a key proinflammatory and proimmune transcription factor in B- and T-cells. NF-κB induces a wide range of cytokines and chemokines, including IL-1, IL-2, IL-6, TNFα, β- and γ- interferons, as well as TCRs α and β (reviewed in McKay, L. I. et al. 1999). The inhibitory effect of glucocorticoids on NF-κB may occur via direct protein-protein interactions between GR and NF-κB (Ray, A. et al. 1994), or via glucocorticoid induction of members of the I-κB family, which act to retain NF-κB in the cytoplasm and hence prevent activation of NF-κB target genes (Auphan, N. et al. 1995; Scheinman, R. I. et al. 1995b). GR also antagonizes gene activation by AP1, which has been shown to induce genes involved in inflammatory diseases such as asthma (Adcock, I. M. et al. 1994; reviewed in Barnes, P. J. et al. 1998). AP1 and NF-κB can act synergistically in the induction of some proinflammatory genes in lung epithelium (reviewed in Barnes, P. J. et al. 1998), and many inflammatory genes suppressed by glucocorticoids lack nGREs in their promoters but do carry binding sites for AP1 as well as for NF-κB (Cato, A. C. B. et al. 1996). Thus, glucocorticoids may further affect NF-κB-mediated immune responses by interfering with AP1 actions.

1.2.5 Developmental effects

Glucocorticoids promote the development and maturation of many tissues. The embryonic adrenal gland begins to synthesize glucocorticoids early in its development; circulating glucocorticoid levels rise steadily and peak at birth (Arai, M. et al. 1993; Nagaya, M. et al. 1995).

Glucocorticoids promote lung maturation by inducing morphological changes and surfactant production in type II pneumocytes (reviewed in Brody, J. S. et al. 1992). Activity of the rate-limiting enzyme in surfactant synthesis, cholinephosphate cytidylyltransferase (CTP), is increased by the synthetic glucocorticoid betamethasone *in vivo* (Mallampalli, R. K. et al. 1994). This effect is apparently a consequence of the metabolic effects of glucocorticoids, which result in increased levels of lipid activators of CTP including phosphatidyl glycerol and fatty acids. Direct glucocorticoid regulation of the genes for the surfactant proteins SP-A, SP-B and SP-C also occurs (McCormick, S. M. et al. 1994; Ballard, P. L. et al. 1996). The actions of glucocorticoids in lung maturation are essential for life. $GR^{null/null}$ mice die within a few minutes of birth due to atalectasis of the lungs (Finotto, S. et al. 1999). In contrast, a small proportion of $GR^{hypo/hypo}$ mice (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b) and all $GR^{dim/dim}$ mice (Reichardt, H. M. et al. 1998) survive to adulthood. In the case of surviving $GR^{hypo/hypo}$ mice, residual activity of the truncated form of GR they express may to be above the threshold level required for induction of the genes required for normal lung function or alternatively that DNA binding and dimerisation is not required for induction of these genes. The fact that all $GR^{dim/dim}$ survive suggests that mutant GR may still bind DNA, since dimerisation of ligand-occupied GR may occur via protein-protein interactions between the ligand binding domains of GR monomers.

Neural crest cells are precursors for a variety of cell types, including autonomic ganglion cells and adrenomedullary cells (Orth, D. N et al. 1998). Neural crest cells have two basic potential differentiation options, depending on whether they are exposed to glucocorticoids (inducing differentiation to a chromaffin phenotype) or neuronal growth factor (inducing differentiation to a neuronal phenotype) (reviewed in Anderson, D. J. 1993). Glucocorticoids exert both positive and negative influences on the phenotype of neural crest cells that have migrated to the developing adrenal gland, thus promoting development of a chromaffin cell phenotype (reviewed in Anderson, D. J. 1993). Chromaffin cells are the peripheral sites for the synthesis and secretion of the catecholamines adrenaline and noradrenaline. Phenylethanolamine-N-methyl transferase (PNMT), the enzyme that converts

noradrenaline to adrenaline, is induced by glucocorticoids in a subset of neural crest cells destined to become chromaffin cells (Teitelman, G. et al. 1982; Jiang, W. et al. 1989; Ross, M. E. et al. 1990; Michelsohn, A. M. et al. 1992) but not those destined to become neuronal (Jiang, W. et al. 1989). Furthermore, glucocorticoids prevent differentiation of neural crest cells to a neuronal phenotype by repressing several neuron-specific genes, including SCG10 (Stein, R. et al. 1988). Very recently, however, analysis of developing adrenals from $GR^{null/null}$ mice has suggested that GR may play no role in chromaffin cell development, since the numbers of chromaffin cells in these mice are normal during gestation (there is no reduction in tyrosine hydroxylase, a chromaffin cell marker) (Finotto, S. et al. 1999). However, PNMT expression is completely abolished in these animals, as expected given the role of GR in expression of the PNMT gene. Furthermore, there appeared to be no switch to a neuronal phenotype. In $GR^{hypo/hypo}$ mice the adrenal medulla is disorganised and PNMT expression reduced (Cole, T. J. et al. 1995a; Cole, T. J. et al. 1995b) but $GR^{dim/dim}$ mice exhibited no changes in PNMT expression or medulla organisation (Reichardt, H. M. et al. 1998), again suggesting GR might still be able to bind DNA in these mice.

1.2.6 Central effects of glucocorticoids

GR are expressed in virtually all brain regions, but expression levels are particularly high in the hippocampus (Arriza, J. L. et al. 1988; Sousa, R. J. et al. 1989). Other brain regions expressing high levels of GR include the cerebellum (Pavlik, A. et al. 1984), amygdala (see below), cortex, thalamus and hypothalamus. Distribution of MR is more restricted, with the majority located in the hippocampus (reviewed in deKloet, E. R. et al. 1998). Since the hippocampus does not express 11β-HSD2 (Robson, A. C. et al. 1998), hippocampal MR effectively function as glucocorticoid receptors.

In addition to its role in HPA axis regulation (section *1.1.2.3*), the hippocampus plays an important role in mood, memory and behaviour (Seckl, J. R. et al. 1995; deKloet, E. R. et al. 1998). Both excessive and inadequate glucocorticoid levels have been

proposed to lead to memory impairment (Luine, V. N. 1997). Therefore, glucocorticoid levels must be maintained within tight limits for normal hippocampal function. Low levels of glucocorticoids (sufficient to activate MR but not GR) or MR agonists enhance memory (reviewed in deKloet, E. R. et al. 1998). Stress, high glucocorticoid levels and GR agonists attenuate memory (reviewed in deKloet, E. R. et al. 1998). Indeed, young rats subject to daily restraint stress for 3 weeks have impaired spatial memory (Luine, V et al. 1994; Luine, V. N. 1997). Importantly, this memory impairment is not permanent. However, *prolonged* exposure to excessive glucocorticoid levels throughout life is likely to contribute to irreversible age-related cognitive dysfunction (Seckl, J. R. et al. 1995). Prolonged glucocorticoid administration or chronic stress may lead to hippocampal neuronal death and adrenalectomy causes death of granule cells in the hippocampus and a reduction in size of dentate gyrus (DG) (Conrad, C. D. et al. 1993; Conrad, C. D. et al. 1995). Exposure to inadequate levels of glucocorticoid impairs spatial memory (Conrad, C. D. et al. 1993; Conrad, C. D. et al. 1995), though paradoxically, glucocorticoid replacement restores spatial memory but does not affect hippocampal morphology (Conrad, C. D. et al. 1995). The importance of hippocampal GR levels in spatial memory is emphasized by studies using transgenic mice; mice expressing antisense GR RNA ubiquitously have impaired spatial memory (Steckler, T. et al. 1999), as do $GR^{hypo/hypo}$ mice (Oitzl, M. S. et al. 1997).

Patients with major depressive disorders hypersecrete cortisol (Murphy, B. E. P. 1991; reviewed in Seckl, J. R. et al. 1995). Depression in old age is often accompanied by cognitive abnormalities and some data suggest that many elderly patients with depression subsequently develop dementia (Emery, V. O. et al. 1992). Antidepressant treatment of depressed patients normalizes HPA axis suppressibility and improves outcome (Holsboer, F. et al. 1996; Barden, N. 1999); the effects of antidepressants on serotonergic neurotransmission are especially interesting, given the potential role of 5-HT in regulation of hippocampal GR (section *1.3*). Indeed, in primary rat hippocampal cultures, long-term antidepressant treatment increases GR mRNA and binding sites (Okugawa, G. et al. 1999). The hippocampus is also a key target for damage in Alzheimer's disease (AD) (Pasquier, F. et al. 1994). Elevated

cortisol levels, increased urinary free cortisol excretion and defective HPA suppressibility are found in many patients with AD (Davis, K. L. et al. 1986; Dodt, C. et al. 1991; Seckl, J. R. et al. 1993) and correlate with hippocampal damage (Deleon, M. J. et al. 1988). Recently, cortisol levels during aging have been shown to correlate with hippocampal atrophy and memory deficits during aging (Lupien, S. J. et al. 1998).

The amygdala plays a key role in behavioural responses to fear and anxiety and these responses are mediated by CRH produced in the amygdala itself, as well as by CRH secreted by the hypothalamus (reviewed in Raber, J. 1998). Glucocorticoids increase CRH expression in the amygdala (Hsu, D. T. et al. 1998), enhancing anxiety whereas adrenalectomy results in decreased expression of CRH (Palkovits, M. et al. 1998). Mice expressing antisense GR RNA ubiquitously exhibit either increased (Korte, S. M. et al. 1996; Strohle, A. et al. 1998) or decreased anxiety (Strohle, A. et al. 1998). These conflicting data may be due to complex effects of the ubiquitous reduction in GR occurring in these animals, which have altered HPA axis function, or differences in housing environments, experimental conditions orr the ages of the animals used (Pepin, M. C. et al. 1992; Barden, N. et al. 1997). Recently, however, mice bearing a conditional GR transgene (controlled by Cre recombinase) targeted to the brain have been generated (Tronche, F. et al. 1999). When GR expression is knocked-out in these mice (in the brain only), they exhibit reduced anxiety (Tronche, F. et al. 1999). Paradoxically, these mice weigh less than normal mice and have reduced abdominal fat deposits (Tronche, F. et al. 1999). The increased HPA activity observed in these mice (section *1.2.2*) would be predicted to lead to increased abdominal fat (reminiscent of Cushing's syndrome). Glucocorticoid actions in the amygdala are associated with the development of obesity (reviewed in Raber, J. 1998). Corticosterone implants in the amygdala activate the amygdala and promote accumulation of abdominal fat (Akana, S. et al. 1997). The reduction in anxiety observed in these mice suggests that CRH is reduced in the amygdala, and this may counteracting the effects of increased HPA axis activity on fat distribution.

When addressing the roles of glucocorticoids in functioning of the central nervous system glial cells are often overlooked. The term "glia" actually covers a very heterogeneous class of cell types that can be broadly split into two groups: macroglia and microglia (reviewed in GarciaSegura, L. M. et al. 1996). Glia and neuronal cells interact in many ways to mutually influence each other's differentiation, development and metabolism (reviewed in GarciaSegura, L. M. et al. 1996). Furthermore, signalling systems and ionic conductances in both cell types are similar (reviewed in GarciaSegura, L. M. et al. 1996). Glial cells maintain synaptic transmission in the hippocampus (Keyser, D. O. et al. 1994), modulate potassium currents in neuronal membranes (Wu, R. L. et al. 1994) and regulates neuronal activity by enhancing glucose utilisation (Pellerin, L. et al. 1994). Neurons reciprocally glial affect electrical, metabolic and transcriptional responses (reviewed in GarciaSegura, L. M. et al. 1996). Specific actions of glucocorticoids in glial cells include potentiation of oligodendrocyte differentiation by regulating the expression of myelin basic protein and proteolipid protein at the post-transcriptional (Kumar, S. et al. 1989) and transcriptional levels (Tsuneishi, S. et al. 1991). *In vitro*, glucocorticoids inhibit astroglia proliferation (Kniss, D. A. et al. 1985) and, *in vivo*, suppress mitosis of astroglia cells in the adult rat dentate gyrus subregion of the hippocampus (Gould, E. et al. 1990). Glucocorticoids may also have adverse effects on neuronal function by inducing necrotic death of glia. Dexamethasone enhances death of C6 glioma cells deprived of serum (Morita, K. et al. 1999); this may be relevant during acute neurological insults such as ischaemia. Local synthesis and metabolism of glucocorticoids by glial cells may also play a role in mediating the effects of glucocorticoids on both glial and neuronal cells (reviewed in GarciaSegura, L. M. et al. 1996).

1.3 Perinatal programming of GR levels

Early life events can permanently program the development of tissues and organs, thus influencing adult physiology and later pathophysiology (the foetal origins hypothesis) (Barker, D. J. P. 1990a).

In humans, low birth weight is correlates with the development of hypertension (Barker, D. J. P. et al. 1990b), glucose intolerance (Hales, C. N. et al. 1991), non-insulin-dependent diabetes mellitus (NIDDM) (McCance, D. R. et al. 1994) and death from ischaemic heart disease (Barker, D. J. P. 1991; Valdez, R. et al. 1994) in adult life. Administration of glucocorticoids to pregnant humans (throughout pregnancy) results in offspring with reduced birth weight (Reinisch, J. M. et al. 1978) and foetal cortisol levels are increased in human intrauterine growth disorder (Goland, R. S. et al. 1993). In rats, reduced birth weight (Reinisch, J. M. et al. 1978) and similar physiological and metabolic changes in adulthood (Nyirenda, M. J. et al. 1998) can be induced by exposure to dexamethasone *in utero*. This suggests that increased glucocorticoid exposure *in utero* may be responsible for programming in humans, as in rats. A major contributing factor to the development of NIDDM appears to be increased hepatic gluconeogenesis (Consoli, A. et al. 1990). In rats, prenatal dexamethasone exposure in the third (i.e. final) week of pregnancy appears to be critical in producing fasting and post-glucose hyperglycaemia in the offspring once adult (Nyirenda, M. J. et al. 1998). Furthermore, levels of PEPCK and GR mRNA expression are permanently elevated in the periportal region of the liver, the major site of hepatic gluconeogenesis in these animals (Nyirenda, M. J. et al. 1998). However, the precise mechanisms involved in this programming effect are unknown.

In utero dexamethasone exposure also permanently programs hippocampal GR levels, with treatment during the third week of pregnancy appearing critical (Levitt, N. S. et al. 1996). In contrast to the effect of prenatal dexamethasone on hepatic GR, hippocampal GR is permanently *decreased*, as is hippocampal MR (Levitt, N. S. et al. 1996). In behavioural tests these animals showed impaired learning and increased anxiety (possibly as a consequence of elevated CRH expression in the amygdala) (Welberg, L. A. M et al. 2000). It would be interesting to examine hippocampal function in aged rats exposed to dexamethasone *in utero* given the role of hippocampal GR in HPA axis regulation and age-related cognitive disorders (section *1.2.6*). Similarly, maternal restraint stress during pregnancy (which increases foetal exposure to maternal glucocorticoids) leads to a long-term reduction in hippocampal GR and impaired post-stress HPA axis feedback in the adult offspring (Henry, C. et

al. 1994), as well as increased anxiety in response to novelty stress (Vallee, M. et al. 1997). *Postnatal* exposure to dexamethasone, in the first week of life, permanently reduces hippocampal GR, but not MR, with no changes in basal corticosterone (Felszeghy, K. et al. 1996). In this study, however, the authors did not study the effects of neonatal dexamethasone treatment on stress responses.

Neonatal handling involves short (15 min) maternal separation of rat pups during the first 3 weeks of life and has profound effects that last for the entire lifetime of the animal. In early adulthood, there are no differences in basal ACTH and corticosterone levels between handled and non-handled rats (Meaney, M. J. et al. 1988; Meaney, M. J. et al. 1989). However, handled animals show blunted ACTH and corticosterone responses to stress, and more effective shut-down of HPA axis activity following stress (Meaney, M. J. et al. 1988; Meaney, M. J. et al. 1989). Compared with aged non-handled rats, aged rats handled in early life have increased GR mRNA and protein in the hippocampus and frontal cortex (Sarrieau, A. et al. 1988; Meaney, M. J. et al. 1989; Henry, C. et al. 1994), maintain low basal corticosterone levels, and show reduced hippocampal cell loss (Meaney, M. J. et al. 1988) and improved spatial memory in old age (Meaney, M. J. et al. 1988). The HPA axis dysregulation, neuronal loss and cognitive deficits in aged non-handled animals may be a consequence of increased exposure to excessive glucocorticoid levels following stress throughout life. Thus, neonatal handling appears to protect the animal from the chronic detrimental effects of stress-activation of the HPA axis. The effects of neonatal handling are the result of altered maternal behaviour, with increased maternal licking and grooming of handled pups, induced by the brief separation (Liu, D. et al. 1997).

Prolonged maternal separation has the opposite effect to the relatively brief separation used in the handling paradigm. When separated from their mother for 3 hours every day for the first 3 weeks of life or 24 hours once at postnatal days 3-4, rats exhibit elevated ACTH levels in response to mild stress in adulthood (Plotsky, P. M. et al. 1993). Furthermore, hippocampal GR is reduced (AvishaiEliner, S. et al. 1999) and spatial memory may be impaired in old age (Meaney, M. et al. 1994;

Meaney, M. J. et al. 1996) compared with non-separated animals. In contrast to handled animals, those exposed to protracted separation lack the reduced anxiety in response to novelty seen in handled animals (Biagini, G. et al. 1998). Thus, maternally separated animals may be less able to "cope" with stress.

The mechanisms underlying the influence of maternal behaviour on development of the HPA axis are incompletely understood, though much progress in their elucidation has been made. In handled pups, circulating levels of thyroxine and its intracellular metabolite triiodothyronine are increased (Meaney, M. J. et al. 1996). In turn, thyroxine (and/or triiodothyronine) alters 5-HT turnover in the hippocampus (Meaney, M. J. et al. 1987; Mitchell, J. B. et al. 1990b). Activation of 5-HT receptors in the hippocampus is essential for the handling effect to occur. *In vivo*, administration of the 5-HT receptor antagonist ketanserin or administration of the 5-HT neurotoxin 5, 7-DHT (which reduces serotonergic input to the hippocampus from the raphe nucleus), decrease the effect of handling on GR expression (Mitchell, J. B. et al. 1990b). In primary cultures of hippocampal neurons, 5-HT increases GR levels (Mitchell, J. B. et al. 1990b; Mitchell, J. B. et al. 1992), but not glial cells (Mitchell, J. B. et al. 1990a). The link between 5-HT receptor activation and induction of GR expression involves an increase in cellular cAMP levels. Hippocampal levels of cAMP are increased in neonatal rats immediately post-handling (Meaney, M. J. et al. 1996). 5-HT treatment of primary hippocampal cultures leads to a four-fold increase in cAMP levels (Mitchell, J. B. et al. 1990a), and treatment with a stable cAMP analogue, 8-bromo-cAMP, increases GR levels (Mitchell, J. B. et al. 1992). Maternal separation and prenatal dexamethasone exposure might decrease GR expression by inhibitory effects on this putative signal cascade.

The early life environment can also program the immune system (reviewed in Chandra, R. K. 1993; Aaby, P. et al. 1993), though whether GR plays a role in setting immune responses is unclear. Maternal malnutrition leads to reduced immmunocompetence in humans and animals, predominantly via a reduction in the number of mature spleen cells (Chandra, R.K. 1974; reviewed in Chandra, R. K.

1993). The immunological effects of malnutrition can persist for years after reinstatement of adequate nutrition (Spirer, Z. et al. 1981).

During development, receptor levels are influenced by the first encounter of receptor with ligand ("hormonal imprinting") (Gaal, A. et al. 1997; Csaba, G. et al. 1998). For example, a single neonatal dexamethasone treatment leads to a prolonged reduction in thymic GR levels (InczefiGonda, A. et al. 1985). The programming effect of prenatal dexamethasone on hepatic GR may also be considered an imprinting effect (Nyirenda, M. J. et al. 1998). Perinatally, molecules similar in structure to the specific ligand can bind receptors and cause false imprinting. *In utero* exposure to a single dose of the pollutant benzpyrene permanently reduces thymic GR (Csaba, G. et al. 1991; Csaba, G. et al. 1992) and this alteration can persist across generations (Csaba, G. et al. 1998). Administration of a single dose of vitamin A to neonatal rats permanently increases thymic GR levels (Gaal, A. et al. 1997); this may be relevant in the relation to the effects of excessive or insufficient vitamin A levels on immune function (reviewed in Chandra, R. K. 1993).

1.4 Transcriptional regulation in eukaryotes

1.4.1 Basal transcription in eukaryotes

In eukaryotic cell nuclei, RNA polymerase II (RNAP-II) transcribes genes encoding mRNAs which are translated into proteins, as well as some small nuclear RNA. RNAP-II is a large multiprotein complex, the exact composition of which remains unclear (reviewed in Aso, T. et al. 1995; Pugh, B. F. 1996; Parvin, J. D. et al. 1998). The basal or general transcription factors (GTFs) (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) interact with the core promoter elements (see below) and are sufficient to allow RNAP-II to bind specifically and transcribe at low levels *in vitro*. RNAP-II directly interacts with many GTFs, mainly through the C-terminal domain of its largest subunit (Zehring, W. A. et al. 1988). Previous models suggested that assembly of the GTFs and RNAP-II on the promoter occurred in a stepwise manner (reviewed in Aso, T. et al. 1995). More recent evidence favours binding of a pre-

assembled holoenzyme complex, though the precise nature of this complex is unclear and may vary depending on the specific promoter and cell type (reviewed in Aso, T. et al. 1995). The first step in transcription initiation is binding of TBP (a component of TFIID) to the TATA box (see below), which induces a large bend and distortion into the DNA (Nikolov, D. B. et al. 1992; Parkhurst, K. M. et al. 1999; Grove, A. et al. 1999; Tsai, F. T. F. et al. 2000). TFIID can also bind to the Initiator element of TATA-less promoters (Purnell, B. A. et al. 1993; Kaufmann, J. et al. 1994), suggesting a universal role for TFIID in transcription initiation. A large number of transcriptional coactivators (e.g. the TAF subunits of TFIID, the USA coactivators and CREB binding protein) act to link sequence-specific factors (e.g. GR) to GTFs to influence transcription initiation.

Several short DNA sequences are associated with the core promoter in most genes (Lewin, B. 2000). The initiator, present in TATA-containing promoters, as well as TATA-less promoters is a short, poorly conserved element that includes the transcription start site. The TATA box (if present), is centred at about –30, (+1 describes the first nucleotide transcribed) which is involved in positioning the transcription machinery; mutations in the TATA box do not prevent initiation but rather lead to variable initiation points (Lewin, B. 2000). Several other promoter elements that bind sequence-specific transcription factors lie around –70 to –100 in some promoters. These include the CAAT box, GC boxes and octamers (Lewin, B. 2000). A vast array of regulatory transcription factors, most of which recognise specific sequences, either directly or indirectly act to enhance or reduce the basal level of transcription (Lewin, B. 2000). Enhancers increase the formation of the transcription initiation complex at the promoter and therefore increase transcription initiation. Enhancer and repressor elements differ from the core promoter region in that they can function in either direction (the core promoter acts unidirectionally to point RNAP-II in the correct direction) and their position relative to the transcription initiation point can vary considerably (Lewin, B. 2000). GR itself is a transcription factor that can either increase or decrease transcription by binding to DNA directly, or by indirect mechanisms (section *1.4.2*).

In vivo, the eukaryotic genome is packaged into chromatin, the basic unit of which is the nucleosome, containing 145 base pairs of DNA wrapped around an octamer of core histone proteins. Packaging of genes into chromatin plays a key role in gene expression, and generally represses transcription by inhibiting the binding of both general and sequence-specific transcription factors to the DNA. Remodelling of chromatin structure (e.g. by the BRG1/BAF complex) allows access of transcription factors to the DNA to activate gene transcription. GR has been shown to interact with chromatin remodelling complexes, leading to activation of transcription (section *1.4.2.1*).

1.4.2 GR is a sequence-specific transcription factor

1.4.2.1 Transcriptional activation

GR itself can recruit and stabilise the preinitiation complex (Freedman, L. P. et al. 1989; Tsai, S. Y. et al. 1990; McEwan, I. J. et al. 1994). GR contains two transactivation regions, one located in the N-terminus that has cell-type and promoter specific activity (Giguere, V et al. 1986), and one in the ligand binding domain that is ligand-dependent (Giguere, V et al. 1986). Transactivation by the N-terminal and the C-terminal domains seems to depend on the formation of an α-helix (McEwan, I. J. et al. 1993; DahlmanWright, K. et al. 1995; DahlmanWright, K. et al. 1996) and the presence of hydrophobic residues, which are likely to be involved in protein-protein interactions (DahlmanWright, K. et al. 1995; Almlof, T. et al. 1998). *In vitro*, GR makes contacts with numerous components of the transcriptional machinery (reviewed in Robyr, D. et al. 2000). These interactions are likely to modulate the effects of GR on target gene expression.

As well as directly interacting with the transcriptional machinery, GR activates transcription through chromatin remodelling. An extensively studied model system to assess transcriptional activation from chromatin templates by GR is the mouse mammary tumour virus long terminal repeat (MMTV LTR) which contains several GREs. Transcriptional activation by GR requires DNA binding, disruption of local

chromatin structure and assembly of a transcription complex, also including NF-1 and Oct-1, at a TATA box (Archer, T. K. et al. 1991). The GR dimer recognises adjacent major grooves on only one face of the DNA (in contrast to many transcription factors that contact both faces of the DNA) (Pina, B. et al. 1990; Archer, T. K. et al. 1991). Since both GR subunits of the activated GR homodimer bind to *one* side of the DNA helix (section *1.1.3*), GR binding to nucleosomal DNA will be less affected by steric hindrance than other transcription factors. Via GR, glucocorticoids cause a small rearrangement of the nucleosome that allows additional binding of the transcription factors NF-1 and Oct-1 (Luisi, B. F. et al. 1991; Archer, T. K. et al. 1992; Truss, M. et al. 1995). The nucleosomal reorganisation of the MMTV promoter may thus be a prerequisite for simultaneous binding of all three factors and maximal transcriptional activation. The key effect of GR during MMTV LTR activation is recruitment of the BRG1/BAF complex (homologous to the SWI/SNF complex in yeast), which is an ATP-dependent chromatin remodelling machine (Muchardt, C. et al. 1993; Farrants, A. K. O. et al. 1997; Fryer, C. J. et al. 1998). Histone hyperacetylation increases transcriptional activation from chromatin templates (Torchia, J. et al. 1998; Bresnick, E. H. et al. 1990), though the actions of histone acetyltransferases, e.g. CBP, appear to be less important in MMTV LTR activation than BRG1/BAF complex recruitment (Bresnick, E. H. et al. 1990; Fryer, C. J. et al. 1998).

1.4.2.2 Transcriptional repression by GR

Direct repression of transcription by GR can occur via several mechanisms (Figure 1.3). Firstly, GR can bind directly to a nGRE on the target gene (Drouin, J. et al. 1990; Drouin, J. et al. 1993; Malkoski, S. P. et al. 1997). In the case of the POMC gene, GR forms a 3 subunit complex at the nGRE: a GR homodimer binds one side of the element (consensus TGACCT), followed by binding of a GR monomer to the other side (Drouin, J. et al. 1993). In contrast to a GRE, GR binding to a nGRE reduces gene transcription, by preventing binding of transcriptional activators. Transient transfection assays in AtT-20 cells showed that a nGRE-luciferase construct is not activated by dexamethasone (Drouin, J. et al. 1993). Furthermore,

mutating the nGRE to a GRE in the POMC promoter leads to transcriptional activation by dexamethasone. The nGRE in the human osteocalcin gene promoter region overlaps the TATA-box suggesting that in this case GR functions as a repressor by competing with a specific TFIID-induced promoter, inhibiting formation of the transcription initiation complex (Meyer, T. et al. 1997) (Figure 1.3). Secondly, GR represses the activity of some transcriptional activators bound to their consensus sites on DNA (e.g. AP1 and NF-κB), and appears to require only direct protein-protein interactions between these factors and ligand-bound GR monomers. These sites are referred to as tethering GREs (Figure 1.3). The concentration of ligand required for this effect is much lower than that required for transcriptional activation by GR homodimers (Vayssiere, B. M. et al. 1997). The interaction between AP1 and GR has been well characterised. AP1 is a ubiquitous transcription factor with diverse actions during cell growth, differentiation and development, and inflammation (Angel, P. et al. 1987; Lee, W. et al. 1987). The AP1 binding site is recognized by Jun homodimers and Jun/Fos heterodimers; binding of Fos is dependent on heterodimer formation (Lee, W. et al. 1987). GR can interact directly, via its DBD, to both homodimers and heterodimers, though Fos appears to be the preferred target (Schule, R. et al. 1990; Kerppola, T. K. et al. 1993). The effects of AP1 on transcriptional activation are thus antagonised by GR, and AP1 reciprocally antagonizes the actions of GR (Schule, R. et al. 1990; Yangyen, H. F. et al. 1990). Similarly, GR can repress, and be repressed by direct interactions with the pro-inflammatory transcription factor NF-κB, without binding to DNA (reviewed in McKay, L. I. et al. 1999). The importance of both of these examples of reciprocal antagonism may be related to the opposing effects of AP1/NF-κB and GR upon inflammatory responses (section *1.2.4* and reviewed in McKay, L. I. et al. 1999).

Finally, at sites termed composite GREs, GR can bind the DNA close to other DNA-bound transcription factors and repress or stimulate transcription depending on the composition of activators bound (Figure 1.3). For example, a 25 base pair region, termed plfG, upstream of the proliferin gene confers 12-*O*-tetradecanoyl phorbol-13-acetate-responsiveness and repression by GR, and footprints both AP1 and GR (Diamond, M. I. et al. 1990). The interaction of GR with AP1 is essential for

hormonal regulation, and Jun homodimers specify activation, and Jun/Fos heterodimers repression by GR (Diamond, M. I. et al. 1990).

A distinct mechanism of repression has also been proposed in which GR represses the activity of NF-κB. Glucocorticoids induce expression of members of the I-κB family, which sequester NF-κB in the cytoplasm, hence preventing activation of NF-κB-target genes (Auphan, N. et al. 1995; Scheinman, R. I. et al. 1995b).

1.4.3 CpG islands and DNA methylation

Overall the CpG dinucleotide is poorly represented in the genome but is clustered in the promoter regions of many genes. The carbon atom at the 5 position in the cytosine of the CpG dinucleotide is the target for methylation by DNA methyltransferase. Generally, tissue-specific genes are methylated in every tissue where they are not expressed, but unmethylated in expressing tissues (reviewed in Mostoslavsky, R. et al. 1997). CpG islands are defined as regions of DNA greater than 200bp with a GC content >0.5 and an observed/expected presence of CpG >0.6 (reviewed in Gardiner-Garden, M. et al. 1987). All housekeeping genes, and possibly the majority of all genes, appear to possess 5' CpG islands (Antequera, F. et al. 1993), yet are unmethylated in all tissues and consitutively active. DNA methylation thus appears to act as a suppressor of gene expression.

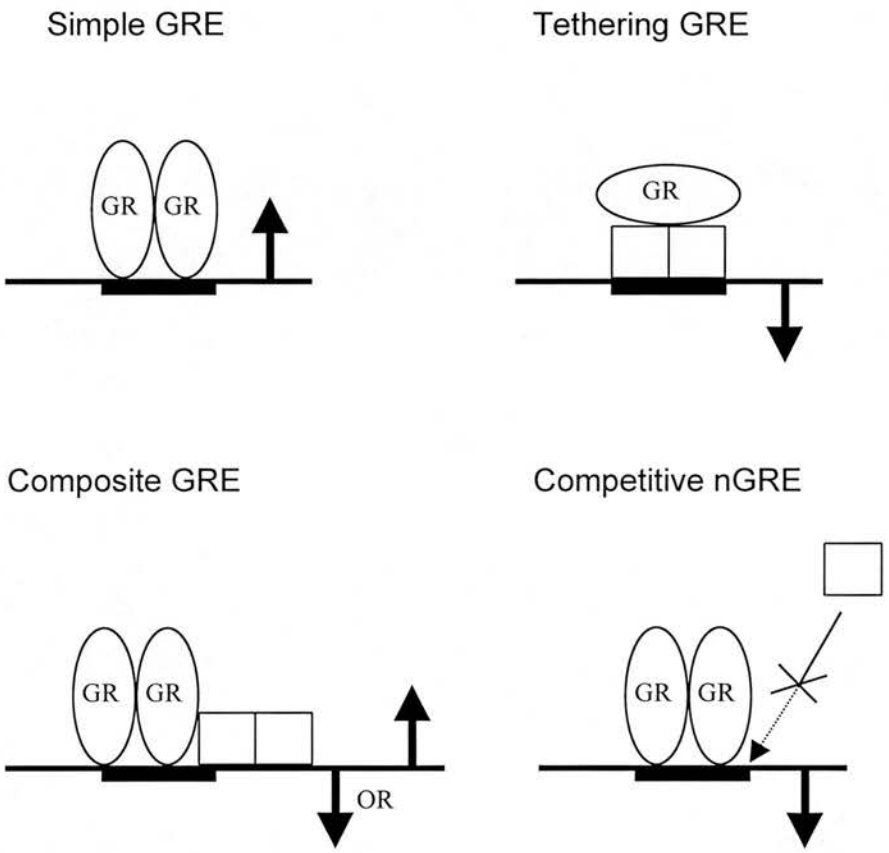


Figure 1.3: *Mechanisms of transcriptional regulation by GR binding directly/indirectly to DNA*

The three proposed mechanisms of transcriptional repression by GR at GREs are depicted (see section *1.4.2* for details). The bold region represents the GRE; boxes represent upstream activating factors e.g. AP1 or basal transcription factors e.g. TBP. Upward arrows represent transcriptional activation by GR and downward arrows represent transcriptional repression by GR. Note that at the composite GRE, GR can either decrease *or* increase gene transcription depending on the non-receptor factors bound. The simple GRE, at which GR activates transcription is also shown.

DNA methylation at CpGs has been shown to be essential for development, since mice with a targeted disruption of the DNA methyltransferase I gene fail to develop past mid-gestation, despite having 30% residual methylation activity (presumably due to activity of alternate DNA methyltransferases) (Li, E. et al. 1992). The overall action of DNA methylation is the repression of gene activity. Methylation may be crucial in suppression of transcriptional "noise" and tissue-specific gene expression and is also important in genomic imprinting. Four DNA methyltransferases, five methyl-CpG-binding proteins (MeCPs) and a candidate demethylase have been identified though the precise functions of all of these proteins *in vivo* are unclear (reviewed in Bird, A. P. et al. 1999).

Only a small number of transcription factors appear to be directly hindered from binding to DNA by methylation. The main mechanism of repression by methylation appears to be due to effects of methylation on nucleosome stability and positioning to deny access of transcription factors to the DNA. Activators or GTFs may be prevented from binding to DNA by binding of MeCPs to methylated CpG sites (Tate, P. H. et al. 1993). MeCP1 appears to bind less tightly to DNA than MeCP2, and may have transient effects on CpG island repression; MeCP2, on the other hand, may be responsible for long term suppression (Ng, H. H. et al. 1999a). Both MeCP1 and MeCP2 are associated with histone deacetylase activity (Ng, H. H. et al. 1999a; Ng, H. H. et al. 1999b). Furthermore, trichostatin A, a specific histone deacetylase inhibitor, is able to induce transcription from methylated genes, even though the genes remain methylated (Ng, H. H. et al. 1999a). Recently, a protein complex, NuRD, with histone deacetylase and nucleosome remodelling properties was identified (Tong, J. K. et al. 1998; Zhang, Y. et al. 1998), suggesting that these two activities are functionally related. NuRD is also targeted to methylated DNA (Zhang, Y. et al. 1999); the effects of this complex on nucleosomal arrangement along with its histone deacetylase activity may explain how CpG methylation leads to gene repression or silencing.

CpG islands normally correspond to promoter regions or other regulatory elements and have the highest density of nonmethylated CpGs in the genome. Many

promoters associated with CpG islands lack TATA boxes and hence exhibit heterogeneous transcription initiation points. During development, there is generalized demethylation in the early preblastula embryo; after implantation most CpGs become methylated, but those in CpG islands are unaltered (reviewed in Mostoslavsky, R. et al. 1997). It is unclear precisely how CpG islands escape methylation. Steric hindrance of DNA methyltransferase (by transcription factors) throughout the entire length of a typical CpG island is unlikely, since CpG islands adopt a relatively "open" chromatin conformation that nucleases can still access (reviewed in Antequera, F. et al. 1999). A recent model proposes that promoters active early in development can exclude methylation by recruiting the molecules that initiate DNA replication (reviewed in Antequera, F. et al. 1999). This results in a stretch of methylation-free DNA that coextends with an initiation loop intermediate. Inactive CpG islands would not be able to form replication origins and would thus be methylated. Later in development, tissue-specific genes are selectively demethylated in their cell-type of expression, or remain permanently silenced (reviewed in Mostoslavsky, R. et al. 1997).

1.4.4 Transcriptional regulation of the GR gene

Surprisingly little is known about the transcriptional regulation of the GR gene itself. The human GR cDNA was cloned by Hollenberg et al in 1985 (Hollenberg, S. M. et al. 1985). Subsequent isolation of the gene identified 9 exons, with a non-coding exon 1. Primer extension, ribonuclease protection analysis (Zong, J. et al. 1990) and S1 nuclease analysis (Encio, I. J. et al. 1991) of GR mRNA has revealed at least four transcription starts for this human cDNA. Furthermore, the regions flanking (up to –860, ATG designated +1) this exon 1 contain no obvious TATA or CAAT boxes, but multiple GC boxes (which are bound by Sp1 and related factors) are present (Zong, J. et al. 1990; Encio, I. J. et al. 1991). Transient transfections of constructs fusing GR genomic DNA to a reporter gene have identified regions important in determining differential promoter activity in a variety of cell lines, and DNase I footprinting and gel mobility shift analysis reveal that AP2 may an important activator of the gene (Nobukuni, Y. et al. 1995). An Sp1 binding site is also present

in the promoter close to the AP2 site (Leclerc, S. et al. 1991; Nobukuni, Y. et al. 1995). Very recent evidence suggests that at least 2 other alternate exons 1 exist within the human GR gene (Breslin, M.B. et al. 1998; V. Lyons personal communication), one of which lies at least 15 kilobases (kb) upstream of the translation start (Breslin, M. B. et al. 1998) (Figure 1.4).

At least 5 alternate exons 1 have been identified in GR mRNAs in the mouse (Strahle, U. et al. 1992; Chen, F. H. et al. 1999a) (Figure 1.4). Four of these alternate exons 1 lie within a CpG island (1B – 1E), while one (1A) lies approximately 32 kb upstream of the translation start. At least three of the alternate exons 1 (1A, 1B and 1C) exhibit considerable heterogeneity in their 5' ends (Strahle, U. et al. 1992) and two of the alternate exons 1 appear to be ubiquitously expressed (1B and 1C). It has been suggested that exon 1A is restricted to T lymphocytes; it is present in T-lymphoma cell lines, but not liver or a limited range of other cell lines (Strahle, U. et al. 1992). Recently, however, a more widespread tissue distribution of exon 1A-containing GR mRNAs has been suggested (Chen, F. H. et al. 1999a). Exons 1D and 1E-containing GR mRNAs are also widely expressed (Chen, F. H. et al. 1999a). Consistent with ubiquitous activity of putative exon 1B and 1C-associated promoters, DNase I hypersensitive sites are associated with regions just upstream of these exons 1 in liver and LTK^- cells (fibroblasts) (Strahle, U. et al. 1992). Exon 1A has been proposed to target GR to the cell membrane, and this may be of relevance in glucocorticoid-induced T cell death (Chen, F. H. et al. 1999a; Chen, F. H. et al. 1999b). However, this remains to be demonstrated conclusively.

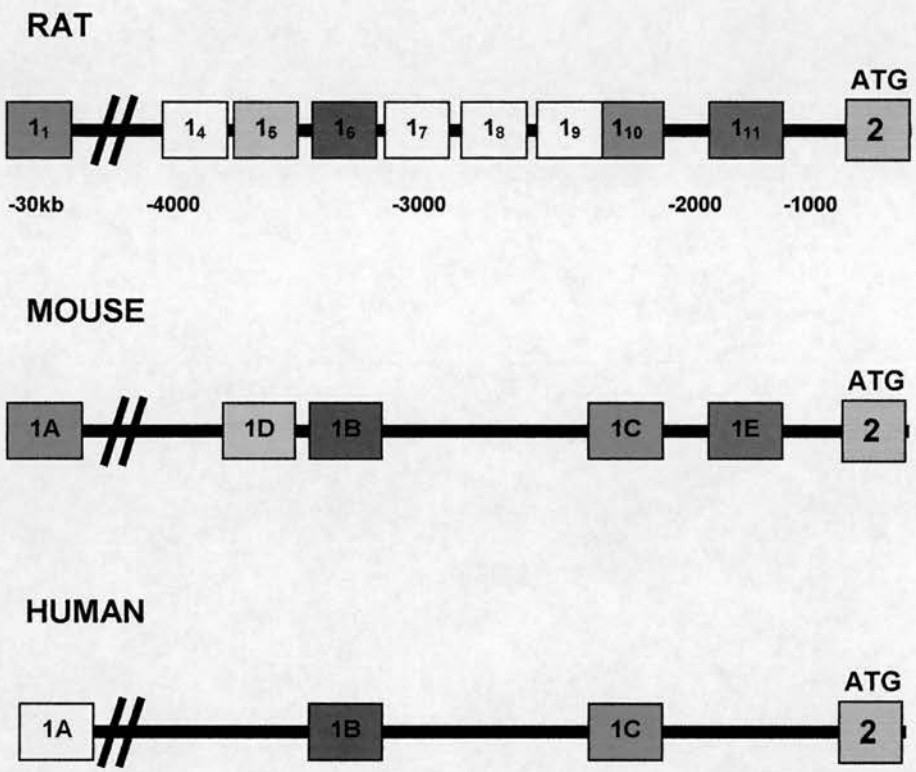


Figure 1.4: *5'-heterogeneity of GR mRNA is conserved across species*

Schematic representation (not to scale) of alternate exons 1 identified to date in the rat ends (Gearing, K. L. et al. 1993; McCormick, J. A. et al. 2000), mouse (Strahle, U. et al. 1992; Chen, F. H. et al. 1999a) and human gene (Hollenberg, S. M. et al. 1985; Breslin, M.B. et al. 1998; V. Lyons personal communication) GR genes (for the rat gene, not all known alternate exons 1 are shown). Homologous exons (including exon 2) are filled in the same colour (except for white filled exons 1, which lack identified homologues). The approximate locations of the exons 1 are shown, relative to the translation start in exon 2 (ATG). The 5' extents of variant exons 1 show considerable heterogeneity that is not represented in this figure.

Rat GR mRNA exhibits considerable heterogeneity at the 5' ends (Gearing, K. L. et al. 1993; McCormick, J. A. et al. 2000). At least 12 alternate exons 1 are be present in the rat GR gene; Lyons, V. personal communication); 5 of these correspond to those identified in the mouse GR gene and one corresponds to that published for the human GR gene (Figure 1.4). Ten of these alternate exons 1 lie in a CpG island spanning 3 kb; three are likely to lie at least 15 kb upstream of the translation start since they are not present in the genomic clone λ208, which contains exon 2 and approximately 15kb of the rat GR gene flanking the 5'-end of exon 2 (McCormick, J. A. et al. 2000).

In each species, each alternate exon 1 is spliced from a fixed 3' donor site to the same 5' acceptor site in exon 2. An in-frame stop codon present immediately upstream of the translation start in exon 2 means that the amino acid sequence of GR itself is unaffected by splicing of alternate exons 1. Thus, alternate exons 1 may reflect alternative promoter usage (section *1.4.5*).

1.4.5 Gene regulation by alternative promoter usage

The use of alternative promoters in gene regulation provides an efficient means of controlling complex patterns of gene expression. The simplest form of alternative promoter usage involves two tandemly arranged promoters within the same exon (e.g. the *myc* gene (reviewed in Marcu, K. B. et al. 1992)). The first exon can be either coding or non-coding. Alternative promoter usage can also result in alternative exons 1 spliced to a common exon 2, for example in the MR (Zennaro, M. C. et al. 1995; Zennaro, M. C. et al. 1996; Vazquez, D. M. et al. 1998), ER (Griffin, C. et al. 1998) and GR (section *1.4.4*) genes, and again in these cases the first exon is non-coding. More complex forms of alternate promoter usage include formation of mRNAs encoding proteins with N-terminal extensions, e.g. where the leader exons are overlapping and mRNAs derived from the upstream promoter contain a translation initiation codon (the shorter isoform may or may not contain a translation start codon). Regulation of the progesterone receptor gene is an example of this type of alternate promoter usage (Kastner, P. et al. 1990).

The use of alternate promoters can affect gene expression in a variety of ways. The human porphobilinogen deaminase gene (PBGD) is an example of a gene containing both a "housekeeping" and a tissue-specific promoter (Chretien, S. et al. 1988). Housekeeping genes are a set of genes expressed ubiquitously and probably required for basal functioning of all cell types. The housekeeping promoter of the PBGD gene has no obvious TATA-box, has multiple Sp1-binding sites, multiple transcription starts and lies within a CpG island, all classic properties of a housekeeping gene. A second, tissue-specific promoter is located 3kb downstream from the housekeeping promoter and is only active in erythroid cells (Chretien, S. et al. 1988). Using promoters of different strengths can influence the level of expression of a gene. For example, the α-amylase gene has a weak downstream promoter, active in liver and a strong upstream promoter, active in the parotid gland (Schibler, U. et al. 1983).

In addition, production of mRNAs with different untranslated 5' leader sequences by alternative promoter usage can affect gene expression by variations in stability or translation efficiency of these mRNAs. Studies of alternate *myc* oncogene mRNAs have shown differences in translational efficiency between mRNA species with different untranslated exons 1 (Kozak, M. 1991). Furthermore, cells transfected with *myc* show discrepancies between *myc* mRNA and protein levels suggesting an element of translational control (reviewed in Marcu, K. B. et al. 1992). The synthesis of mRNAs with GC-rich 5' leaders may have a profound influence on translational efficiency due to the formation of secondary structures. Gene products containing alternate 5'-untranslated regions with low free energy are inefficiently translated (Kozak, M. 1991). The presence of short open reading frames (sORFs) within the leader sequence may also be important (reviewed in Geballe, A. P. et al. 1994). For example, the 5' leader of the human cytomegalovirus gp48 mRNA contains a 22 codon upstream open reading frame that represses translation of the downstream cistron (Cao, J. H. et al. 1996). Translational repression in this case involves ribosomal arrest at the end of the upstream sORF, as opposed to an effect of higher initiation efficiency at the upstream sORF. In other cases, differences in initiation efficiency at upstream sORFs might lead to differences in translational

efficiency between mRNAs (Geballe, A. P. et al. 1994). Translational regulation can also occur by interactions between mRNAs and proteins e.g. the iron regulatory element/iron regulatory factor (Theil, E. C. 1994).

1.5 Aims

This thesis examines the transcriptional regulation of the rat GR gene. GR are involved in many physiological processes, and exert important effects during development. Their role in the hippocampus may be especially important, since changes in GR density alters HPA axis regulation, which can subsequently affect all GR-mediated functions in the organism. During development, environmental effects can permanently program GR levels. Paradoxically, a prenatal manipulation (dexamethasone exposure) that permanently increases hepatic GR levels permanently reduces hippocampal GR. Hippocampal GR can also be permanently increased by another perinatal manipulation, neonatal handling. All these perinatal programming effects influence adult physiology, and may lead to the development of pathology in later life. An understanding of the transcriptional regulation of the GR gene is thus important not only in general physiological terms, but also to elucidate the mechanisms of GR programming.

There were several key aims of the work described in this thesis. Firstly, to gain an insight into general transcriptional regulation of the GR gene by determining the tissue distributions of alternate exon 1-containing GR mRNAs. For the purpose of this thesis, I shall define a promoter as a region of DNA that directs RNAP-II to initiate transcription from initiation points resulting in mRNAs containing a particular alternate GR exon 1. With this definition in mind, differences in the tissue abundances of alternate exon 1-containing GR mRNAs might reflect differences in promoter usage between tissues. Next, to determine if promoter activity is indeed associated with alternate exons 1, and if so to identify important regulatory regions. Finally, to examine programming effects on alternate exon 1-containing GR mRNAs. Specifically, to determine whether prenatal dexamethasone treatment affects the abundance of exon 1-containing GR mRNA (to identify a promoter responsible for

the increase in hepatic GR following this treatment), and examine the effects of 5-HT in primary hippocampal cultures, which mimics the effect of neonatal handling.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from BDH Chemicals Ltd, Magna Park, Lutterworth, Leicestershire LE17 4XN or Sigma Chemicals Ltd, Fancy Road Poole, Dorset, BH17 7NH unless otherwise stated.

Agarose and low melting point agarose Caesium chloride Agar	Life Technologies Ltd, 3 Fountain Drive, Paisley PA3 9RF
Nucleotide triphosphates	Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks HP7 9NA
Ethanol	Hayman Ltd, 70, Eastways Industrial Park, Witham, Essex CM8 3YE
Bactotryptone Yeast extract Agar	Beckton Dickinson, Between Towns Road, Cowley, Oxford OX4 3LY
Beetle luciferin	Promega Ltd, Delta House, Chilworth Research Centre, Southampton SO1 7NS
D19 developer Amfix fixative	H.A. West Ltd, 41 Watson Crescent, Edinburgh EH11 1ES

2.1.2 Radiochemicals

All radiochemicals were supplied by Pharmacia Amersham International plc (see above).

Compound	Specific activity
[α-^{32}P]-GTP	3000 Ci/mmol
[α-^{32}P]-UTP	3000 Ci/mmol
[α-^{33}P] ddNTPs	500 Ci/mmol

2.1.3 Enzymes

All enzymes, with the exception of those listed below, were supplied by Promega Ltd (see above).

Alkaline phosphatase	Roche Diagnostics Ltd, Bell Lane, Lewe,
High activity DNA ligase	East Sussex BN7 1LG

2.1.4 Miscellaneous

DNA size markers (1kb ladder)	Life Technologies Ltd (see above)
TRIzol reagent	
Glassmax DNA isolation system	
5'-RACE system version 2.0	
Autoradiographic film	H.A. West Ltd (see above)
NICK columns	Pharmacia Amersham Biotech (see above)

Reverse transcription system	Promega (see above)
Galacto Light Plus assay kit	Tropix Ltd, 47, Wiggins Ave, Bedford MA, USA
Pico-fluor 40 scintillant fluid	Canberra Packard, Brook House, 14 Station Road, Pangbourne, Berkshire RG8 7DT
Hybaid Recovery DNA purification kit II	Hybaid Ltd, Action Court, Ashford Road, Ashford, Middlesex TW15 1XB
Hybspeed RPA kit RPAII kit	AMS Biotechnology, Milton Park, Abingdon, Oxfordshire
Vectastain Elite ABC Kit	Novacastra, Benton Lane, Newcastle-upon-Tyne NE12 8EW

2.1.5 General buffers and solutions

Unless stated, distilled water was used to prepare all solutions. All solutions, except those marked * were sterilized in an autoclave before use.

DEPC water	0.5ml DEPC was added to 500ml ultrapure water and left for at least 1h before autoclaving
DNA loading buffer*	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol
0.5M EDTA (pH 8.0)	800ml water was added to 186.1g $Na_2EDTA.2H_2O$. pH was adjusted to

	8.0 with NaOH and the volume adjusted to 1000ml
Formamide loading buffer*	34.03% bromophenol blue, 0.03% xylene cyanol, 20mM EDTA in deionised formamide
GTE	50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA
Alkaline SDS solution*	0.2M NaOH, 1% SDS
1M Tris-HCl, pH 8.0	121.1g Tris base in 800ml water. pH was adjusted to 8.0 with concentrated HCl and the volume adjusted to 1000ml with water.
5M potassium acetate	245.6g potassium acetate was dissolved in 300ml water. The volume was adjusted to 500ml by adding 57.5ml glacial acetic acid and 142.5ml water
PBS	8g NaCl, 0.2g KCl, Na_2HPO_4, 0.24g KH_2PO_4 in 800ml water. pH was adjusted to 7.4 and the volume adjusted to 1000ml with water
TBE (10x)	108g Tris base, 55 g boric acid, 20ml 0.5M EDTA, made up to 1000ml in DEPC water
TE (1x)*	10mM Tris-HCl (pH 8.0), 1mM EDTA

Glycerol tolerant buffer (20x)	216g Tris base, 72g taurine, 4g $Na_2EDTA.2H_2O$, dissolved in 1000ml water
Probe elution buffer (50x)	2M Tris, 1M glacial acetic acid, 50mM EDTA (pH 8.0), 10% lauryl sulphate. Made up in DEPC water
Denaturing solution for RNA preps*	4.73g guanidium thiocyanate, 0.318ml 0.75M, sodium citrate (pH 7), 0.476ml 10% sarcosyl, 0.072ml β-mercaptoethanol, 5.540ml DEPC water
2M Sodium acetate pH 4.0	272g sodium acetate.$3H_2O$ was dissolved in 800ml water. The pH was adjusted to 4.0 with glacial acteic acid and the volume made up to 1000ml
MEA buffer (10x)	200mM MOPS acid, 50mM Sodium acetate, 10mM EDTA, pH to 7.2
0.1M Tris phosphate pH 7.8	900µl 18.1M phosphoric acid was added to 500ml water. The pH was adjusted to 7.8 by adding Trizma base solid
Transfection lysis buffer*	25mM Tris phosphate pH 8.0, 2mM DTT, 1% Triton X-100, 10% glycerol
Luciferase assay buffer (2x)*	40mM Tricine, 67mM DTT, 0.2mM $Na_2EDTA.2H_2O$, 2mM $MgSO_4$,

	0.25mM coenzyme A, pH adjusted to 7.8

2.1.6 Molecular biology buffers (excluding restriction enzyme buffers)

All enzymes and buffers were supplied by Promega unless stated

Large scale *in vitro* transcription buffer (1x) (made in lab)	80mM Hepes-KOH, 24mM $MgCl_2$, 2mM spermidine, 40mM DTT
Transcription optimized buffer (1x)	40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl
T4 polynucleotide kinase buffer (1x)	70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM DTT
T4 DNA ligase buffer (1x)	30mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM DTT, 1mM ATP
Reverse transcription buffer (1x)	10mM Tris-HCl pH 8.8, 50mM KCl, 0.1% Triton X-100

2.1.7 Restriction enzyme buffers

All supplied by Promega unless stated

Universal restriction buffer (1x) (prepared in lab)	500mM NaCl, 500mM Tris-HCl pH 8.0, 110mM $MgCl_2$, 60mM

	β-mercaptoethanol, 1mg/ml bovine serum albumin
*EcoR*I, *Pst*I restriction buffer (1x)	90mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl, 1mM DTT. (pH 7.5 at 37°C)
*Nco*I, *Sal*I restriction buffer (1x)	6mM Tris-HCl, 6mM $MgCl_2$, 150mM NaCl, 1mM DTT. (pH 7.9 at 37°C)
*Sph*I restriction buffer (1x)	10mM Tris-HCl, 10mM $MgCl_2$, 150mM KCl. (pH 7.4 at 37°C)
*Ssp*I restriction buffer (1x)	6mM Tris-HCl, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT (pH 7.5 at 37°C)

2.1.8 Cells and animals

2.1.8.1 Bacteria for cloning

E. coli strains HB101 and JM109 were supplied by Promega (see above)

2.1.8.2 Cell lines

B103 cells were kindly provided by Professor David Schubert of the Salk Institute
All other cell lines were already held in this laboratory

2.1.8.3 Animals

Animals were supplied by Charles River UK Ltd, Margate, Kent, UK

2.1.9 Bacterial media

Luria-Bertoni broth (LB)	1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl
LB-agar	LB with 1.5% (w/v) agar. To pour plates, LB-agar was melted in a microwave, and allowed to cool until warm before pouring into 100mm petri dishes. If required, ampicillin was added (100μg/ml) before pouring plates.

2.1.10 Cell Culture Media

Unless indicated, all media and additives for maintenance of cell lines were supplied by Life Technologies (see above).

Modified Eagle Medium α medium (MEM α medium)

Dulbecco's modified Eagle medium (DMEM)

Nutrient Mixture F-10 (HAM)

Hank's Balanced Salts Solution

Trypsin/EDTA (10x)

Hepes (1M) (Sigma)

D-(+)-Glucose solution (45%) (Sigma)

Penicillin/Streptomycin (10000U/ml:10000μg/ml)

Foetal calf serum

Horse serum

L-glutamine (200mM)

2.1.11 DNAs

2.1.11.1 Oligonucleotides

All oligonucleotides were supplied by Oswel DNA Service, University of Southampton, Southampton S016 7PX

5'-RACE PCR

GSP1: 5'-AAGGGATGCTGTATTCA-3'
Anchor Primer:
5'-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'
GSP2: 5'-ACTCCAAATCCTTCAAGAGGTCA-3'
M6002: 5'-AAGCCTGTTTCACTGTCCAT-3'
M6003: 5'-CAACAGATCTGATCTCCAGG-3'
UAP: 5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3'
GSP3: 5'-TTGGAATCTGCCTGAGAAGC

Sequencing

5'-RACE PCR products: used GSP3
Sp6: 5'-TGTAATACGACTCACTATAG-3'
T7: 5'-TGTAATACGACTCACTATAG-3'
Transfection construct 1_{7b}: 5'-CTGCAGACACGCCCTCT-3'

Oligonucleotides for pGL3-Basic modification

A5351: 5'-GAGCTCGGTACCGCATGCAGATCTGCGATCTAAGTG-3'
A5352: 5'-CATGGTGGCTTTACCAACAGTACCGGAATCCCACTT-3'
A6594: 5'-AGCTCCTGCAGTCGACAAGCTTGACGTCGGATCCGTAC-3'
B4697: 5'-GGATCCGACGTCAAGCTTGTCGACTGCTG-3'
B4698: 5'-AGATCGCAGATCTGCATGCGGTACCG-3'

B4699: 5'-GGATTCCGGTACTGTTGGTAAAGCCAC-3'

RT-PCR

Exon 2: 5'-CATGGACAGTGAAACGGC-3'
Exon 1_7: 5'-AAAGAAACTCGGTTTCCCTC-3'
Exon 1_{10}: 5'-GTTGACTTCCTTCTCCGTGA-3'
1_6-specific primer: 5'-ACCTGGCGGCACGCGA-3'

2.1.11.2 Plasmids

Cloning

pGL3-Basic	All Promega (see above)
pGL3-Promoter	
pGEM-T	
pGEM T-Easy	

Expression plasmids

pSV2L	Promega
pRSVluc	Amersham Pharmacia Biotech Ltd
pCH110	Amersham Pharmacia Biotech Ltd
C/EBPα and C/EBPβ	Gift from S. L McKnight
WT-1	Gift from R. Davies
NGFI-A	Gift from J. Millbrandt
HNF1α	Gift from M. Pontoglio and M. Yaniv
pKC275	Gift from K.E. Chapman

2.1.11.3 Construction of plasmids

To generate constructs for transient transfections, fragments of pVL152, pVL154 and pVL156 (subclones of λ208, a genomic clone encoding exon 2 and approximately 15kb of the rat GR gene flanking the 5'-end of exon 2 kindly supplied by M. Jacobson and K. Yamamoto) were ligated into pGL3-Basic with a modified polylinker (pGL3-BM) digested with appropriate restriction enzymes (constructs are summarised in Figure 4.4). All digests and fragment recoveries were performed as describe in sections *2.2.5.1* and *2.2.5.2*.

The polylinker in pGL3-Basic was modified to simplify construct generation. The modification was performed by Val Lyons. Six overlapping oligonucleotides (section *2.1.10.1*) were ligated into pGL3-Basic digested with *Kpn*I and *Nco*I and purified through a low-melting point agarose gel (section *2.2.5.2*). Oligonucleotides A5352 and B4699 were 5'-phosphorylated to enable ligation; for each oligonucleotide, a 30µl reaction contained 9µg oligonucleotide, 100mMM Tris-HCl pH 8.0, 10mM $MgCl_2$, 5mM DTT, 0.3mM ATP and 5U T4 polynucleotide kinase. Reactions were incubated at 37°C for 45min, then 65°C for 10min. Pairs of oligonucleotides (A5351/B4698, A5352/B4699 and A6594/B4697) were annealed by incubating 1.5µg of each at 65°C for 10min and cooling to room temperature slowly. The three annealed pairs of oligonucleotides were combined, and 7.5µl of this mix ligated (section *2.2.9.1*) to 1µl of vector in a 10µl reaction; 0.5U T4 DNA ligase was used. This ligation reaction was used in a transformation with HB101 cells, and a clone containing the modified polylinker identified by appropriate restriction digests. It should be noted that the intended *Pst*I restriction site was not present in the original clone identified and used to generate the majority of constructs since the sequence of the A6594 oligonucleotide was incorrectly transcribed when purchased. The corresponding B4697 oligonucleotide, however, contained the correct sequence for this site, so DNA repair mechanisms would repair this mismatch to the correct *Pst*I site in 50% of clones. The original ligation was used to transform HB101 cells again and a clone containing the correct *Pst*I site identified.

P2 (-4572 to -9) was a *Hind*III/*Ssp*I fragment; P2 (reversed) contained the same fragment in the reverse orientation (-9 to -4572); $P1_5$ (-4572 to –3552) a *Hind*III/*Sph*I fragment; $P1_6$ (-4572 to -3336) a *Hind*III/*Pst*I fragment; $P1_7$ (-4572 to -2927) a *Hind*III/*Bgl*I fragment; $P1_8$ (-4572 to –2803) a *Hind*III/*Kpn*I fragment; $P1_{9/10}$ (-4572 to 2532) a *Hind*III/*Sac*I fragment; $P1_{10}$ (-4572 to -2318) a *Hind*III/*Hinc*II fragment; $P1_{11}$ (-4572 to -1767) a *Hind*III/*Pst*I fragment; P0, (-4572/-796) a *Hind*III/*Hind*III fragment, and H3 (-871/-9) a *Hind*III/*Ssp*I fragment (Figure 4.4 for a summary). All numbers relate to the translation start site, +1.

Subclones of $P1_7$ were generated to allow more detailed analysis of elements conferring activity to $P1_7$ in transient transfections (Figure 4.9). $P1_{7a}$ (-3552/-2927) an *Sph*I/*Bgl*II fragment; $P1_{7b}$ (-3341/-2927) a *Pst*I/*Bgl*I fragment, and $P1_{7c}$ (-3207/-2927) a *Pst*I/*Bgl*I fragment.

The 134bp *Pst*I/*Pst*I fragment (-3341/-3207) was subcloned into pGL3-BM and pGL3-Promoter (pGL3-P) in both orientations. First, it was subcloned into pGL3-BM digested with *Pst*I: all clones obtained contained the insert in the incorrect. To generate a construct containing the insert in the correct orientation (P134C) *Hinc*II/*Sst*I fragment from BM134C was subcloned into pGL3-P digested with *Sma*I/*Sst*I. To obtain the fragment in the incorrect orientation in pGL3-P (P134I), BM134I was digested with *Hinc*II/*Bgl*II and the insert subcloned into pGL3-P digested with *Sma*I/*Bgl*II. P134C digested with *Kpn*I/*Bgl*II provided the insert for generation a construct with the 134bp fragment in pGL3-BM in the correct orientation (BM134C).

For all constructs, the presence and orientation of inserts was checked by appropriate restriction digests and/or cycle sequencing. Plasmid DNAs used in transfections were purified by CsCl density gradient centrifugation.

2.2 Methods

2.2.1 Animals

Animals were maintained under controlled lighting (lights on 07.00 to 19.00h) and temperature (22°C) with water and food available *ad libitum*. For RNA isolation adult (3-8 months) male Wistar rats were killed, tissues immediately dissected, frozen on dry ice and stored at -80°C.

Animals treated *in utero* with dexamethasone were male offspring of female Wistar rats. Time-mated female rats (5 females per group) were given dexamethasone (100μg/kg per d) subcutaneously during the third week of pregnancy; controls received vehicle (4% ethanol-0.9% saline) throughout pregnancy. Male offspring, selected at random from a total of 5 litters, were used when adult (8 months). Dr. Moffat Nyirenda treated, killed and collected tissues from these animals. For primary hippocampal cultures, female Wistar rats were mated and killed on day 18 of pregnancy. Pups were dissected from the uterus and killed by placing on ice in a 50ml universal container.

2.2.2 Primary hippocampal cultures

Hippocampi were dissected and immediately placed in 1.5ml Hank's buffer/0.015M Hepes pH 7.4 (H/H) in a sterile 6ml tube. Hippocampi were washed with 2ml H/H and the H/H removed with a sterile plastic pasteur pipette, along with any floating particles. This wash step was repeated twice. The hippocampi were then dissociated by adding 1ml of trypsin 1.25mg/ml and incubating at 37°C for 15min. The tube was gently tapped every 5min to mix. Trypsin was removed and 1.5ml medium (Modified Eagle Medium α supplemented with 0.015M Hepes, 10μg/ml penicillin and streptomycin, 200mM KCl and 0.5% w/v glucose) added. After 1min, medium was removed and the hippocampi washed 3 times with H/H. Hippocampal cells were dispersed by triturating 20 times in the final H/H wash using a plastic pasteur pipette, followed by three gentle passes through a 25G needle. Dispersed

hippocampal cells were transferred to 5ml of medium in a 50ml universal tube and centrifuged for 5min at 500rpm, room temperature. Cells were resuspended in medium allowing 1ml medium per 2 hippocampi. Cell density was determined using an Improved Neubauer haemocytometer (Hawksley) and adjusted to 1.5-2 x 10^6 cells/ml. Cell viability was determined by adding 100µl cells to 100µl 0.4% (w/v) Trypan blue and counting the number of cells taking up dye (dead cells) and those not. Viability was typically 80-90%. 3ml of suspended cells were plated on 60mm culture dishes (pre-coated for at least 1h with 1ml poly-D-lysine (0.25mg/ml), which was removed before adding cells), and placed in an incubator at 37°C with 5% CO_2, where they were maintained for the entire culture period. 2d after plating, the medium was aspirated from the cells and 3ml fresh medium containing 20µM uridine and 20µM 5-fluorodeoxyuridine was added. After a further 3d, the medium was aspirated and fresh medium containing either 100nM 5-HT or vehicle (water) added. Cells were harvested after 4d further incubation with 5-HT for RNA extraction.

2.2.2.1 Immunocytochemistry

To ensure that primary hippocampal cultures were predominantly neuronal in cell population, immunocytochemistry was carried out using the Vectastain Elite ABC Kit (Novocastra). Primary hippocampal cultures were established as described above and maintained in culture for 6 days before testing. All washes and incubations were performed on an orbital shaker, set to low speed, at room temperature unless stated. Each dish of cells was washed with 1ml PBS, followed by incubation with 1ml 40% paraformaldehyde for 20min. Cells were then washed with 1ml PBS, incubated with 1ml 0.1 Triton X-100/PBS for 5min, incubated with 0.25% BSA/PBS for 1h. Primary antibodies were diluted 1 in 1,000 in 0.25% BSA/PBS and 1ml of these dilutions were added to the cells. The antibodies used were anti-glial fibrillary acidic protein (GFAP), to identify glial cells, and anti-neuron-specific enolase (NSE), to identify neuronal cells. As a negative control, 1ml of 0.25% BSA/PBS was added to cells. Each test was performed in duplicate. Cells were incubated with primary antibodies at 4°C overnight. Cells were washed with 0.25% BSA/PBS for 10min. Secondary antibody (anti-rabbit IgG) was diluted 1 in 200 in 10ml PBS and 150µl of

blocking serum added. 1ml of this solution was added to each dish of cells and incubated at room temperature for 45min. Cells were washed with 1ml PBS for 10min and incubated with Vectastain (200µl each of solution A and solution B in 10ml PBS made 30min before use) for 30min. Cells were washed for 10min with PBS, incubated for 2min with AEC substrate (150µl 3-amino-9-ethyl carbazole (AEC), 100µl buffer, 100µl hydrogen peroxide and 5ml distilled water), and given a final wash with water for 5min. Cells were then examined using a Nikon at x10 magnification and photographed.

2.2.3 Gel electrophoresis

2.2.3.1 Analytical agarose gels

Agarose gels were prepared by adding solid agarose to 0.5x TBE 0.8-2.5% (w/v) followed by boiling in a microwave oven. 50µg/ml of ethidium bromide was added to the gel mix which was then poured into a gel mould with an appropriate comb after mixing. Once set the gel was submerged in 0.5x TBE in a Horizon 58 (30ml gel) or Horizon 11.54 (100ml gel) electrophoresis tank (Life Technologies). 1kb DNA markers and samples containing 1µl DNA/RNA loading buffer were then loaded into the wells and electrophoresed at 100-200V until the DNA fragments were resolved. DNA was visualised on a UV transilluminator at 254nm, imaged using an Appligene Imager and recorded on Seikosha video printer paper.

2.2.3.2 Preparative agarose gels

To gel purify DNA fragments (e.g. in subcloning procedures) a 1% (w/v) agarose gel was prepared as above except low melting point agarose (Life Technologies) was used and the gel poured in a cold room (4°C). Samples were electrophoresed at 100V until the desired fragment was resolved. DNA was visualized on a UV transilluminator at 365nm to prevent UV damage to DNA, the required band excised with a scalpel and DNA purified as described in section *2.2.5.2.*

2.2.3.3 RNA gels

Integrity of total RNA (for RNA isolation see section *2.2.4*) was verified by denaturing agarose gel electrophoresis. Gel trays, tanks and combs were scrubbed in hot soapy water, soaked in 0.1M NaOH for 30min and rinsed with ultrapure water before use. Typically, a 1% (w/v) gel was prepared by weighing out 0.25g agarose, adding 18ml DEPC water and boiling in a microwave for 30sc. Once cooled slightly, 4.5ml 40% (w/v) formaldehyde solution (Sigma) and 2.5ml 10x MEA was added, and the gel was cast. Once set the gel was aged by submerging in a Horizon 58 electrophoresis tank in 1x MEA for 15min. Meanwhile, 1-2µl of RNA sample was denatured by adding to a mix of 2.5µl 40% (w/v) formaldehyde, 2.5µl 10x MEA, 10µl deionised formamide and incubating at 65°C for 15min. 2µl of a 1:5 mix of 10mg/ml ethidium bromide and gel loading buffer was added to the denatured samples which were then loaded onto the gel and resolved for 20-30min at 100V. RNA was visualised on a UV transilluminator at 254nm and photographed as before.

2.2.3.4 Denaturing polyacrylamide gel electrophoresis

Prior to use, glass plates used in polyacrylamide gel electrophoresis were scrubbed with detergent, rinsed with dH_2O then rinsed with absolute ethanol. "Sequencing gels" contained 6% polyacrylamide. To check cRNA probe quality a 5% gel was used; for gel purification of cRNA probes and in analysis of RNase protection assays, a 4% gel was used.

For sequencing gels, two clean glass plates (45cm x 35cm) had 0.3mm spacers inserted between them; after clamping with bulldog clips the edges were sealed with 2% agarose (w/v). 42g of urea was dissolved in 15ml 40% acrylamide:bis-acrylamide (19:1), 4ml 20x glycerol tolerant buffer, 600µl 10% ammonium persulphate (w/v), made up to 100ml with DEPC water and filtered through Whatman #1 filter paper. Polymerisation was started by adding 40µl TEMED, and the gel mix was cast immediately. For DNA sequencing, the flat side of a comb was inserted to allow use of a shark-tooth comb once the gel was set; for other gels a 20

tooth comb was inserted. The gel was left overnight to polymerise before assembly into the electrophoresis tank (Kodak Biomax STS 45I) with 0.8x glycerol tolerant buffer as running buffer. The gel was pre-run at 1800V for 30min and the wells flushed with buffer before loading samples denatured in formamide loading buffer at 95°C for 3min. Gels were typically run at 1800V until the bromophenol blue had reached the bottom of the gel, but to read more 5' in sequencing experiments a second loading of sample was sometimes performed at this point. In such cases the gel was run until the bromophenol blue of the second loading reached the bottom of the gel. After electrophoresis the gel was transferred to Whatman 3MM paper and dried under vacuum at 80°C on a BioRad 583 gel drier. The gel was exposed to autoradiographic film overnight and then developed; if necessary the gel was then re-exposed for longer.

5% gels were made by dissolving 3.6g urea in 1.25ml 40% acrylamide:bis-acrylamide (19:1), 1ml 10x TBE, 100µl 10% ammonium persulphate (w/v) and making up to 10ml with DEPC water. Two glass plates (8cm x 10cm) cleaned as described above were assembled with 1mm spacers to make the gel cast. Polymerisation was started by adding 10µl of TEMED and the gel was poured immediately. A 10 tooth comb was inserted and the gel allowed to polymerise for at least 30min before use. 5% gels were run using a vertical electrophoresis tank (Sigma) with 1x TBE; samples were denatured in formamide loading buffer at 95°C for 3min before loading and the gel run at 20mA for 15min. After electrophoresis the gel was transferred to Whatman 3MM paper, wrapped in clingfilm and exposed to autoradiographic film for 1h.

For a 4% gel, two glass plates (15cm x 17cm) were cleaned as described, assembled with 0.8mm spacers and sealed with 2% agarose (w/v). 21g of urea was dissolved in 5ml 40% acrylamide:bis-acrylamide (19:15) 5ml 10x TBE, 300µl 10% ammonium persulphate (w/v) and made up to 50ml with DEPC water. 100µl TEMED was added to start polymerisation and the gel poured immediately. A 20 tooth comb was inserted and the gel allowed to polymerise for at least 2h before assembly into the electrophoresis tank (Model V16 vertical gel electrophoresis system, Life

Technologies). Wells were flushed with buffer before loading samples denatured in formamide loading buffer at 95°C for 3min. The gel was run at 30mA until the bromophenol blue just ran off the bottom of the gel. The gel was then transferred to Whatman 3MM paper and dried under vacuum at 80°C on a BioRad 583 gel drier. The gel was exposed to autoradiographic film overnight and then developed; if necessary the gel was then re-exposed for longer. For analysis using the phosphorimager, after an initial exposure to film the gel was then exposed to the phosphorimager screen for 1-5d depending on the autoradiography result.

2.2.3.5 Autoradiography

Dried 6% gels were exposed to DuPont NEF 485 autoradiographic film in a cassette containing a single intensifying screen (Kodak). ^{32}P gels were exposed at -80°C to take advantage of the intensifying screen. After an appropriate exposure time the film was developed in D19 developer for 2min and fixed in Amfix fixative (1 in 5 dilution), rinsed in tap water and left to dry before analysis.

2.2.3.6 Phosphorimagery

Gels were exposed to a Molecular Dynamics or Fuji phosphor screen for 1 to 7 days depending on autoradiography results and then analysed using a Molecular Dynamics or Fuji Film FLA-2000 phosphorimager.

2.2.4 Ribonucleic acid (RNA) Preparation

2.2.4.1 Sample preparation

Total RNA was prepared using either TRIzol reagent or guanidium isothiocyanate extraction. For extraction from cells in culture, each dish of cells was washed twice with 3ml PBS, the appropriate volume of TRIzol reagent or denaturing solution added and cells harvested using a cell scraper; extractions were performed immediately. For extraction from tissues except hippocampus, material was

disrupted by grinding under liquid nitrogen in a pestle and mortar, aliquoted into 1.5ml eppendorf tubes in approximately 100mg aliquots and stored at -70°C until needed. Hippocampus was disrupted in TRIzol or denaturing solution using syringes (section *2.2.4.2*) without prior disruption in liquid nitrogen.

2.2.4.2 TRIzol extraction

1ml of TRIzol reagent was used to extract RNA from 100mg of ground tissue or one hippocampal half. After addition of TRIzol reagent, sample were disrupted using syringes with needles of decreasing gauge (19, 23, and 25 gauge) and incubated for 5min at room temperature. 0.2ml of chloroform was added, samples incubated at room temperature for 3min and centrifuged in a microcentrifuge at 14,000rpm for 15min at 4°C. The aqueous phase was transferred to a fresh eppendorf tube and RNA precipitated by adding 0.5ml isopropanol. Samples were incubated at room temperature for 10min and centrifuged in a microcentrifuge at 4°C for 10min at 14,000rpm. The supernatant was removed with a drawn-out glass pasteur pipette and pellets washed with 1ml cold (4°C) 75% ethanol. After vortexing, samples were centrifuged in a microcentrifuge at 4°C for 5min at 7500rpm. The supernatant was removed with a drawn-out glass pasteur pipette and pellets allowed to air-dry for 5min at room temperature. Pellets were resuspended in 10-150µl DEPC water, depending on pellet size, by incubating at 65°C for 15min and vortexing vigorously.

2.2.4.3 Guanidium isothiocyanate extraction

0.5ml denaturing solution was used to extract RNA from one 60mm dish of cells (4.5 to 6 x 10^6 cells), 100mg of ground tissue or one hippocampal half. Samples were disrupted using needles as before, then 50µl of 2M Sodium acetate (pH 4.0) added followed by vortexing. 500µl of phenol saturated with 0.1M citrate buffer, pH 4.3 was added, followed by vortexing. 100µl chloroform:isoamyl 24:1 mix was added, samples vortexed vigorously for 10s and placed on ice for 15min. Samples were then centrifuged at 14,000rpm for 20min in a microfuge and the supernatants immediately transferred to fresh 1.5ml eppendorf tubes. RNA was precipitated by

adding 200µl isopropanol and incubating at -20°C for at least 1h. RNA was collected by centrifuging at 14,000rpm for 20min in a microfuge, the supernatant was removed with a drawn out glass pasteur pipette, and the pellets were resuspended in 60µl denaturing solution. RNA was pelleted by adding 60µl isopropanol, incubating at -20°C for at least 1h and centrifuging at 14,000rpm for 10min. Pellets were washed with 200µl 75% ethanol and centrifuged at 14,000rpm for 10min. The supernatant was aspirated with a drawn out glass pasteur pipette and the pellet air dried for a few minutes, until the edges began to turn translucent. Pellets were resuspended in 10-150µl DEPC water, depending on pellet size, by incubating at 65°C for 15min and vortexing vigorously. The concentrations of RNA samples were determined using the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech) and integrity checked on a 1% agarose denaturing gel (section *2.2.3.3*).

2.2.5 Manipulation of DNA

2.2.5.1 Restriction digests

Plasmid DNA was digested using 5-15U of restriction endonuclease in 1x appropriate restriction buffer in a final volume of 10-40µl at 37°C for 1-2 h. To digest "minipreps" approximately 0.5µg of DNA was digested; for subcloning and for linearisation of cRNA probe templates, 10µg of DNA was used. Digestion was verified by agarose gel electrophoresis. Where the insert and vector to be ligated had been cut with the same enzyme (that generated an overhang), an equal volume of TE and 1U alkaline phosphatase was added followed by incubation at 37°C for 1h, followed by 15min at 65°C to inactivate the alkaline phosphatase. Where a blunt end was required for ligation, the 5' overhang was blunted by adding 5U Klenow fragment, 1/10vol 2mM dNTPs and incubating at room temperature for 37°C for 10min followed by 65°C for 10min. Note that if a blunt end and overhanging end were required Klenow treatment was performed after digestion by the first restriction enzyme, then the second enzyme was added to the reaction that was then incubated

for a further 2h at 37°C. For minipreps the whole digest was run; for subcloning and template linearisation, a 1µl aliquot of digest was electrophoresed.

2.2.5.2 DNA fragment recovery

DNA fragments for subcloning were purified using a Hybaid Recovery DNA Purification Kit II. Fragments were resolved on a 1-2% low melting point agarose gel run at 100V, visualised with a UV transilluminator at 365nm (to prevent damage to DNA) and the required fragments excised. Gel slices were placed in a spin filter to which 400µl of resuspended Binding Buffer had been added. The sample was placed at 55°C for 5min, the tube flicked to mix and then centrifuged in a microcentrifuge for 30s at 14,000rpm. 500µl of Wash Solution was added to the spin filter and centrifuged as before. This wash step was repeated, the pellet dried by a further 1min centrifugation and the spin filter transferred to a new catch tube. 18µl of Elution Solution was added, the binding matrix/DNA resuspended by flicking and DNA recovered by centrifugation as before. Recovery of DNA was checked by electrophoresing a 1-2µl aliquot on a 1% agarose gel.

2.2.5.3 Purification of templates for cRNA probes

Template linearisation was verified by agarose gel electrophoresis, the DNA purified by phenol-chloroform extraction. DEPC water was added to give a working volume of 100µl. An equal volume of TE-saturated phenol-chloroform:isoamyl (25:24:1) mix was added followed by brief vortexing. The sample was centrifuged at 14,000rpm in a microcentrifuge for 2min and the supernatant transferred to a fresh eppendorf tube taking care to avoid the interface. A tenth vol of 5M NaCl was added, the tube vortexed and then 2.5 vol ethanol added. The tube was vortexed and placed at -70°C for 20min. The sample was centrifuged at 14,000rpm for 5min and the supernatant removed. The pellet was washed with 1ml cold 70% ethanol (made with DEPC water) and centrifuged at 14,000rpm for 5min. The supernatant was removed and the pellet air-dried for 15min before resuspending in 10µl DEPC water.

Recovery was checked by electrophoresis of an aliquot on a 1% agarose gel and the linearised plasmid DNA stored at -20°C until needed.

2.2.6 Ribonuclease Protection Assays

2.2.6.1 Synthesis of cRNA probes

Probes for RNase protection assays were synthesized using corresponding 5'-RACE clones as templates (Table 3.2), with the exception of the exon 1_6 probe whose template was made by subcloning an RT-PCR product generated from total rat liver RNA using GSP3 and 1_6-specific primer (section *2.1.10.1*). All templates, except that for the exon 1_4 probe, were linearised with *Nco*I and probes transcribed with SP6 phage polymerase. The exon 1_4 template was linearised with *Sal*I and the probe transcribed with T7 phage polymerase. A reaction was set up containing approximately 1µg linearised DNA template, 1x transcription optimized buffer, 3mM each of ATP, CTP and GTP or UTP, 10µM DTT, 0.5µl 10mg/ml bovine serum albumin and 20U RNasin RNase inhibitor. 5µl of [α-^{32}P]-UTP or [α-^{32}P]-GTP (3000 Ci/mmol) was added (if unlabelled UTP or GTP respectively had been used in the reaction mix) followed by 20U of SP6 or T7 phage polymerase. Reactions were incubated at 37°C for 2h, 1U DNase added followed by a further 15min incubation at 37°C. Probes were purified through Sephadex G-50 DNA grade ("NICK" columns) equilibriated with 3ml TE or DEPC water. The reaction was added to the column and allowed to enter the gel bed. 400µl TE or DEPC water was added and the eluate collected and discarded. A further 400µl TE or DEPC water was added to the column and the eluate collected. 1µl of eluate (purified probe) was added to 1ml of Pico-fluor 40 scintillant fluid and counted for 1min in a Wallac 1450 Microbeta plus liquid scintillation counter. Probe integrity was verified by electrophoresing 10^5 cpm of probe on a 5% denaturing polyacrylamide gel (section *2.2.3.4*). Typically, probes with over 2 x 10^5 cpm/µl were used in experiments. Due to the high GC content of the DNA templates, several bands were often observed after autoradiography, suggesting "stalling" and dissociation of RNA polymerase; if the full-length product did not predominate, the probe was not used.

2.2.6.2 Large scale in vitro transcription of "sense" RNA transcripts

To synthesize "sense" exon 1_{10} and exon 1_{11}-containing transcripts for optimization of RNase protection assays, a large scale *in vitro* transcription reaction was performed. Templates for exon 1_{10} and exon 1_{11} synthesis were pJIM2 digested with *Pvu*II and pVL132 digested with *Pvu*II respectively, purified by phenol chloroform extraction (section *2.2.5.3*). In both cases, all the recovered template (approximately 10μg DNA) was used in the transcription reaction. The volume of template DNA was adjusted to 40μl with DEPC water and a 100μl reaction was set up containing 40μl template DNA, 20μl reaction buffer, 30μl of 25mM NTPs (25mM each ATP, CTP, GTP, TTP) and 100-200U T7 RNA phage polymerase. The reaction was incubated at 37°C for 4h. 9U of DNase I was added to the reaction, followed by a further 15min incubation at 37°C. The "sense" RNA was then recovered by phenol chloroform extraction (*2.2.5.3*, except citrate-saturated phenol was used) and aliquoted into 5μl aliquots and stored at -70°C. The concentration of the recovered "sense" RNA was determined using the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech) and integrity checked on a 1% agarose denaturing gel (section *2.2.3.3*).

2.2.6.3 Gel purification of cRNA probes

The exon 1_6-exon 2 cRNA probe was found to successfully hybridize to target mRNA transcripts only if the cRNA was completely full length, making it necessary to gel purify the exon 1_6 probes. This may have been a consequence of the C_8 sequence close to the 3' end of exon 1_6 that could lead to the formation of a large number of self-interactions. Other probes made at the same time as the exon 1_6+exon 2 cRNA probe which were also gel purified, showed no difference in the quality of the RPA results (data not shown). Accordingly, only the exon 1_6-exon 2 cRNA probe was gel purified.

Probes synthesised at the same time as the exon 1_6 probe were gel purified in tandem. After probe synthesis (section *2.2.6.1*), 50μl of formamide loading buffer

was added to the entire purified probe sample which was then electrophoresed on a 5% denaturing acrylamide gel with enlarged wells (6 teeth of the 20 tooth comb were taped together) for 1.5h. The gel was exposed to autoradiographic film for 10min with the position of the film in relation to the gel carefully marked. After developing the film the full-length probe band was located on the gel, excised and placed in a 1.5ml eppendorf tube. The gel was then re-exposed to autoradiographic film for 10min to check the correct band had been taken. Probes were eluted from the gel slice using a Bio-Rad Model 422 Electro-Eluter. Membrane Caps were soaked for at least 1h at 60°C in 1x probe elution buffer. The apparatus was assembled according to the manufacturer's instructions and elution performed in 1x probe elution buffer at 10mA/glass tube for 1 hour. After elution, the polarity was reversed for 1min to remove probes from the dialysis membrane. After draining the upper buffer chamber the buffer left in the glass tube was removed down to the level of the frit and discarded. The silicone adaptor and membrane cap were removed and the buffer in the membrane cap (approximately 400µl) containing purified probe transferred to a 1.5ml eppendorf tube. Membrane caps for individual probes were stored in 0.05% sodium azide. 1µl of recovered probe was counted in a scintillant counter and integrity verified on a 5% denaturing polyacrylamide gel (section *2.2.3.4*).

2.2.7 Ribonuclease Protection Assays

2.2.7.1 Ribonuclease Protection Assays

Ribonuclease protection assays were performed using the HybSpeed RPA kit (Ambion). 50µg of total RNA (experimental) or 20µg yeast tRNA (positive and negative controls) was co-precipitated with 10 x 10^5 cpm cRNA probe (5 x 10^5 cpm if gel purified) by adding 1/10 vol 5M ammonium acetate, 2.5 vol ethanol and placing at -20°C for 15min. The precipitate was collected by centrifugation in a microcentrifuge for 15min at 14,000rpm and allowed to air dry for 2min. Pellets were resuspended in 20µl hybridization buffer (provided in kit) by incubating at 95°C and vortexing several times. Resuspension was confirmed by pipetting the sample from the eppendorf tube and holding next to a Geiger-Muller counter: if

approximately 70% of the counts were in the pipette tip, the sample was considered resuspended. Samples were incubated at 95°C for a further 3min and quickly transferred to 68°C for 1h. 100µl of a 1/25 dilution of RNase A/T_1 (supplied in kit) was added (for positive controls digestion buffer without RNase A/T_1 was used) and reactions incubated at 37°C for 30min, vortexing after RNase addition and after 15min incubation. 150µl RNase Inactivation/Precipitation solution (provided with kit) was added, reactions vortexed and placed at -20°C for 15min. RNA pellets were recovered by centrifugation at 14,000rpm for 15min, resuspended in 8µl gel loading buffer (provided with kit) and resolved on a 4% denaturing polyacrylamide gel. Positive control samples were diluted so the amount of radioactivity loaded on the gel was equivalent to that of the most radioactive experimental sample.

2.2.7.2 Analysis of data

Data were quantified using a Molecular Dynamics or Fuji Film FLA-2000 phosphorimager, after exposure of the dried gel to a phosphor screen for 1 to 7 days, depending on the strength of signal obtained on the corresponding autoradiograph. Background was set for individual bands by placing a line just above the band of interest and measuring intensity (i.e. pixels). Band areas were enclosed with an oval and the intensity within this area determined by 3D densitometry. Background was subtracted and values corrected for size differences (Table 3.2). The abundance of each exon 1 was then expressed as a percentage of total GR mRNA transcripts: exon 1/exon 2 band intensity/exon 1/2 band + exon 2 band intensities x 100. When performed, Student T-tests for independent variables were carried out using the Statistica software package (version 5) with $p < 0.05$.

2.2.8 PCR-based techniques

2.2.8.1 5'-RACE PCR

2.2.8.1.1 First strand DNA synthesis

5'-RACE PCR was performed using a kit obtained from Life Technologies. 2.5pmol GSP1 (section *2.1.10.1*) was annealed to 5µg total RNA in a total volume of 15µl by denaturing at 70°C for 7min and then placing on ice for 1min. First strand cDNA synthesis was carried out in a 25µl reaction mixture containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 3mM $MgCl_2$, 10mM DTT, 400µM dNTPs (all supplied with kit), incubating at 42°C for 2min, adding 200U SuperScript II reverse transcriptase (RT) and incubating for a further 30min. The reaction was incubated at 68°C for 15min to inactivate RT, and 1µl RNase mix added followed by incubation at 37°C for 30min.

2.2.8.1.2 cDNA purification

GR cDNA was purified using a GlassMAX DNA Isolation Spin Cartridge (Life Technologies). 100µl of ultrapure water was equilibriated to 65°C for use later and the binding solution (6M NaI) was equilibriated to room temperature. 120µl of binding solution was added to the RT reaction and the cDNA/NaI solution transferred to a GlassMAX spin cartridge. The spin cartridge was centrifuged at 14,000rpm for 20s and the flowthrough discarded. 400µl of cold (4°C) 1x wash buffer was added to the spin cartridge followed by centrifugation at 14,000rpm for 20s. This wash step was repeated two additional times. The cartridge was washed in the same way twice with 400µl of cold (4°C) 70% ethanol and centrifuged at 14,000rpm for 1min after the final wash. The spin cartridge was transferred into a fresh sample recovery tube and 50µl of the water pre-equilibriated to 65°C added. cDNA was eluted by centrifugation at 14,000rpm for 20s.

2.2.8.1.3 Terminal deoxynucleotidyl transferase (TdT) tailing of cDNA

10µl of purified cDNA was transferred to a 0.5ml eppendorf tube for use as an "untailed" cDNA control in PCR; the remainder was dried using a Speedvac (Savant), resuspended in 10µl ultrapure water and used in a 25µl reaction that contained 10mM Tris-HCl (pH 8.4), 25mM KCl, 1.5mM $MgCl_2$ (supplied as 5x tailing buffer with kit), and 200µM dCTP. The reaction mix was incubated for 2min at 94°C, chilled on ice for 1min and 10U TdT added. After mixing gently, the reaction was incubated for 10min at 37°C; TdT was inactivated by incubating at 65°C for 10min.

2.2.8.1.4 Polymerase chain reaction

For each RNA sample tested, two rounds of PCR were performed to increase specificity. For the first round of PCR 10µl tailed cDNA was used in a reaction with 400nM UAP "anchor" primer (section *2.1.10.1*) and 400nM primer GSP2, 20mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, and 200µM dNTPs. Positive control reactions contained 10µl "untailed" cDNA and 400nM primers 6002 and 6003 section (section *2.1.10.1*) instead of "anchor" primer and GSP2. Negative controls contained no cDNA. Reactions were overlaid with 2 drops of mineral oil and incubated at 94°C for 5min before adding 2.5U *Taq* DNA polymerase (Promega) and starting the cycling program. 35 cycles of PCR amplification (96°C, 45sec; 45°C, 40sec; 72°C, 1.5min) were performed. Nested PCR reactions were carried out on the products from the "tailed" cDNA and negative control reactions, using the same conditions with 400nM each of primers UAP and GSP3, instead of "anchor" primer and GSP2. PCR products from nested PCR on "tailed" cDNA were cloned in pGEM-T easy (section *2.2.9.1*) and if of sufficient length, sequenced for analysis.

2.2.8.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

First strand cDNA synthesis was performed using the Reverse Transcription System (Promega). 2µg total RNA was reverse transcribed in a 40µl reaction mixture

containing 5mM $MgCl_2$, 1x reverse transcription buffer, 1mM each of dATP, dCTP, dGTP and dTTP, 40U RNasin, 1μg oligo$(dT)_{15}$ primer, and 30U AMV-RT was prepared (made up to a total volume of 40μl with DEPC water) and incubated for 45min at 42°C. 5μl of reaction product was then used in a PCR reaction containing 200nM of each primer (section *2.1.10.1*), 20mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, and 200μM dNTPs. A negative control reaction containing water was performed in parallel. Reactions were overlaid with 2 drops of mineral oil and incubated at 94°C for 5min before adding 2.5U *Taq* DNA polymerase and starting the cycling program. 35 cycles of PCR amplification (96°C, 45s; 45°C, 40s; 72°C, 1.5min) were performed. To determine whether primary hippocampal cultures expressed exon 1_7-containing GR mRNA (Chapter 5), the PCR conditions were 35 cycles, 95°C, 5min; 50°C, 45s; 72°C, 1.5min. PCR products were analysed by electrophoresing on a 1% agarose gel.

2.2.8.3 Cycle sequencing

DNA sequencing was done using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). Termination mixes were prepared by mixing 2μl of dGTP nucleotide master mix and 0.5μl of [α-^{33}P] ddNTP (G, A, T, or C; one of each per sequencing reaction) in 0.5ml eppendorf tubes to produce a termination mix for each ddNTP. A reaction mix was made by mixing 2μl reaction buffer, 1μl DNA, 0.05pmol primer (section *2.1.10.1*), 13μl distilled water and 8U Thermo Sequenase polymerase. 4.5μl of reaction mix was added to each termination tube and the tubes transferred to a pre-heated thermal cycling block. A cycling program with the following parameters was performed: 40 cycles of 95°C, 30s; 45°C, 30s; 72°C, 1 min. Upon completion of the cycling program, 4μl of stop solution was added and reactions stored at -20°C for a maximum of 3d. Samples were resolved on 6% glycerol tolerant sequencing polyacrylamide gels.

2.2.9 Cloning of DNA

2.2.9.1 DNA ligation

Fragments recovered from low melting point agarose gels were ligated using 1-5U of T4 DNA ligase in 1x supplied buffer in a final volume of 10µl. Typically, 2-4µl (approximately 50ng) of insert DNA was used, and 0.5-1µl (approximately 100ng) vector DNA. Reactions were incubated at 4°C overnight before transforming into competent *E.coli*.

2.2.9.2 Preparation of competent E.coli

For general cloning procedures, the *recA* HB101 strain was used. Transformation-competent cells were prepared by inoculating 1ml of Luria broth was inoculated with a single colony of bacteria and incubating in a rotator at 37°C overnight. 50ml of LB was then inoculated with this culture and incubated at 37°C in a shaking incubator for 1.5 to 2h so the cells were still in the late log phase of multiplication when made competent. The culture was then centrifuged in a Beckman J2-MC centrifuge at 7000 rpm for 5min and the pellet resuspended in 20ml of cold 0.1M $CaCl_2$. Cells were left 10min to 2h on ice, centrifuged at 7000rpm for 5min and resuspended in 2ml of cold 0.1M $CaCl_2$. The resulting competent cells were either used immediately or stored up to 4d on ice at 4°C, with daily changes of ice. For cloning of 5'-RACE PCR products, commercially bought competent JM109 *E.coli* were used.

2.2.9.3 Transformations

For general cloning of DNA, HB101 cells were used; super-competent commercially obtained JM109 cells were used for cloning of 5'RACE-PCR products.

To transform competent HB101 cells, 200µl of cells were placed in a pre-chilled 1.5ml eppendorf tube on ice. 200-500ng of DNA was added to the cells and left on ice for 15min. The cells were then heat shocked for 2min at 42°C and left on ice for

2min to recover. The entire contents of the tube were then plated onto LB agar plates left to air dry for 10min and then placed upside down in an incubator at 37°C overnight.

To transform JM109 cells, cells were removed from storage at -80°C and allowed to thaw on ice. The cells were gently flicked to mix, and 100µl transferred to a pre-chilled 1.5ml eppendorf tube. 200-500ng of DNA added and the mixture gently flicked to mix. The reaction was placed on ice for 10min, heat shocked at 42°C for 50s and allowed to recover on ice for 2min. 400µl of room temperature LB was added and the cells placed in a rotating incubator at 37°C for 30min prior to plating 200µl on LB agar (containing 40µl 50mg/ml X-GAL, 40µl 0.1M IPTG and 100µg/ml ampicillin). The plates were then allowed to air dry for 10min before being placed upside down in an incubator at 37°C overnight.

2.2.9.4 Screening of clones: minipreps

Screening of transformants was carried out by small-scale preparation of plasmid DNA followed by appropriate restriction digests and agarose gel electrophoresis. Following transformation, single transformant colonies were picked and used to seed 2ml of LB containing 100µg/ml ampicillin. After overnight incubation in a rotating incubator at 37°C, cultures were transferred into 1.5ml eppendorf tubes, centrifuged in a microcentrifuge at 14,000rpm for 1min and the pellets resuspended in 100µl GTE. 200µl fresh 0.2M NaOH/1% SDS (w/v) was added, the tubes vortexed and placed on ice for 2min. 150µl 5M potassium acetate (pH 4.8) was added, the tubes vortexed and placed on ice for 5min. The tubes were then centrifuged in a microcentrifuge at 14,000rpm for 5min and the supernatant transferred to fresh eppendorfs tubes. 225µl chloroform/isoamyl alcohol (24:1) and 225µl Tris-HCl saturated phenol was added, the tubes vortexed and centrifuged in a microcentrifuge for 2min. The supernatant was transferred to a fresh eppendorf tube, 2 volumes of absolute ethanol added and the tubes vortexed prior to incubation at room temperature for 5min. The tubes were centrifuged at 14,000rpm in a microcentrifuge

for 5min, the supernatant removed with a drawn-out glass pasteur pipette and the pellet left to air dry for 10min. The pellet was then resuspended in 50µl TE containing 50ng RNase A and stored at -20°C until needed.

2.2.9.5 Large scale plasmid DNA preparation: CsCl density gradient centrifugation

Single colonies of transformants containing the required plasmid were used to innoculate 2ml of LB containing 100µg/ml ampicillin and grown for 8h at 37°C in a rotating incubator. This culture was transferred to 500ml LB containing 100µg/ml ampicillin and grown overnight at 37°C in a shaking incubator. Cells were pelleted at 6000rpm for 5min at 4°C in a Beckman J2-MC centrifuge using a JA-14 rotor. The pellet was resuspended in 12ml of GTE, mixed with 24ml of fresh 0.2M NaOH/1% SDS (w/v), and placed on ice for at least 10min. 16ml of cold 5M potassium acetate was added, mixed gently, and placed on ice for 10min, prior to centrifugation at 6000rpm for 10min at 4°C. The supernatant was filtered through gauze into fresh centrifuge pots, 32ml of isopropanol added and the DNA precipitated at room temperature for 30min. The DNA was pelleted by centrifugation at 10,000rpm for 3min at 4°C. The pellet was air-dried and resuspended in 2.2ml TE, then 3g of CsCl added and dissolved. 100µl of ethidium bromide (10mg/ml) was added and the DNA solution transferred into a 3ml Beckman Quickseal tube and centrifuged for either 4h at 100,000rpm, or for 16 to 20h at 70,000rpm in a TLA100.3 rotor in a Beckman Optima TLX Ultracentrifuge. The plasmid DNA band was removed using a needle and syringe through the tube wall and transferred to a fresh tube. CsCl/TE (1g + 1ml) was added to fill the tube and the tube centrifuged as before. Closed circular plasmid DNA was removed and the ethidium bromide extracted using isopropanol until no pink colour was observed. Plasmid DNA was then dialysed against three changes of 2L TE at 4°C. Concentration and quality (absorbance ratio A_{280}/A_{260}) of plasmid DNA was determined using a GeneQuant spectrophotometer (Pharmacia Biotech). Plasmid DNA was stored at -20°C until needed.

2.2.10 Transient transfection studies

2.2.10.1 Maintenance of cell lines

HepG2 (human hepatoma), C6 (rat glioma) and B103 (rat neuroblastoma) cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 100μg/ml penicillin/streptomycin and 2mM L-glutamine. GH_3 (rat pituitary tumour) cells were maintained in Nutrient Mixture F-10 (HAM) supplemented with 15% heat-inactivated horse serum and 2.5% heat-inactivated foetal calf serum, 100μg/ml penicillin/streptomycin and 2mM L-glutamine. HepG2 and C6 were routinely split 1:5 when confluent; GH_3 and B103 cells reached approximately 50% confluence and were split 1:5 and 1:20 respectively. To harvest and split cells, they were washed with 8ml serum-free DMEM, then treated with 1.5ml 1x trypsin/EDTA in HBSS for 1-2min to release the cells then resuspended in 9ml DMEM with serum. Cells were then diluted as appropriate in DMEM with serum. To freeze stocks of cells for storage under liquid nitrogen, cells from a single confluent flask were resuspended in 3ml freezing medium (10% DMSO, 90% heat inactivated foetal calf serum), and transferred in 1ml aliquots to cryotubes (Nalgene). The tubes were then sandwiched in polystyrene and placed at -70°C overnight before storing under liquid nitrogen.

2.2.10.2 Transient transfections: calcium phosphate precipitation method

24h before transfection cells were seeded on 60mm dishes, allowing 3 dishes per transfection. Cells were harvested by centrifugation at 1000rpm for 5min and resuspended in sufficient medium to allow 1ml of resuspended cells/60mm dish. Cells were counted using an Improved Neubauer haemocytometer (Hawksley) and if necessary diluted further to give a cell count of 5-7 x 10^5 cells/ml for HepG2 and C6 cells or 2 x 10^5 cells/ml for B103 cells. 1ml of cells was added to 60mm dishes containing 3ml of medium and incubated at 37°C with 5% CO_2 for 24h before transfection.

All solutions used were equilibriated to room temperature prior to use. Medium on cells was replaced with 3ml of fresh medium at least 1h prior to transfection. For each construct DNA solutions (300μl total) were set up in triplicate in filter-sterilised water and contained 37μl filter-sterilised 2M $CaCl_2$. Control DNA solutions consisted of (i) 10μg pGEM3 (inert DNA) and (ii) 5μg pSV2 luciferase, 1μg pCH110 (containing the β-galactosidase gene) and 4μg pGEM3 to give a total of 10μg DNA. Due to the large variation in the insert lengths of the test plasmids, the amounts used were corrected so equimolar amounts were used; 1μg pCH110 was used in each transfection and the amount of pGEM3 adjusted accordingly to give a total of 10μg DNA. For cotransfections with expression plasmids encoding transcription factors, 0.5μg of plasmid encoding the relevant factor was added before adjustment to 10μg DNA. DNA solutions were briefly vortexed to mix. 20-30min before transfection, DNA solutions were added dropwise to 300μl 2x HEPES-buffered saline with slow agitation and incubated at room temperature for 20min. After briefly vortexing, the DNA solutions were added slowly to the dishes of cells with agitation. 24h after transfection the medium on the cells was replaced with 3ml fresh medium; to treat cells with 10^{-7}M dexamethasone, 1mM dexamethasone (in ethanol) was diluted into medium and added to cells. 24h later, cells were harvested for assays. Medium was aspirated, HepG2 and C6 cells were washed with 3ml phosphate-buffered saline (B103 cells became detached if washed) and 300μl of lysis buffer added to the dishes. After incubation at room temperature for 15min, cells were scraped and pipetted into eppendorf tubes; cell debris was pelleted by centrifugation at 14,000rpm for 2min in a microcentrifuge. All transfections were performed using two independently prepared plasmid DNAs.

2.2.10.3 Transient transfections: electroporation method

Cells were harvested as described above and resuspended in medium to give 7.5 x 10^6 cells/ml (3 x 10^6 cells/sample were required). For each sample, 400μl of cells were placed in a 50mm x 4mm cuvette (Equibio). Due to the large variation in the insert lengths of the test plasmids, the amounts used were corrected so equimolar amounts were used (N.B. double the amounts of all DNAs were used for

electroporation); 2μg pKC275 (encoding the *lacz* gene controlled by the RSV LTR promoter) was used and the amount of pGEM3 adjusted accordingly to give a total of 20μg DNA. Control DNA solutions consisted of (i) 10μg pGEM3 (inert DNA) and (ii) 10μg pRSV luciferase, 2μg pKC275 and 4μg pGEM3 to give a total of 10μg DNA. All transfections were performed in triplicate. The DNA solutions were added to the cells which were then subjected to a discharge of 200V, 950μF in a Gene Zapper 450/2500 (IBI Kodak). Samples were then incubated for 10min at room temperature before adding to 60mm culture dishes containing 3ml medium. Cells were harvested for luciferase and β-galactosidase assays 24h later.

2.2.10.4 Luciferase assays

Since luciferase is labile, luciferase assays were performed in duplicate on the same day that cells were harvested. All solutions were equilibriated to room temperature, then 40μl of sample was added to 100μl 2x assay buffer with 5μl 0.1M ATP in a 5ml borosilicate tube (Starstedt). Luciferase activity was assayed using a Lumat LB9501 luminometer that injected 105μl 1mM beetle luciferin. Values recorded were the means of the duplicate measurements performed.

2.2.10.5 β-galactosidase assays

β-galactosidase activity was assayed using a Galacto-Light Plus kit obtained from Tropix and all samples were assayed in duplicate. All solutions used were equilibriated to room temperature prior to use. Galacton-Plus substrate was diluted 1:100 with Reaction Buffer Diluent to make the reaction buffer which was then dispensed in 67μl aliquots into 5ml Rohren tubes. 10μl of sample was added, left to incubate at room temperature for 15-60min and then assayed using a Lumat LB 9501 luminometer (Berthold) which injected 105μl Light Emission Accelerator. Values recorded were the means of the duplicate measurements performed.

2.2.10.6 Data analysis

Data were analysed using a Microsoft Excel spreadsheet. The means of the background luciferase and β-galactosidase assay values (those for transfections with pGEM3 only) were subtracted from experimental values and promoter activity expressed as luciferase activity/β-galactosidase activity, thus controlling for differences in cell number and transfection efficiency. The mean value obtained for empty vector (pGL3-BM) was then set to a value of 1 and mean experimental activities expressed relative to this value. The value obtained with pSV2 luciferase confirmed successful transfection; transfections in which low pSV2 luciferase activities were obtained were excluded from analysis. The Statistica software package (version 5) was used to analyse data. For experiments involving multiple groups, ANOVA analysis was performed, followed by a *post-hoc* Fisher test with p set to <0.05. For other experiments Student t tests for were performed with p set to <0.05. For clearer graphical representation, data was expressed relative to the whole promoter region (P2), which was set at 100% activity, unless stated.

Chapter 3: Tissue-specific distribution of alternate exon 1-containing GR mRNA transcripts

3.1 Introduction

The importance of glucocorticoid actions is reflected by the presence of GR in virtually every tissue (Ballard, P. L. et al. 1974). Levels of GR vary between (Kalinyak J. E. et al. 1987; Whorwood, C. B. et al. 1992) and within tissues (Herman, J.P. et al. 1989b), and are also dynamic within tissues depending on the stage of development (Cole, T. J. et al. 1995b) and environmental conditions (Olsson, T. et al. 1994). In liver, glucocorticoid activation of GR regulates many genes involved in glucose homeostasis (section *1.2.3*). In thymus, glucocorticoids play a role in T-lymphocyte maturation, with an excess of GR activation causing death of cells whose T cell antigen receptors have inadequate avidity for self-antigen/MHC (reviewed in Ashwell, J. D. et al. 1996; Tolosa, E. et al. 1998; Vacchio, M. S. et al. 1998). GR activation in hippocampus has many effects including alterations of HPA axis activity and effects on memory and cognition (section *1.2.6*).

The wide variation in GR expression both spatially and temporally suggests complex regulation of the GR gene. Work from this laboratory (McCormick, J. A. et al. 2000) has demonstrated the existence of multiple alternate exon 1-containing GR mRNAs, which might reflect transcription initiated at alternative promoters. The 3' ends of these alternate exons 1 that are fused to exon 2 are shown in Table 3.1.

The experiments described in this chapter were designed to determine the relative abundance of alternate exon 1-containing GR mRNA transcripts within various tissues. Tissue-specific differences in alternate exon 1 usage would suggest tissue-specific regulation of the GR gene. The tissues examined were hippocampus, thymus and liver, in which glucocorticoids have distinct actions (section *1.2*).

Exon	source	3'-boundary	position
1_1	Thymus	...CTGGGATGAAG.....	5' of -15000
1_2	Thymus	...AGGGCGACCGG.....	5' of -15000
1_3	Hippocampus	...GCAACAGCAAG.....	5' of -15000
1_4	Hippocampus	...CCGAGGGGCAGgt....	-4238 to -4011
1_5	Hippocampus	...TTCCTCCGAGTgt....	-3592 to -3538
1_6	Liver*	...CCGGGCTCACAgt....	-3322 to -3269
1_7	Hippocampus	...TCCCAGGCCAGgt....	-2981 to -2929
1_8	Hippocampus	...GTCGCCGACAGgt....	-2838 to -2738
1_9	Hippocampus	...ACGGATTCTAAgt....	-2557 to -2479
1_{10}	Hippocampus, thymus	...CGGGTGCTGAGgt....	-2417 to -2217
1_{11}	Hippocampus	...GAACTCAACAGgt....	-1812 to -1705

Table 3.1: *5'-RACE reveals at least 11 alternative exons 1 in GR mRNA*

A total of 10 different alternative exons 1 were found in GR mRNA from hippocampus or thymus. 54 independent 5'-RACE clones were isolated from a total of 8 different 5'-RACE reactions carried out on hippocampal RNA and a further 4 clones were isolated from thymus RNA. *Exon 1_6 (present in the published rat cDNA sequence (Miesfeld, R. et al. 1986)) was not detected by 5'-RACE PCR, but is included for completeness. Taken from McCormick, J. A. et al. 2000.

3.2 Methods

3.2.1 Experimental design

To determine the relative abundance of alternate exons 1 in rat tissues, ribonuclease protection assays (RPA) were employed. The assay was designed so that the levels of individual exons 1 could be expressed as a percentage of total GR mRNA transcripts and is outlined in Figure 3.1. Templates for cRNA probes (with the exception of the exon 1_6 probe, see below) were derived from 5'-RACE PCR clones, which contained 186 nucleotides of exon 2 and a unique exon 1 sequence, depending on the clone. The length of exon 1 varied between clones. Probe fragments protected by GR mRNA transcripts could therefore be of two lengths; 186 nucleotides representing protection by GR transcripts containing exon 2 (common to

all GR mRNA transcripts) but not the specific exon 1, and a longer fragment representing protection by transcripts containing the specific exon 1 in the probe in addition to the common exon 2 (Figure 3.1). When resolved on a denaturing polyacrylamide gel and analysed using a phosphorimager, the proportion of GR mRNAs containing the specific exon 1 of interest can be expressed as a proportion of total GR mRNAs. The predicted sizes of protected fragments containing both exon 2 and the specific exon 1 of interest are shown in Table 3.2.

Exon 1	Plasmid name	Vector	Size of protected fragment (nucs)
1_1	pVL166	pGEM T-easy	228
1_2	pVL167	pGEM T-easy	238
1_4	pVL133	pGEM T	336
1_5	pVL135	pGEM T	242
1_6	pJIM5	pGEM T	221
1_7	pVL134	pGEM T	245
1_{10}	pJIM2	pGEM T-easy	306
1_{11}	pVL132	pGEM T	247

Table 3.2: *Predicted sizes of cRNA probe fragments protected by alternate GR exon 1-containing mRNAs by RNase protection analysis*

3.2.2 Generation of template for exon 1_6 cRNA probe

Exon 1_6 was not detected amongst the products of 5'-RACE PCR on hippocampal RNA, possibly as a result of the hybridization of the 5'-RACE UAP-anchor primer (which contains a $G(GGIIG)_3$ sequence) to a C_8 sequence close to the 3' end of exon 1_6; 5'-RACE products likely to contain only very short amounts of exon 1 sequence were not analyzed by sequencing. To synthesise an exon 1_6+exon 2 cRNA probe it was therefore necessary to generate a template by performing RT-PCR on total rat liver RNA (see Chapter 2), subcloning products into pGEM T-easy and identifying an exon 1_6-containing transformant. The resulting probe contained 186 nucleotides

of exon 2 (as for those derived from 5'-RACE PCR clones) and 35 nucleotides of exon 1_6.

3.2.3 Optimisation of experimental system

Initial experiments were performed using the RPA II kit (Ambion). Preliminary data suggested that exon 1_{10} accounted for approximately 30% of GR mRNA transcripts in total RNA from liver and hippocampus (Figure 3.2). In addition to the expected fragments, however, several fragments were obtained with sizes intermediate to those expected for the exon 2 and exons 1+2 protected fragments (186 nucleotides and 306 nucleotides respectively). These products may have resulted from protection of the probe by mRNA transcripts produced from additional initiation points or may merely have been artefacts. If they were real, they would have to be included in the analysis, so it was necessary to resolve this question. A plasmid containing the exon 1_{10} insert, pJIM2, was linearised with *Pvu*II in order to synthesise a "sense" exon 1_{10} transcript. The template was transcribed with T7 phage polymerase as for probe synthesis (section *2.2.6.1*) except non-radiolabelled UTP was used. After purification, 50µl (1/8) of the product was phenol-chloroform extracted, resuspended in 5µl DEPC water and electrophoresed on a 1% denaturing agarose gel. From this gel it was estimated that the yield in 50µl was between 0.1ng and 1ng. The "sense" exon 1_{10} was used in a range of dilutions (neat to 10^{-6}) in an RPA against radiolabelled "antisense" exon 1_{10} probe (Figure 3.3). This experiment was performed using the HybSpeed RPA kit (Ambion) which involves a much shorter hybridization time (10 minutes instead of overnight as for the RPA II kit). The data obtained showed that the multiple protected fragments observed with total liver RNA (lane L) are obtained when the "sense" exon 1_{10} transcript is used (lanes N to 10^{-2}). Since the "sense" exon 1_{10} transcript is initiated from a single promoter (the T7 promoter in pGEM T-easy) this confirms that the protected fragments apparent between 186 and 306 nucleotides in length are artefacts, most likely due to secondary structure in the probe, which has a high GC content. These data did, however, raise another problem; in this experiment, exon 1_{10}-containing transcripts appeared to represent the majority of GR mRNA, in contrast to the 30% estimated using the RPA

II kit. This difference could have been due to the increased sensitivity, the reduced hybridization time, or varying reagent components between the 2 kits.

A comparison of the two RPA kits, both supplied by the same manufacturer (Ambion) was performed to determine which provided more accurate quantitation of the relative abundance of GR mRNAs. This was carried out using a mixture of two "sense" probes, encoding exon 1_{10}+exon 2 and exon 1_{11}+exon 2, mixed in known proportions. The plasmid pVL132, which provided the template for an exon 1_{11} probe, was linearised with *Pvu*II in order to synthesise "sense" exon 1_{11} transcripts. To generate large amounts of "sense" transcripts which could be quantified accurately, a large scale *in vitro* transcription reaction was used to synthesise "sense" exon 1_{10} and 1_{11} transcripts (both of which also contained 186 nucleotides of exon 2) (section *2.2.6.2*). The concentrations and integrity of these transcripts were determined and they were then mixed in equal amounts to give a concentration of approximately 6μg/μl of each. This mixture was diluted to give a stock solution containing approximately 0.25ng/μl of each transcript. RPAs were then performed using dilutions of this stock solution against [^{32}P]-UTP-labelled exon 1_{10} and 1_{11} cRNA probes with the RPAII and HybSpeed RPA kits. The data obtained with the RPAII kit were uninterpretable since the samples had degraded in the overnight incubation (data not shown); those obtained with the HybSpeed RPA kit (in which hybridization was only 10 minutes) are shown in Figure 3.4. When incubated with a mixture of sense exon 1_{10} and 1_{11} transcripts, the sizes of protected fragments of the exon 1_{10}-containing cRNA probe will be 384 nucleotides (i.e. protection of the exon 1_{10}+exon 2 containing portion of the probe by the sense exon 1_{10} transcript) and 264 nucleotides (i.e. the exon 2 portion protected by the sense exon 1_{11} transcript). Similarly, these sense transcripts will protect 258 (protection of the exon 2 portion by the sense exon 1_{10} transcript) and 377 (protection of the exon 1_{11}+exon 2 containing portion of the probe by the sense exon 1_{11} transcript) nucleotides of the probe. Since the sense transcripts were equimolar, the two protected fragments obtained using each probe should be of equal intensity (after adjustment for fragment size). In practice, the intensity of the 384 nucleotide fragment of the exon 1_{10}-containing cRNA probe was much greater than that of the 264 nucleotide fragment (Figure 3.4,

lanes 1_{10} 10^{-1}, 10^{-2}). Using the exon 1_{11}-containing cRNA probe, the 258 nucleotide fragment was more intense than the 377 nucleotide fragment (Figure 3.4, lanes 1_{11} 10^{-1}, 10^{-2}). These data suggested that the HybSpeed RPA kit is inaccurate at estimating the relative abundances of these transcripts. 10min hybridization time was possibly insufficient to reach equilibrium; indeed the HybSpeed RPA kit probe/RNA hybridization kinetics are biphasic (Ambion, personal communication).

Optimization of hybridization time was done by performing an RPA using a 10^{-2} dilution of the "sense" transcript mix against ^{32}P-UTP-labelled "antisense" an exon 1_{11}+exon 2 probe at different time-points (Figure 3.5). The results showed that when the "antisense" exon 1_{11} probe was used, 60min of hybridization gave protected fragments of 258 nucleotides and 377 nucleotides with approximately equal intensity compared to other hybridization times which were far from equal intensity (Figure 3.5). The results of this experiment therefore suggested an optimal hybridisation time of 60 min, and this hybridisation time was used for all subsequent RPAs.

3.3 Results

3.3.1 Exon 1_{10}-containing transcripts account for the majority of GR mRNA transcripts in a screen of rat tissues

RPAs performed using total RNA from various rat tissues showed levels of exon 1_{10}-containing transcripts accounted for between 56% and 87% of total GR mRNA transcripts (Figure 3.6 and Table 3.2). In liver 76% of GR mRNA transcripts contained exon 1_{10}, in hippocampus 63%, and in thymus 56% (Figure 3.6 and Table 3.2). Several tissues were only screened for exon 1_{10} transcript levels and contained heart, 75%; kidney, 79%; lung, 87% and testis, 80% (Figure 3.6).

3.3.2 Exon 1_6-containing transcripts show widespread tissue distribution

The exon 1_6+exon 2 cRNA probe was found to successfully hybridize to target mRNA transcripts only if the cRNA was completely full length, making it necessary

to gel purify the exon 1_6 probes. Exon 1_6-containing transcripts, were expressed in hippocampus (19% of GR transcripts), liver (10%) and thymus (21%) (Figure 3.7 and Table 3.2).

3.3.3 Other exon 1-containing GR mRNAs exhibit tissue-specific distributions

Other exon 1-containing GR transcripts exhibited more limited, tissue-specific distributions. Exon 1_1, identified in a 5'RACE clone from thymus was present only in thymus RNA (27% of GR mRNA) and not in liver and hippocampus (Figure 3.8 and Table 3.3). It should be noted that the exon 1_1-protected cRNA probe consists of two distinct fragments both of which appear to be specific (Figure 3.8); this may result from transcription initiation from two different starts close to one another or secondary structure forming in the mRNA. Exon 1_5-containing mRNA transcripts accounted for 8% of GR mRNA in hippocampus but were not detected in thymus or liver. (Figure 3.9 and Table 3.3). Exon 1_7-containing mRNAs were also hippocampus-specific and accounted for 8% of hippocampal GR transcripts (Figure 3.10 and Table 3.3). Exon 1_{11}-containing transcripts were relatively more abundant in hippocampus than in liver and were not present in thymus (Figure 3.11 and Table 3.3). Levels of exon 1_2 and exon 1_4-containing GR mRNA transcripts, identified by 5'-RACE PCR of thymic and hippocampal RNA respectively, were below the limit of detection of the RPA (Figure 3.8, Table 3.3 and data not shown).

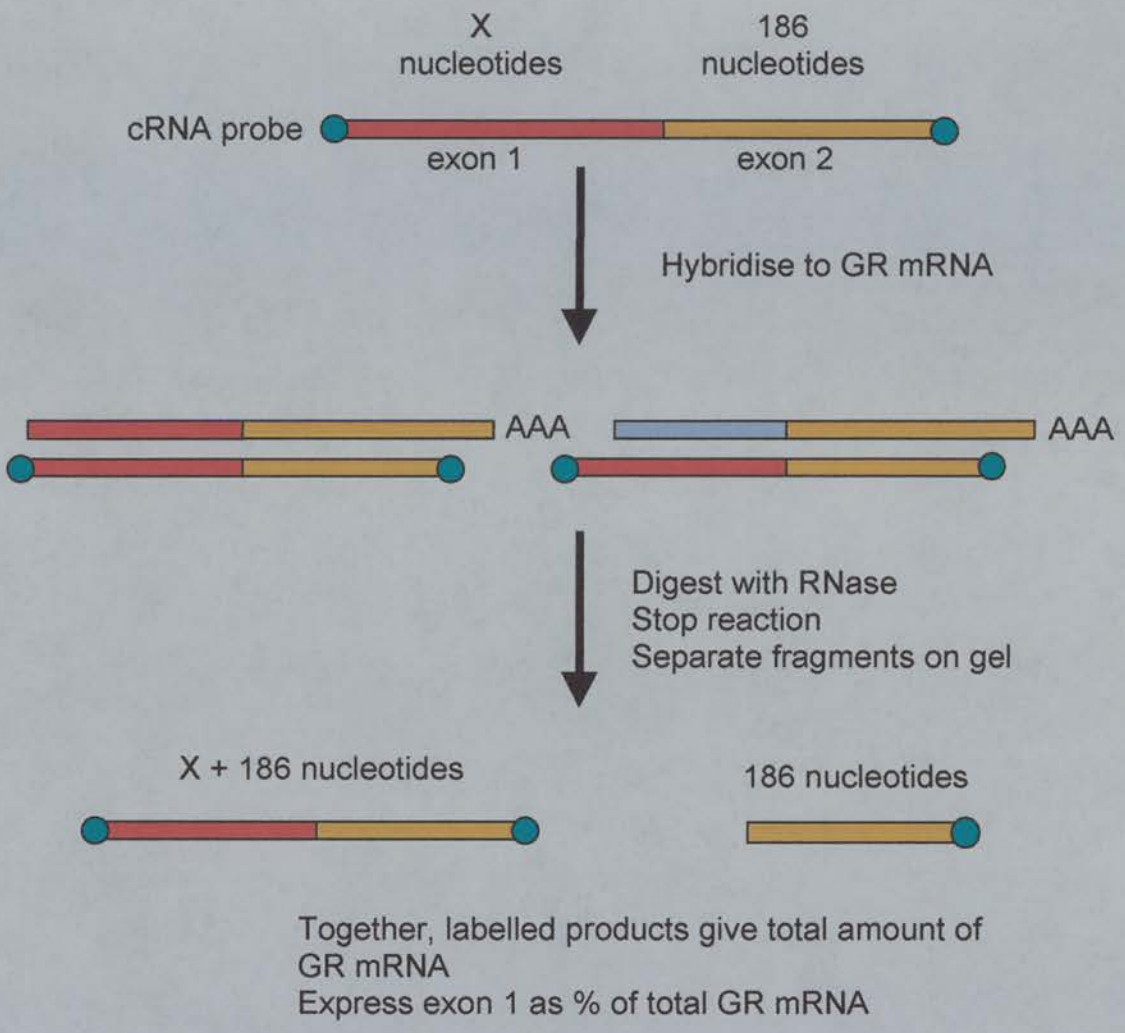


Figure 3.1: *Experimental design for RNase protection analysis*

A radiolabelled cRNA probe is incubated with total RNA isolated from the tissue of interest. 186 nucleotides, complementary to the 5' end of exon 2 can hybridise with all GR mRNAs. A region at the 3' end of the probe can also hybridise with the specific exon 1 to which it is complementary, if that variant GR mRNA is present in the sample. Regions of a probe molecule hybridised to a target mRNA are protected from degradation by RNase, and following RNase treatment can be resolved on a denaturing polyacrylamide gel. Probe molecules hybridising with GR mRNAs lacking the specific exon 1 complemented by the full-length probe give rise to protected fragments 186 nucleotides in length. Probe molecules hybridising with GR mRNAs containing the exon 1 of interest give rise to longer protected fragments of defined length (see Table 3.2). The sum of the intensities of these two fragments gives the total amount of GR mRNA in the sample; the amount of the specific exon 1 of interest can then be expressed as a percentage of total GR mRNAs.

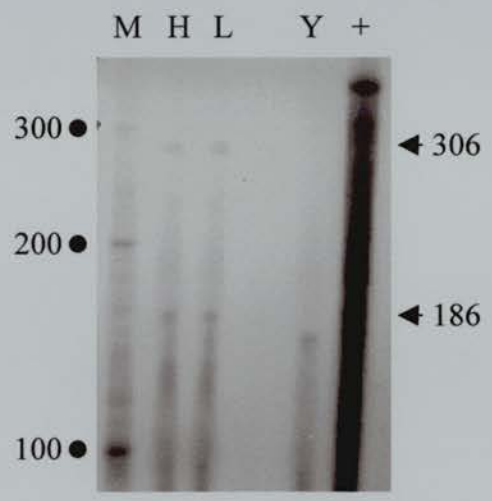


Figure 3.2: *RNase protection analysis of the abundance of exon 1_{10}-containing GR mRNAs in rat hippocampus and liver using the RPA II kit (Ambion)*

An RNase protection assay was carried out on 50μg total RNA from adult male rat hippocampus (H) and liver (L). Lanes marked Y contained yeast RNA, +, undigested probe and M, markers. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{10}, and the 306 nucleotide fragment protected by transcripts containing exon 1_{10} and exon 2.

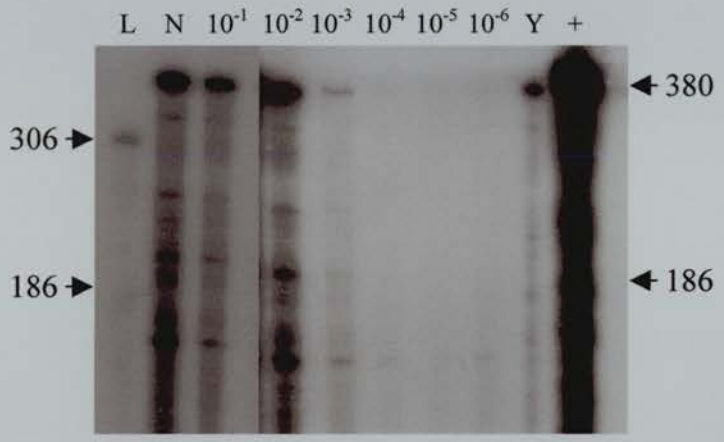


Figure 3.3: *RNase protection analysis using "sense" exon 1_{10}-containing GR RNA to establish which fragments represent exon 1_{10}-containing GR mRNA transcripts <u>in vivo</u>*

An RNase protection assay was carried out using the HybSpeed RPA kit (Ambion) with a [α-^{32}P]-GTP-labelled cRNA probe containing exons 1_{10} and 2. "Sense" exon 1_{10}-containing transcripts were synthesised from pJIM2 linearised with *Pvu*II with T7 phage RNA polymerase by the same method used to synthesise the radiolabelled cRNA probe (section *2.2.6.1*). "Sense" exon 1_{10}-containing transcripts were used in the assay at the dilutions indicated, ranging from neat (N) to a 10^{-6} dilution; 10µg yeast RNA was added to normalise samples. 50µg total RNA from adult male rat liver (L) was used as a control to confirm protection. Lanes marked Y contained yeast RNA only and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{10}, the 380 nucleotide fragment protected by synthetic "sense" exon 1_{10} transcripts and the 306 nucleotide fragment protected by transcripts containing exon 1_{10} and exon 2. Note that lanes L, N and 10^{-1} are from a shorter exposure (2d compared with 10d) of the same experiment.

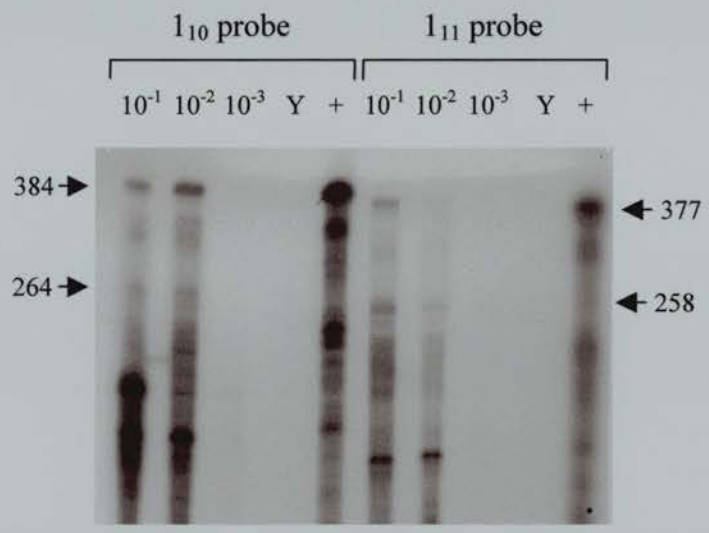


Figure 3.4: *RNase protection analysis to determine quantitative accuracy of the HybSpeed RPA kit*

An RNase protection assay was performed using exons 1_{10} and 1_{11}-containing cRNA probes against a mixture of "sense" transcripts containing exons 1_{10} and 1_{11}. "Sense" transcripts were synthesised using a large-scale *in vitro* transcription method (section *2.2.6.2*) and quantified using a Genequant (Pharmacia Biotech) spectrophotometer. A stock solution of 6μg/μl with respect to each "sense" transcript was prepared and diluted to give a 0.25ng/μl solution. This solution was diluted 10^{-1}, 10^{-2}, and 10^{-3}. 2μl of these dilutions were added to 10μg yeast RNA and used in the RNase protection assay. Lanes marked Y contained yeast RNA and +, undigested probe. Arrowheads indicate the sizes of protected fragments.

Figure 3.5: *RNase protection analysis to determine optimal hybridisation time for alternate exon 1-containing GR cRNA probes with the HybSpeed RPA kit*

RNase protection assays were performed using an exon 1_{11}-containing cRNA probe against a mixture of "sense" transcripts containing exons 1_{10} and 1_{11}. 10pg of "sense" transcript mixture (5pg each transcript) was used, with 10μg yeast RNA added to normalise. 50μg total RNA from adult male rat liver (L) was used as a control to confirm protection. Hybridisation times (in minutes) were varied as indicated in the figure. Lanes marked Y contained yeast RNA, +, undigested probe, and 10^{-3}, a 10^{-3} dilution of probe not subjected to the RNase protection procedure. Arrowheads indicate sizes of protected fragments.

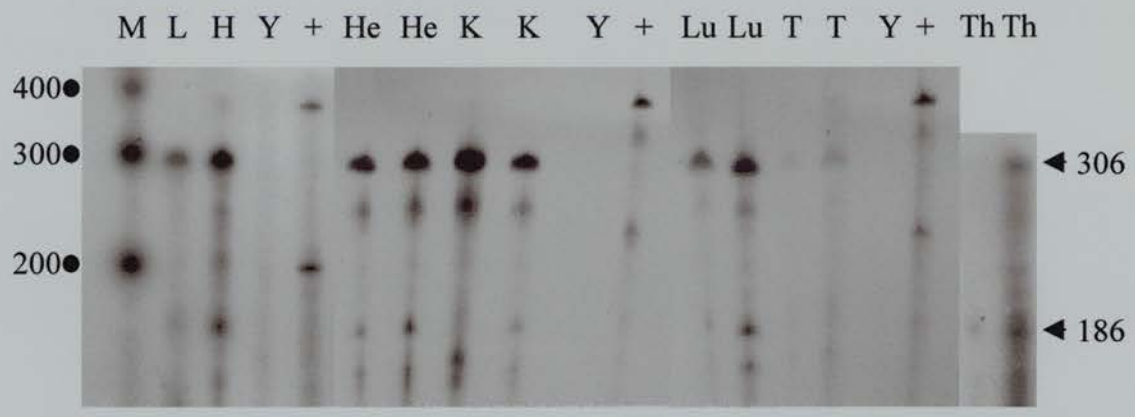


Figure 3.6: *RNase protection analysis of the abundance of exon 1_{10}-containing GR mRNA in various rat tissues*

RNase protection assays were carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L), heart (He), kidney (K), Lung (Lu), Testis (T) and thymus (Th). Lanes marked Y contained yeast RNA, +, undigested probe and M, markers. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{10}, and the 306 nucleotide fragment protected by transcripts containing exon 1_{10} and exon 2. The data presented is a composite of several experiments run under identical conditions.

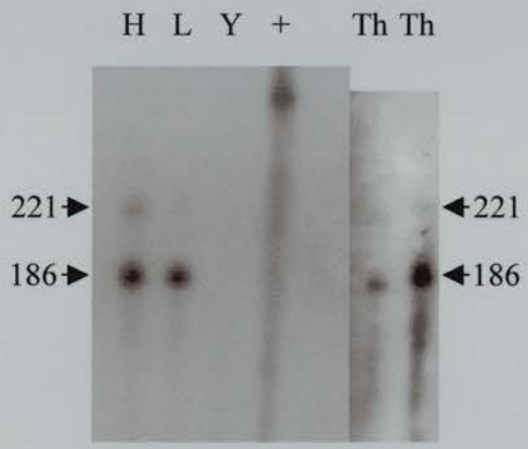


Figure 3.7: *RNase protection analysis of the abundance of exon 1_6-containing GR mRNA in rat liver, hippocampus and thymus*

RNase protection assays were carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L) and thymus (Th). Lanes marked Y contained yeast RNA, and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_6, and the 221 nucleotide fragment protected by transcripts containing exon 1_6 and exon 2. The data presented is a composite of two experiments run under identical conditions.

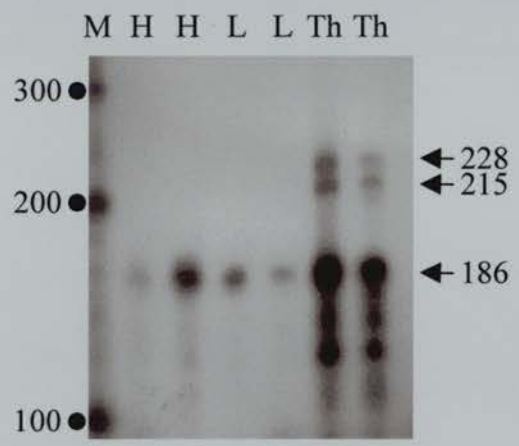


Figure 3.8: *RNase protection analysis of the abundance of exon 1_1-containing GR mRNA in rat liver, hippocampus and thymus*

An RNase protection assay was carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L) and thymus (Th). Lanes marked Y contained yeast RNA, and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_1, and the 228 nucleotide fragment protected by transcripts containing exon 1_1 and exon 2. Note that, for quantitation, the 215 nucleotide band (which probably also contains exon 1_1 spliced to exon 2) was included.

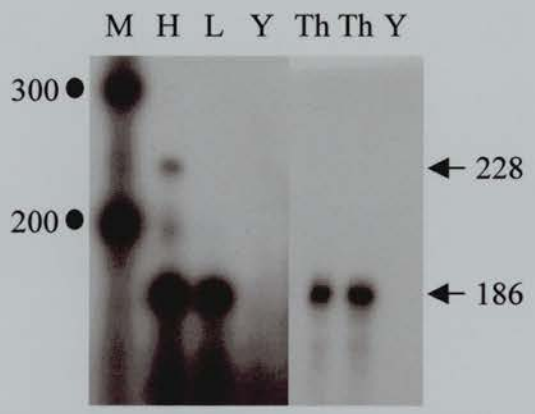


Figure 3.9: *RNase protection analysis of the abundance of exon 1_5-containing GR mRNA in rat liver, hippocampus and thymus*

RNase protection assays were carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L) and thymus (Th). Lanes marked Y contained yeast RNA, and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_5, and the 228 nucleotide fragment protected by transcripts containing exon 1_5 and exon 2. The data presented is a composite of two experiments run under identical conditions.

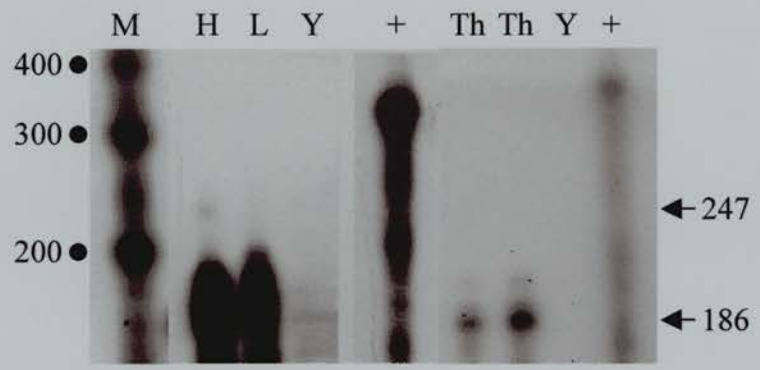


Figure 3.10: *RNase protection analysis of the abundance of exon 1_7-containing GR mRNA in rat liver, hippocampus and thymus*

An RNase protection assay was carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L) and thymus (Th). Lanes marked Y contained yeast RNA, and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_7, and the 247 nucleotide fragment protected by transcripts containing exon 1_7 and exon 2. Data presented is a composite of two experiments performed under identical conditions.

Figure 3.11: *RNase protection analysis of the abundance of exon 1_{11}-containing GR mRNA in rat liver, hippocampus and thymus*

An RNase protection assay was carried out on 50μg total RNA from adult male rat hippocampus (H), liver (L) and thymus (Th). Lanes marked Y contained yeast RNA, and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{11} and the 247 nucleotide fragment protected by transcripts containing exon 1_{11} and exon 2. Note that for quantitation an approximately 230 nucleotide band (which probably also contains exon 1_{11} spliced to exon 2) that was sometimes seen (not in this example) was included. Data presented is a composite of two experiments performed under identical conditions.

Exon	Hippocampus	Liver	Thymus
1_1	0	0	25.6%, 27.1%
1_2	0	0	0
1_4	0	0	0
1_5	8.1%, 7.4%	0	0
1_6	17.0%, 20.7%	10.0 ± 1.3% (6)	21.9%, 19.9%
1_7	8.0% ± 3.7% (3)	0	0
1_{10}	61.3%, 63.8%	74.8%, 77.5%	52.7%, 58.9%
1_{11}	12.5%, 7.9%	2.1%, 1.7%	0

Table 3.3: *Relative abundance of alternative exon 1-containing GR mRNA in rat hippocampus, liver and thymus*

Summary of RNase protection assays performed on total RNA from rat liver, hippocampus and thymus (see text for description of quantitation technique). In each case, the amount of exon 1 is expressed as a percentage of the total amount of GR mRNA in that particular tissue. Where individual values are shown, the results for each of two independent experiments are given. Where the experiment was carried out more than twice, values shown represent the mean ± SEM (n). Levels of exon 1_4- and exon 1_2-containing mRNA were below the limits of detection (~1%) in the three tissues examined in each of two experiments.

3.4 Discussion

RPAs have shown that of at least 12 alternate GR exons 1 identified in cDNA clones isolated from libraries (exon 1_6) or by 5'RACE-PCR, at least 6 of the alternate GR exons 1 are present *in vivo*: exons 1_1, 1_5, 1_6, 1_7, 1_{10} and 1_{11}. Other exon 1-containing mRNAs (identified by 5'-RACE PCR on hippocampus (exon 1_4) or thymus (exon 1_2)) (McCormick, J. A. et al. 2000) were not detected, presumably because they were below the limits of detection of the RPA. There are several other alternate exons 1 which have been identified (McCormick, J. A. et al. 2000) but which were not assayed: exons 1_3, 1_8 and 1_9 from hippocampus (McCormick, J. A. et al. 2000) and exon $1_{4.5}$ from liver (Lyons, V., personal communication). Exon 1_3-, 1_8- and 1_9-containing GR mRNAs are unlikely to be present at significant levels in hippocampus under basal conditions since they were poorly represented in the 5'-RACE PCR. Furthermore, the sum of the exon 1 variants of GR that were examined

was close to 100%, suggesting that the identified exon 1-containing GR mRNA transcripts probably account for the total of GR mRNA in hippocampus. Similarly, it is unlikely that any other alternate exon 1-containing transcripts will be of significance in thymus, since the total of those examined was close to 100%. In contrast only 88% of GR transcripts were accounted for in liver, suggesting either untested exon 1 variants are present or further novel variants exist.

In liver, hippocampus and thymus, as well as in heart, kidney, lung and testis, transcripts containing exon 1_{10} accounted for the majority of GR mRNAs (56%-87% of total GR mRNA). Exon 1_{10}-containing transcripts also account for the majority of GR mRNA transcripts in rat skeletal muscle (Cleasby, M., personal communication). Although 5'RACE-PCR is not a quantitative technique, it is worth noting that the number of clones containing exon 1_{10} obtained with this technique (55% in hepatoma cell line RNA, Jacobson, M. and Yamamoto, K.R., personal communication; 57% in hippocampus and 50% in thymus, Lyons, V. and Chapman, K.E., personal communication) support the finding that exon 1_{10} is the predominant exon 1 in GR mRNA in the rat. These data suggest that exon 1_{10}-containing GR mRNAs are expressed ubiquitously and may be derived from transcription initiated from a constitutively active promoter. GR mRNA transcripts containing exon 1_6 were also present in all 3 tissues examined (11-21% of total GR transcripts). Although their presence in other tissues remains to be determined, these data suggest that transcripts containing exon 1_6 may also be products of a constitutively active promoter. Both exons 1_{10} and 1_6 have been previously identified in rat; exon 1_{10} in liver GR mRNA (Gearing, K. L. et al. 1993) and exon 1_6 in the published rat GR cDNA sequence (Miesfeld, R. et al. 1986). In rat liver, 4 putative alternate exons 1 were identified by 5'-RACE PCR, but only one (exon 1_{10}) was detectable by RNase protection analysis on total liver RNA (Gearing, K. L. et al. 1993). Exon 1_6 was also identified, but was not detectable by RPA, possibly for the same reasons that made detection difficult in the studies presented here. Of the two remaining "exons 1" identified previously, one corresponded to genomic sequence immediately upstream of exon 2; the final one is contained *within* exon $1_{4.5}$ (Gearing, K. L. et al. 1993).

Exon 1_1 was present in 21% of GR mRNA in thymus, but was absent from liver and hippocampus. Exon 1_1 corresponds to exon 1A identified in mouse T-cell lines (Dieken, E. S. et al. 1990; Chen, F. H. et al. 1999a), (70% identity). Exon 1A-containing transcripts are absent from liver and brain (Strahle, U. et al. 1992). Previous work has shown that exon 1A is present in 50% of GR transcripts in mouse thymus, that the T-lymphoma cell lines S49 and WEHI-7 contain exon 1A, and exon 1A-containing transcripts are derived from a promoter limited to T-cells (Cole, T. J. et al. 1995a). This last conclusion is strengthened by the observation that a DNaseI hypersensitive site, situated over the promoter, is only detected in T-lymphocyte cell lines (Strahle, U. et al. 1992).

Hippocampal RNA exhibited the greatest heterogeneity in alternate exon 1 population with 5 different exons 1 accounting for close to 100% of GR transcripts. The contributions of exons 1_{10} and 1_6 have already been described and together make up 82% of total GR transcripts. There are significant levels of GR mRNA containing exons 1_5 and 1_7, which were undetectable in liver or thymus (these are detectable, however, by the more sensitive technique of RT-PCR (section *4.4*). Exon 1_5-containing mRNA transcripts accounted for 8% of GR mRNA in hippocampus but were not detected in thymus or liver despite being identified in a rat hepatoma cell line by 5'-RACE PCR (Jacobson, M. and Yamamoto, K.R., personal communication). Furthermore, exon 1_{11}, which was present at low levels in liver and absent from thymus, represented 10% of GR mRNA in hippocampus.

In mouse, 5 different promoters have been implicated in transcription of the GR gene, resulting in 5 alternate exons 1 spliced onto a common exon 2 (Strahle, U. et al. 1992; Chen, F. H. et al. 1999a) (see Figure 1.4). Exon 1A corresponds to exon 1_1 in rat; exon 1B corresponds to exon 1_6 and exon 1C to exon 1_{10} (Strahle, U. et al. 1992). Exons 1B and 1C are located in a large CpG island, highly conserved with the CpG island present in the rat GR gene (Chapter 4), and have been shown by PCR and RPAs to be ubiquitous (Dieken, E. S. et al. 1990). Recently, two additional exons 1 have been identified in mouse (Chen, F. H. et al. 1999a). Exon 1D corresponds to the rat exon 1_5 and 1E to the rat exon 1_{11} (they match exactly at their

3' ends). Exon 1D-containing GR mRNAs were found by RNase protection analysis to be ubiquitous, with high expression in liver, and exon 1E-containing transcripts were expressed highly in liver and muscle, with lower levels of expression in kidney, spleen, brain and pancreas (Chen, F. H. et al. 1999a). The reliability of these data is questionable, however, since exon 1A-containing GR mRNAs were found to be expressed at high levels in T lymphoma cell line S-49, liver and muscle, and present in all other tissues examined except thymus. The authors discuss the data of Strahle et al., but do not offer an interpretation of their own data, which directly contradicts that of Strahle et al, and also disagrees with the data presented here for the homologous rat exon 1.

Having established the existence of alternate exons 1 with tissue-specific abundances, the question remains as to their biological relevance. It has been suggested that regulation of GR by promoter 1A is linked to the role glucocorticoids play in T-lymphocyte apoptosis and maturation (Cole, T. J. et al. 1995a; Chen, F. H. et al. 1999a; Chen, F. H. et al. 1999b). It is, however, debatable as to whether exon 1_1 really is T-cell-specific. RPAs performed in our laboratory have shown that exon 1_1 is equally expressed in a similar proportion of GR mRNA (~20-25%) in thymic epithelium, thymocytes and mature $CD4^+$ T-cells (Dammermann, A., personal communication). These data do not necessarily mean that promoter 1A (presuming exon 1_1-containing transcripts are derived from a homologous rat promoter) is not important in regulation of GR in the immune system, but suggest the role of promoter 1A is more general in immune tissues. Further experiments on other immune tissues such as lymph nodes and peripheral blood mononuclear cells would help to resolve these issues. Indeed, experiments described in Chapter 5 indicate that exon 1_1 is detectable at a level of ~1% of GR mRNA in liver. This exon 1_1-containing population of mRNAs is likely to be from lymphocytes present in the relatively large volume of blood perfusing the liver.

Glucocorticoids are widely used in the treatment of leukaemias and lymphomas (Csoka, M. et al. 1997) and their mode of action in these conditions has been attributed to activation of a membrane-associated GR (Gametchu, B. et al. 1994;

Sackey, F. N. A. et al. 1997). Recently, it has been suggested that exon 1A may be responsible for the expression of the membrane-associated GR (Chen, F. H. et al. 1999a; Chen, F. H. et al. 1999b). The membrane-associated form of GR derived from exon 1A-containing transcripts was found to be of higher molecular weight than GR, though it is unclear how exon 1A could contribute to protein targeting. It is unclear how a membrane form of GR could signal following binding of ligand, and although a membrane-associated receptor has been reported for MR, it is the product of an independent gene and is structurally totally different to MR (Wehling, M. 1997; Schmidt, B. M. W. et al. 1998). Most importantly, the in-frame stop codon at the beginning of exon 2 means that the GR protein derived form exon 1A-containing transcripts will be identical to all other GR proteins.

In human, only a single exon 1 has been described in the literature (Zong, J. et al. 1990) corresponding to rat exon 1_{10}. However, recent work from this laboratory and others has identified additional exon 1 sequences present in human GR mRNA including one homologous to exon 1_6 (Lyons, V. and Chapman, K.E., personal communication; Breslin, M. B. et al. 1998) and an additional human exon 1 (Breslin, M. B. et al. 1998). RPAs have shown that in humans, exons 1 homologous to rat exons 1_{10} and 1_6 are present in the majority of GR mRNA (in approximately equal amounts) in human liver and peripheral blood mononuclear cells (Nelson, R., personal communication). The presence of alternate exons 1 in GR mRNA may therefore be a universal characteristic in mammals.

The hippocampus consists of 5 major subregions: dentate gyrus, and the CA1, CA2, CA3 and CA4 pyramidal cell fields. The distribution of alternate exon 1-containing GR mRNA transcripts *within* the hippocampus has been examined in our laboratory by *in situ* mRNA hybridisation analysis. Similar to distribution seen for "pan" GR probes (encoding part of exon 2 or exons 6-9), exon 1_{10} (present in 63% of GR transcripts in whole hippocampus) is highly expressed in the dentate gyrus, with intermediate expression in CA1 and much lower expression in CA3 and CA4 (McCormick, J. A. et al. 2000). In contrast, GR mRNA transcripts containing exons 1_5, 1_7, or 1_{11} showed a more homogenous distribution, although in each case

expression was highest in the dentate gyrus and CA1 region of hippocampus. A possible explanation for the great variety of alternate exons 1 in hippocampus compared with other tissues is that the hippocampus is a complex and highly plastic region of the brain, exhibiting high sensitivity to both endocrine and neurochemical signals (Sapolsky, R. M. et al. 1984a). Hippocampal GR can vary greatly depending on genotype. For example, Brattleboro rats, congenitally deficient in vasopressin, have reduced GR in hippocampus but normal levels in the rest of the brain (Veldhuis, H. D. et al. 1982). Furthermore, hippocampal GR can be both upregulated and downregulated by various manipulations, including neonatal handling which permanently increases hippocampal GR (section *1.3*). There is evidence that GR mRNA may exhibit similar 5'-heterogeneity in mouse brain, though only whole brain has been examined and not hippocampus specifically. The previously published mouse exons 1B and 1C have been found in GR mRNA isolated from whole mouse brain (Strahle, U. et al. 1992) and RT-PCR from this laboratory suggests that homologues to rat GR exons 1_4, 1_5 and 1_{11} also exist in mouse brain (Freeman, A. and Whiteley, L., personal communication). In combination with data suggesting the existence of multiple human GR exons 1 (Lyons, V., Nelson, R. and Chapman, K.E., personal communication) these data add further weight to the hypothesis that regulation of the GR gene by multiple promoters is universal, as one might predict.

There are many examples of genes whose products contain multiple 5'-untranslated regions. The existence of these alternate exons 1 is usually attributed to transcription from alternative promoters. Alternative promoters are commonly used to express the same gene product at different stages of development or in different cell types. MR, which like GR, is highly expressed in hippocampus is also derived from mRNAs exhibiting 5'-heterogeneity, with 3 alternate exons 1 (Kwak, S. P. et al. 1993). The 3 variant MR mRNAs, differ in relative abundance in hippocampus and kidney (Kwak, S. P. et al. 1993). Furthermore, relative abundance varies within subregions of the hippocampus in adult rats, (Kwak, S. P. et al. 1993) and also within hippocampal subregions, during development and in response to adrenalectomy (Vazquez, D. M. et al. 1998). At least 5 alternate exons 1 for the human oestrogen receptor α exist

and these differ in their relative abundances between tissues and between sexes (Flouriot, G. et al. 1998). Other genes whose products contain multiple 5'-UTRs indicative of alternate promoter usage include ovine growth hormone receptor (Adams, T. E. 1995), γ-glutamyl transpeptidase (Sepulveda, A. R. et al. 1994) and insulin-like growth factor (Holthuizen, P. et al. 1990) genes. In common with many of these genes, the GR gene is expressed in a wide variety of tissues at varying levels, and is developmentally and differentially regulated.

The use of multiple and tissue-specific promoters provides a flexible means of regulating levels of GR, which are widely but variably expressed. There are several ways by which GR levels could be subtly altered, with biologically significant consequences, by the production of mRNAs with alternate exons 1 from multiple promoters. Firstly, if multiple promoters do indeed govern transcription of alternate rat GR exons 1, activity of each promoter will depend on the transcription factors present in a cell/tissue as well as the signal transduction pathways activated within the cell. Additionally, there are two means by which alternate exons 1 could regulate amounts of GR at the level of translation. The alternate exon 1-containing GR mRNA transcripts may vary in their translational efficiency, due to differences in the degree of secondary structure they contain. Gene products containing alternate 5'-untranslated regions with low free energy are inefficiently translated (Kozak, M. 1991). Since the alternate GR exons 1 are GC rich there is a high probability that translationally unfavourable secondary structures will exist, with some exons 1 possessing secondary structures more unfavourable than others. Sequence analysis of the CpG island revealed the existence of an sORF lying within exon 1_{10}, located at –2140 to –2119, and two starting in exon 1_4 (at –4219 and –4708) that terminate at –12 in exon 2. Similar sORFs in, for example, GCN4 or the BCR/ABL oncogene mRNAs have been shown to be involved in the translational control of their expression (Mueller, P. P. 1986; Muller, A. J. et al. 1989). However, since only a few sORFs were identified in the rat GR promoter region, in the most commonly found exon 1 in GR mRNAs (exon 1_{10}), and in the minor variant exon 1_4 sequence, it is unlikely that this is an important control mechanism. Finally, there may be differences in the stabilities of alternate exon 1-containing mRNAs, resulting in

lower usage of some exon 1-containing transcripts as translational templates before degradation.

GR mRNA transcripts exhibit extensive 5'-heterogeneity in their 5'-untranslated regions. At least one of these classes of exons 1 (1_{10}) is ubiquitous, present in the majority of GR mRNAs and may result from transcription from a constitutively active promoter; other exons 1 exhibit tissue-specific distributions (with the probable exception of exon 1_6). Since it has been shown in mouse that the GR gene is regulated by multiple promoters producing mRNAs containing alternate 5'-untranslated regions and this phenomenon has been described for many other genes, it is likely that the rat GR gene is regulated in the same manner. A functional analysis of the putative promoter regions is described in Chapter 4.

Chapter 4: *In vitro* regulation of GR gene transcription

4.1 Introduction

GR mRNA shows great variation in abundance between tissues. For example, levels are very low in testis, high in lung and moderate in liver and brain (Kalinyak, J. E. et al. 1987; Whorwood, C. B. et al. 1992). Adrenalectomy increases GR mRNA in brain and kidney, but does not alter liver and lung GR mRNA; in contrast dexamethasone treatment decreases GR mRNA by 40-60% in a wide variety of tissues (Kalinyak, J. E. et al. 1987). Dexamethasone-induced downregulation also occurs in cultured cells, including HeLa S_3 (Cidlowski, J. A. et al. 1981) and AtT-20 mouse pituitary tumour cells (Svec, F. et al. 1981). In adult rats, administration of corticosterone or chronic stress down-regulate GR mRNA and protein levels in the hippocampus in a site-specific manner (Sapolsky, R. M. et al. 1984b; Sapolsky, R. M. et al. 1985). Down-regulation of hippocampal GR mRNA occurs primarily at the level of transcription of the GR gene (Rosewicz, S. et al. 1988; Hoeck, W. et al. 1989), though post-transcriptional events may also be important (Dong, Y. et al. 1988). Hippocampal GR mRNA can also be upregulated, as observed when neonatal rats are handled (Sarrieau, A. et al. 1988; Meaney, M. J. et al. 1988; Meaney, M. J. et al. 1996).

The data presented in Chapter 3 show that GR mRNA species differing in their untranslated exons 1 exhibit tissue-specific differences in distribution. Sequence analysis of 5kb of DNA flanking the 5' end of exon 2 of the rat GR gene revealed that most (10) exons 1 lie within a CpG island (68% CG, with a CG/GC ratio of ≥0.8 between –1620 and –4520 relative to the translation start at +1) highly conserved between rat, mouse and human. CpG islands are frequently associated with multiple transcription initiation sites, resulting in transcripts with alternate exons 1 spliced at the same 3' splice donor site onto exon 2 (Koller, E. et al. 1991; Ye, K. et al. 1993). It is also likely that the exons 1 upstream of the CpG island are differentially regulated, since at least one (exon 1_1) is present in GR mRNAs in thymus but absent for hippocampus and liver (section *3.3.3*). Thus, the tissue-specific differences in alternate GR exon 1-

containing mRNAs may reflect tissue-specific promoter usage both within the CpG island and further upstream, providing a mechanism for the variation in levels of GR mRNAs between. Furthermore, the existence of multiple promoters may explain how adrenalectomy and dexamethasone induce changes in GR mRNA levels, the degrees of which vary between tissues (Kalinyak, J. E. et al. 1987), and even within subregions of the hippocampus (Sapolsky, R. M. et al. 1984a; Sapolsky, R. M. et al. 1985).

The focus of the work described in this chapter was to begin to dissect the means by which the rat GR gene is transcriptionally regulated. Initial studies to examine transcriptional regulation of the GR gene involved 5'-RACE PCR, which led to the isolation of a large number of exon 1_{10}-containing (approximately 60) clones ((McCormick, J. A. et al. 2000) and Table 5.2). 5'-RACE PCR is one procedure for mapping the 5' of mRNA, and hence the transcription initiation point, though ideally more than one approach should be used to map transcription starts to avoid erroneous mapping due to artefacts. Therefore, RNase protection analysis was used to determine transcription initiation points for exon 1_{10}. To determine whether promoter activity is associated with alternate exons 1, a series of genomic constructs was made and used in transient transfection assays. Since tissue-specific differences in the relative abundances of alternate GR exons 1 were previously observed (Table 3.3), constructs were tested in relevant cell lines. Some of these constructs were then cotransfected with expression plasmids encoding various transcription factors predicted to bind to sites within the CpG island in the GR promoter region to identify possible regulators of GR promoter activity. The effects of dexamethasone on promoter activity were also studied to assess whether the GR promoter is autoregulated.

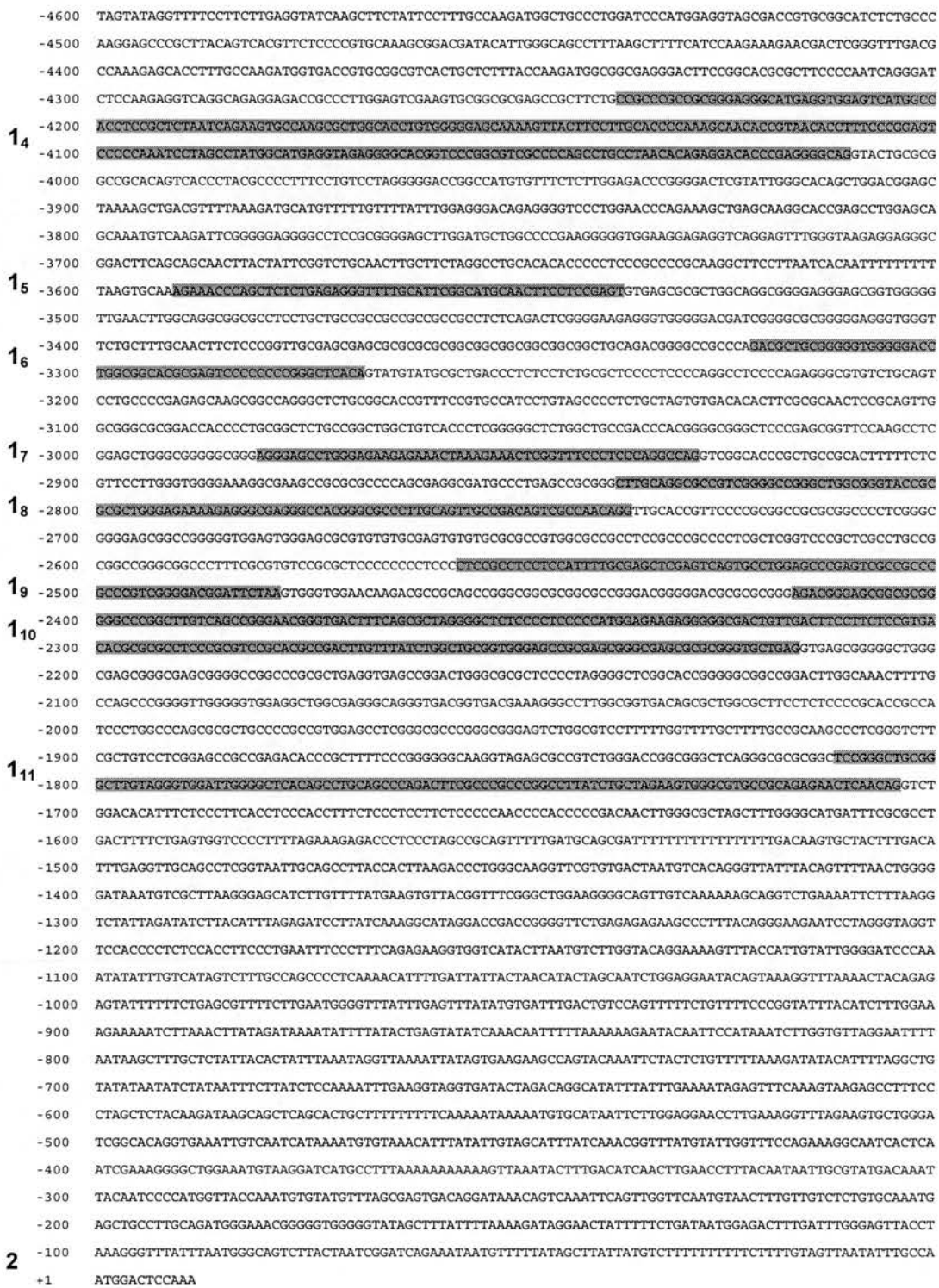


Figure 4.1: *Sequence of 5'-DNA flanking exon 2 of the rat GR gene*

The legend for this figure is given on page 117.

Figure 4.1: *Sequence of 5'-DNA flanking exon 2 of the rat GR gene*

Numbering is with respect to the translation start, at +1. Shaded regions indicate exon 1 sequences found in 5'-RACE PCR clones amplified from hippocampal GR mRNA. In the case of exon 1_6 (which was not represented among the 5'-RACE PCR clones), the bold nucleotides are those present in the published rat cDNA sequence (Miesfeld, R. 1986). Note that exons 1_9 and 1_{10} have since been shown to overlap (section 4.3.1) The start of exon 2 is at –13. Adapted from (McCormick, J. A.et al. 2000).

4.2 Experimental design

The region spanning the entire CpG island in the putative GR gene promoter region used to generate the constructs described in this chapter is shown in Figure 4.1.

4.2.1 Mapping of Exon 1_{10} transcription start points

To determine the positions of transcription starts for exon 1_{10}, RNase protection analysis was employed. A cRNA probe was synthesised from pVL163, a plasmid containing genomic sequence extending 631bp 5' of the 3' splice donor site in exon1_{10}, into exon 1_8 (Figure 4.2). This probe was used in an RNase protection assay as described in Chapter 2, hybridising for 10min against 50μg rat liver total RNA. To provide markers to estimate the sizes of any protected fragments and hence the 5' extent and transcription initiation sites of exon 1_{10}-containing transcripts, pVL163 was sequenced with a primer complementary to the T7 RNA polymerase promoter present in the plasmid.

Attempts to map transcription starts by primer extension proved unsuccessful: control experiments using yeast tRNA revealed the same pattern of extension products as liver RNA, suggesting a problem with primer design (data not shown). Two different primers gave the same data, suggesting the GC-rich nature of the region of interest was interfering with the experiments.

4.2.2 Transfection studies

A series of genomic constructs was made by subcloning fragments of pVL154 into pGL3-BM (pGL3-Basic, containing a modified polylinker, described in *2.1.10.3*). The constructs effectively formed a 3' deletion series of the rat GR promoter fused to luciferase. The 5' end (5' of exon 1_4 and the CpG island) was invariant; the 3' extent of the constructs was variable (Figure 4.4). P2 was fused to luciferase within exon 2; the activity of P2 therefore reflects the activity of the whole promoter region since all transcripts initiated can be spliced to exon 2, resulting in luciferase gene transcription (Figure 4.4). P2 (rev) contained the same sequence as P2 but was fused to the luciferase gene in the reverse (3' to 5') orientation. Luciferase activity detected using other constructs reflects activity of any promoter immediately 5' of the exon 1 fused to the luciferase gene. Although other promoters on the same genomic fragment may be active, transcripts initiated at more 5' putative promoters are not transcriptionally fused to luciferase (there is no 3' splice acceptor) and will not, therefore result in luciferase activity. Cell lines used were B103 (rat neuroblastoma), C6 (rat glioma) and HepG2 (human hepatoma). A limited number of transfections were carried out with GH_3 (rat pituitary tumour) cells. Apart from GH_3 cells, which were transfected by electroporation (section *2.2.10.2*), all cells were transfected by the calcium phosphate precipitation method (section *2.2.10.3*). Note that, for all transfections, DNA solutions for each dish of cells was prepared independently (representing an n value of one). For experiments involving multiple groups, ANOVA analysis was performed, followed by a *post-hoc* Fisher test with p set to <0.05. For other experiments Student t tests for were performed with p set to <0.05.

4.3 Results

4.3.1 Mapping of Exon 1_{10} transcription start points

RNase protection analysis of total liver RNA using a cRNA synthesised from the genomic clone pVL163 resulted in a large number of protected fragments (Figure 4.2).

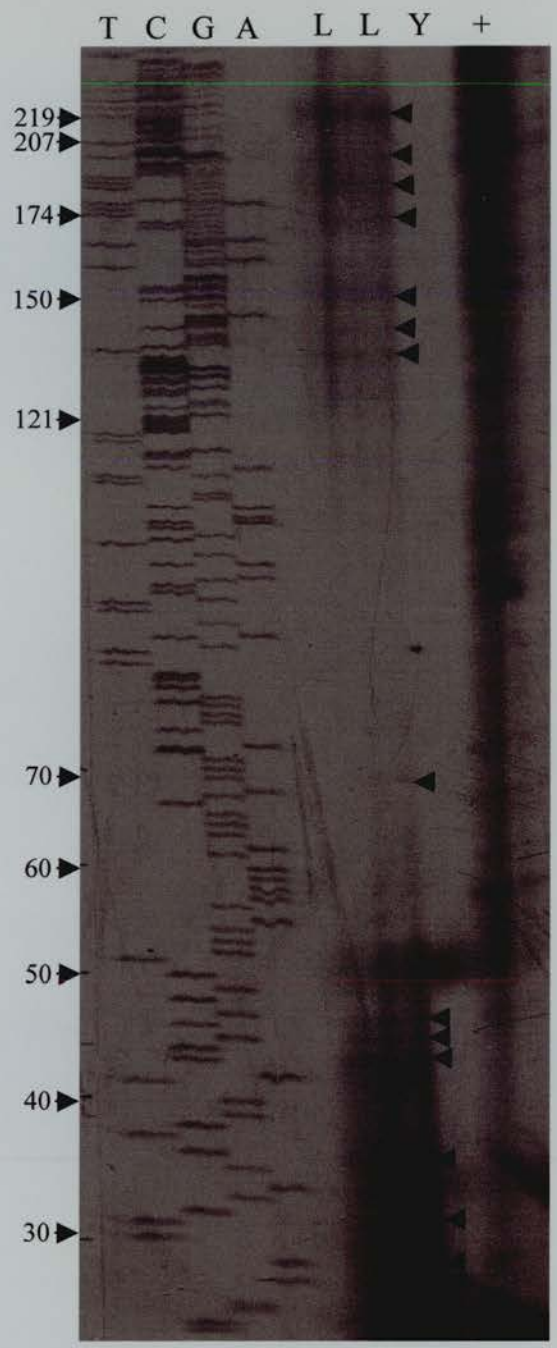


Figure 4.2: *RNase protection analysis reveals considerable heterogeneity in transcription starts for exon 1_{10}-containing GR mRNAs in liver*

An RNase protection assay was carried out on 50μg total RNA from adult male rat liver (L). The template used to generate the cRNA probe (pVL163) extended 631bp upstream of the 3' splice donor site in exon 1_{10}-containing GR mRNA. Lanes marked Y contained yeast RNA, +, undigested probe. A sequencing reaction of pVL163 was used as size markers (T, C, G and A). Arrowheads indicate protected fragments; these were mapped onto exon 1_{10} (Figures 4.3 and 4.13).

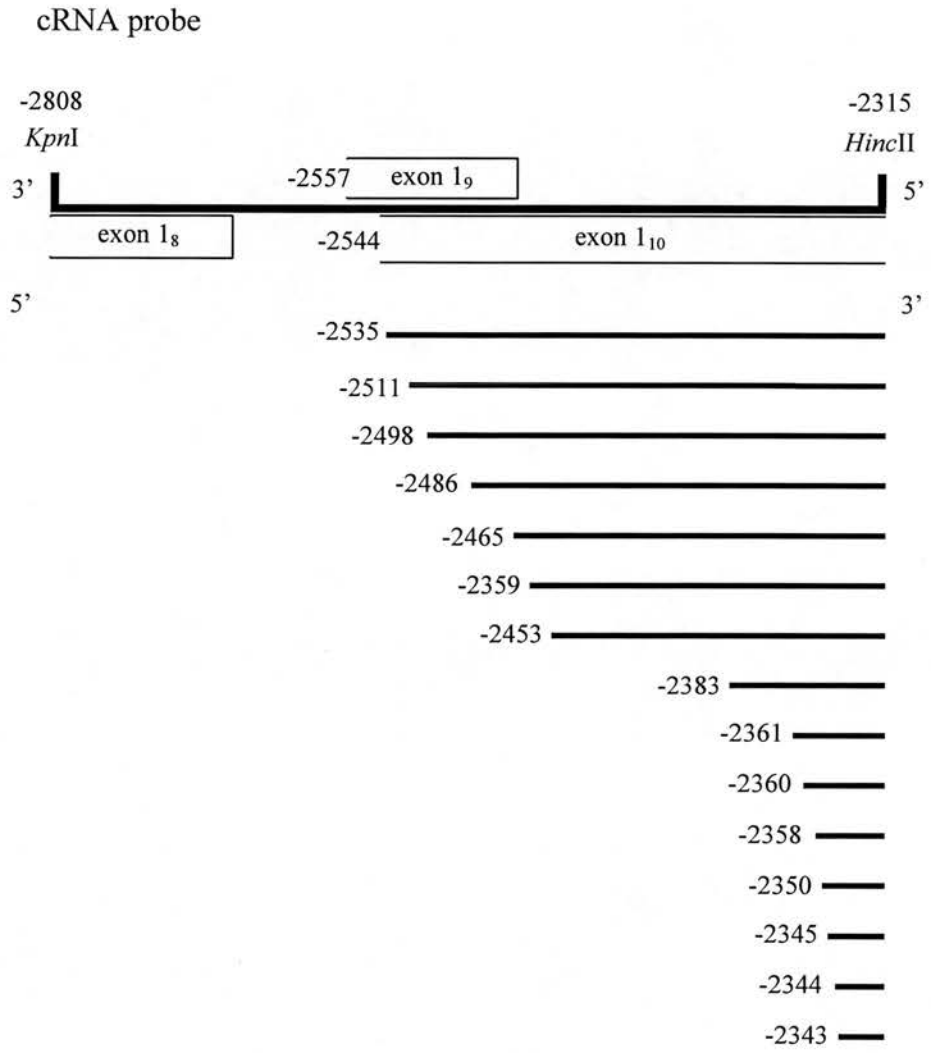


Figure 4.3: *Mapping of fragments detected by RNase protection analysis onto the GR gene*

The protected fragments detected by RNase protection analysis (Figure 4.2) using a cRNA probe synthesised from pVL163 were mapped onto exon 1_{10}. The cRNA extended from the *Hinc*II site to the *Kpn*I sites marked. The positions of exon 1_8, 1_9 and 1_{10} are marked by boxes. Closed ends indicate the known 3' ends of exons 1_8 and 1_9; open ends indicate either the most upstream transcription start known (at –2557 for exon 1_9 and at –2544 for exon 1_{10}), or the fact that the 5' and 3' ends of the probe do not extend into regions known to be contained in exons 1_8 and 1_{10}. Protected probe fragments are marked 5' and 3' to reflect the corresponding stretch of mRNA.

The sizes of protected fragments were 220, 196, 183, 171, 150, 144, 138, 70, 46, 45, 43, 35, 30, 29 and 28 nucleotides. The most prominent protected fragments were those ranging in size from 28 to 46 nucleotides and the one at 220 nucleotides. All fragments were mapped onto exon 1_{10} in relation to the 3' end of the *Hinc*II restriction site which lies at the 5' end of the cRNA probe (Figure 4.3 and summarized in Figure 4.13). This analysis revealed considerable heterogeneity in the 5' ends of exon 1_{10}-containing mRNAs.

4.3.2 GR CpG island constructs exhibit cell line-specific promoter activity

Transient transfection studies using a series of deletion constructs spanning the GR gene CpG island (Figure 4.4) showed that sequences within this region were able to drive luciferase gene transcription (Figure 4.5). Activities, however, varied considerably both within and between cell line. P2, which spans the entire CpG island (-4572/-9) and is fused to luciferase within exon 2, showed the highest activity in all cell lines in which it was tested (Figure 4.5). When compared to promoterless vector, P2 activity was approximately 20 fold higher than vector in B103 and C6 cells; in HepG2 it was approximately 55 fold higher. The same fragment in the reverse orientation with respect to luciferase had no significant activity in any cell line (Figure 4.5). To make comparisons between cell lines easier, activities of constructs were expressed relative to P2 activity, nominally set at 100%. The most active construct in HepG2 cells after P2 was $P1_{9/10}$; activity of this construct was also considerable in both B103 and C6 cells (Figure 4.5). $P1_{10}$ had significant activity in B103 and HepG2 cells, but in C6 cells was not significantly different to vector (Figure 4.5). The $P1_7$ construct showed the highest activity of any individual promoter in B103 and C6 cells; this construct showed lower but significant activity in HepG2 cells (Figure 4.5). $P1_6$ and $P1_8$ showed significant activity in all cell lines (Figure 4.5). $P1_{11}$ activity was only significant in HepG2 cells (Figure 4.5). Activity of $P1_5$ was not significant in C6 or HepG2 cells and was significant but very low in B103 cells (Figure 4.5). H3, fused to luciferase upstream of exon 2 but not within an identified exon 1 was significantly active in B103 and HepG2 cells but was not significantly active in C6 cells. Surprisingly, a region containing no

identified exon 1 but fused to luciferase within exon 2 (P0 construct) also exhibited substantial activity in all cell lines (Figure 4.5).

4.3.3 A $P1_{9/10}$ construct exhibits promoter activity in a pituitary tumour line

Transient transfection of GH_3 cells proved difficult. The calcium phosphate precipitation is ideally suited to rapidly dividing cells: GH_3 cells reached 50% confluence after 10 days and tests confirmed that this technique was unsuitable for these cells. Experiments using Lipofectin (Life Technologies) also proved unsuccessful so cells were transfected by electroporation. $P1_8$, which was active to varying degrees in B103, C6 and HepG2 cells had no activity in GH_3 cells (Figure 4.6). $P1_{9/10}$, the most active construct other than P2 in HepG2 cells, had significant activity in GH_3 cells, with activity 5 fold greater than promoterless vector (Figure 4.6). No other constructs were available for testing when these initial transfections were performed.

4.3.4 Overexpression of HNF-1α, C/EBPα or C/EBPβ does not increase P2 activity in HepG2 cells

The GR gene contains putative binding sites for HNF-1α, C/EBPα and C/EBPβ. In order to test if these transcription factors could potentially regulate GR gene transcription, cotransfections of P2 with plasmids encoding candidate transcriptional regulators of GR were performed in HepG2 cells. None of these transcription factors increased P2 activity significantly (Figure 4.7). As a positive control, a plasmid encoding 11-β hydroxysteroid dehydrogenase (pVL108) was cotransfected with C/EBPα, resulting in induction (approximately 20-fold) of pVL108 activity, as expected (Williams, L. J. S. et al. 2000) (Figure 4.7).

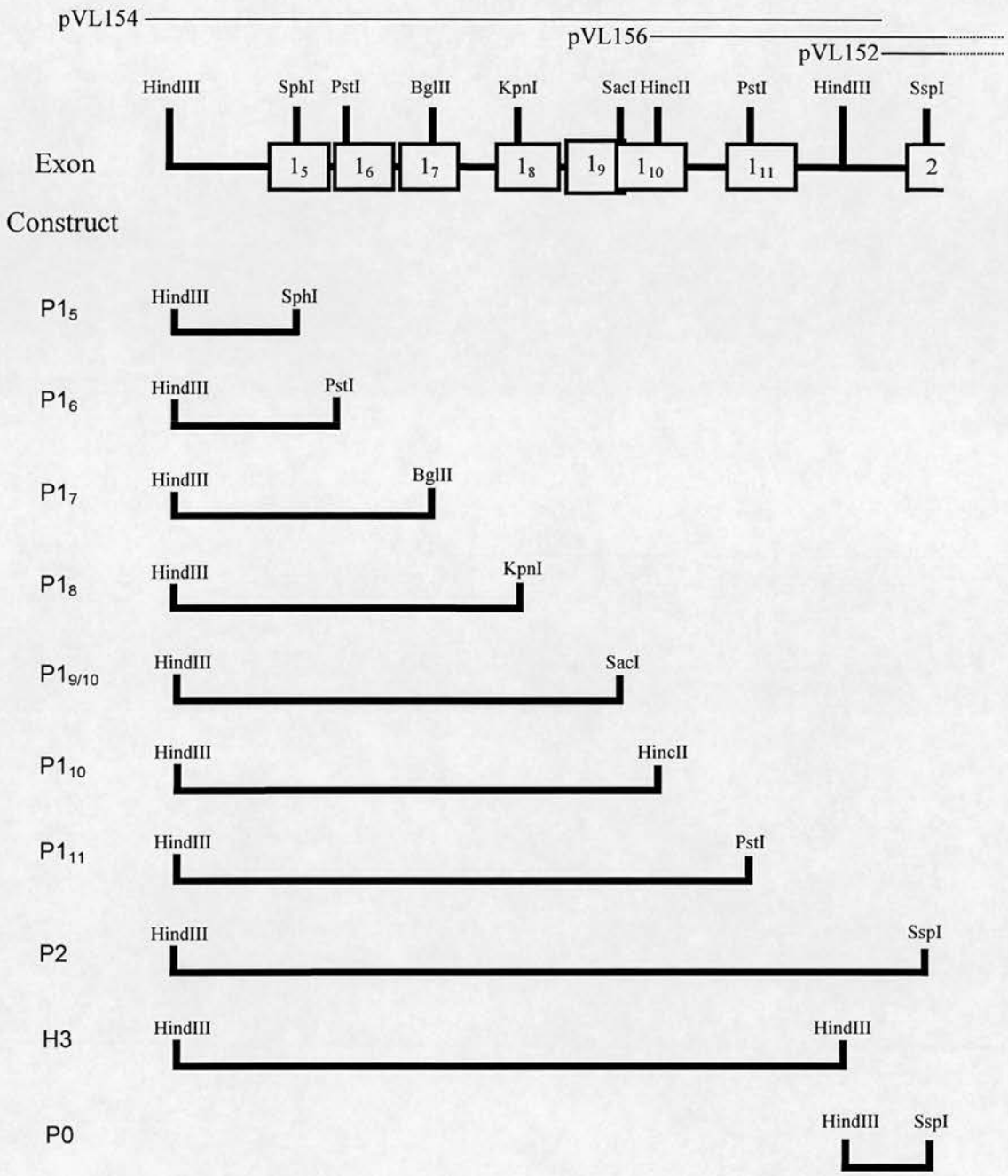


Figure 4.4: *Design of transfection constructs*

To determine whether promoter activity was associated with alternate GR exons 1, a series of constructs in which rat genomic DNA fragments were fused to a luciferase reporter gene (section *2.1.10.3*), were used in transient transfection assays. All constructs with the exception of P0 (which had a 5' end downstream of exon 1_{11}) had the same 5' end but differed in their 3' ends. The 3' end of P2 was fused to luciferase within exon 2 while other constructs were fused within known exons 1, or in the case of H3 and P0 at sites not within any known exons 1. Genomic DNA present in the parent plasmids used to generate the constructs (pVL152, pVL154 and pVL156) is also indicated.

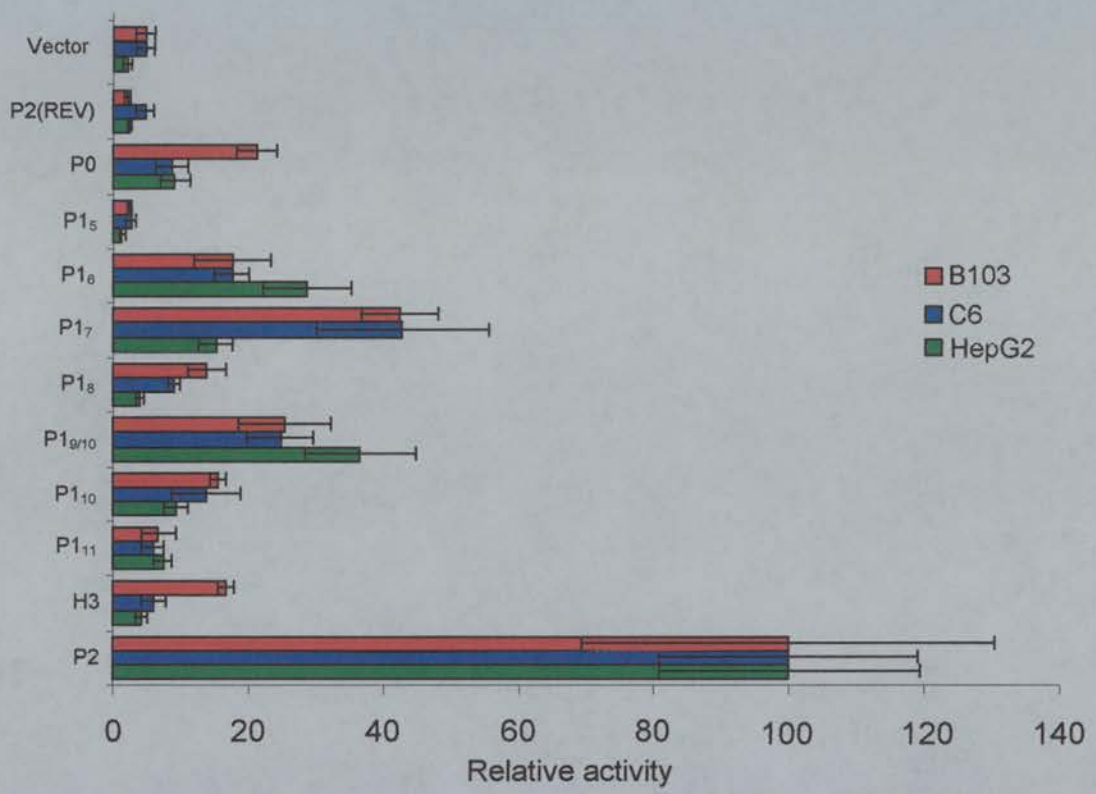


Figure 4.5: *Cell line-specific activity of GR gene constructs*

Transient transfection analysis of regions of the GR gene was carried out in HepG2 (human hepatoma), C6 (rat glioma) and B103 (rat neuroblastoma) cells. Activity of P2 (spanning the whole CpG island, fused to luciferase within exon 2) was nominally set at 100% for each cell line and activity of the other constructs expressed relative to this value. n=6-9 (2 independent plasmid DNA preparations), means ± SEM. Two-way ANOVA analysis revealed significant differences in activities between constructs ($F_{10,2,20}$=35, p=0.0001) but no significant differences in activities between cell lines (see text for details of differences).

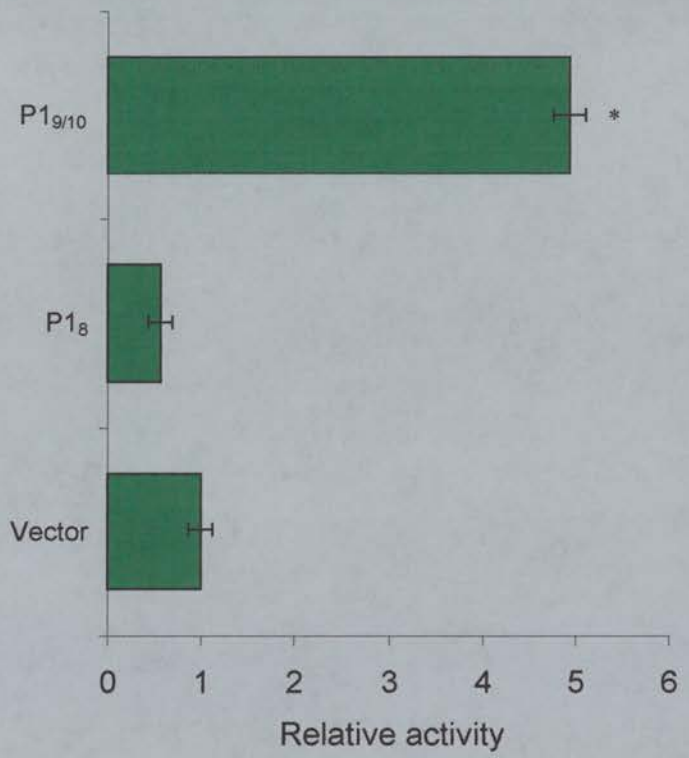


Figure 4.6: *$P1_{9/10}$ exhibits promoter activity in GH_3 (rat pituitary tumour) cells*

Transient transfection analysis of $P1_8$ and $P1_{9/10}$ constructs was carried out in GH_3 cells by electroporation. Activity was expressed relative to vector (PGL3-BM). n=3, means ± SEM. One-way ANOVA analysis revealed significant differences between groups ($F_{2,6}$=266, p=0.0001). * $p<0.05$, *post-hoc* Fisher PLSD test.

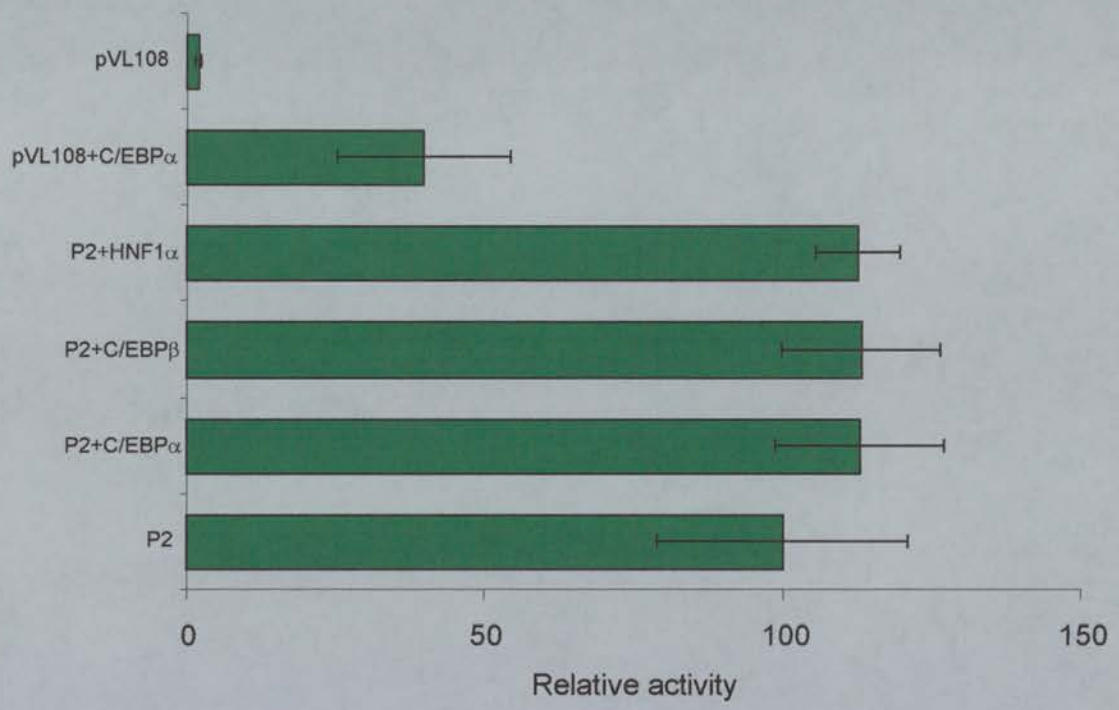


Figure 4.7: *Overexpression of HNF-1α, C/EBPα or C/EBPβ does not increase P2 activity in HepG2 cells*

The effects of overexpression of HNF-1α, C/EBPα and C/EBPβ on P2 activity in HepG2 cells were determined by transient transfection analysis. Activity of P2 was nominally set at 100% and activity of P2 cotransfected with transcription factors expressed relative to this value. The effect of C/EBPα on pVL108 activity (a construct containing the 11β-hydroxysteroid dehydrogenase I promoter region fused to luciferase) was used as a positive control. n=3, means ± SEM. One-way ANOVA analysis revealed no significant differences between groups.

4.3.5 Overexpression of NGFI-A, HNF-1α, WT-1 and C/EBPα does not increase $P1_7$ activity in B103 cells but HNF-1α overexpression leads to a small decrease in P2 activity

NGFI-A has been implicated in the increase in hippocampal GR following neonatal handling, and handling specifically increases exon 1_7-containing GR mRNA levels (McCormick, J. A. et al. 2000). WT-1 is a member of the zinc finger family of transcription factors that includes NGFI-A and Sp1 (reviewed in Little, M. et al. 1999). To begin to understand how the cell line-specific activity of $P1_7$ shown in Figure 4.5 is regulated, $P1_7$ was cotransfected with plasmids encoding NGFI-A, HNF-1α, WT-1 and C/EBPα in B103 cells. P2 was also cotransfected with these transcription factors in B103 cells. No significant induction of $P1_7$ was observed with any of the transcription factors used (Figure 4.8). HNF-1α overexpression led to a small but significant reduction (15% decrease) in P2 activity in B103 cells while the other factors had no significant effect on P2 activity (Figure 4.8).

4.3.6 A 134bp region of $P1_7$ is responsible for its cell line-specific activity

Transfection of the 3' deletion series shown in Figure 4.4, showed that a construct fused to luciferase within exon 1_7 had considerably higher activity in B103 and C6 cells than in HepG2 cells (Figure 4.5). In order to identify the sequence elements responsible for conferring cell line-specific activity on $P1_7$, a 5' deletion series of the $P1_7$ construct was generated by subcloning appropriate restriction fragments (Figure 4.9 and section *2.1.10.3*) to create $P1_{7a}$ (-3575/-2931), $P1_{7b}$ (-3341/-2931), and $P1_{7c}$ (-3207/-2931). These 3 constructs, with P2 and $P1_7$, were assayed in transfected HepG2 and B103 cells. In HepG2 cells, P2 and $P1_7$ had high activity relative to vector (Figure 4.10a), as observed previously (Figure 4.5). $P1_{7a}$ and $P1_{7b}$ had similar activity to $P1_7$, and $P1_{7c}$ activity was close to vector (Figure 4.10a). $P1_{7b}$ activity was significantly lower than $P1_7$ activity. In B103 cells, P2 and $P1_7$ constructs had high activity relative to vector (Figure 4.10b), as observed previously (Figure 4.5). Removal of a 997bp piece of DNA upstream of $P1_7$ ($P1_{7a}$) had no significant effect on activity (Figure 4.10b). Further deletion, including removal of exon 1_6 ($P1_{7b}$) increased activity relative to $P1_{7a}$ (but not

$P1_7$) while further deletion ($P1_{7c}$) virtually ablated promoter activity in B103 cells (Figure 4.10b). There were no significant differences between $P1_7$ activity and $P1_{7a}$ and $P1_b$ activity. In both cell lines, activities of $P1_{7a}$ and $P1_{7b}$ were significantly higher than that of $P1_{7c}$.

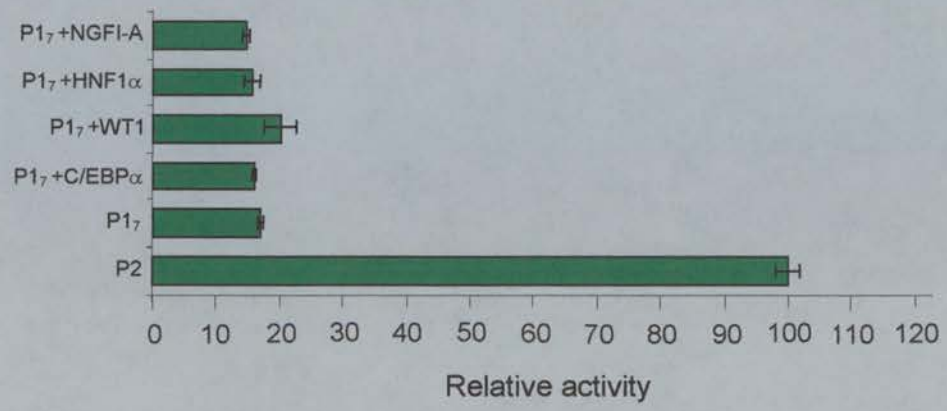
4.3.7 A 134bp region acts as an orientation-independent enhancer in neuroblastoma cells but not in hepatoma cells

The 134bp region of $P1_7$ that resulted in the large increase in luciferase activity in B103 cells was subcloned into both pGL3-BM and pGL3-P (which contains an SV40 promoter), in both orientations to give the constructs BM134C (pGL3-BM, correct orientation), BM134I (pGL3-BM, incorrect orientation), P134C (pGL3-P, correct orientation) and P134C (pGL3-P, incorrect orientation). These constructs were transiently transfected into B103 cells and HepG2 cells. In both cell lines, activity of pGL3-P was 25 times greater than that of pGL3-BM (Figure 4.11). In B103 cells, constructs BM134I had significantly higher activity (11-fold higher) than pGL3-BM, but BM134C did not (Figure 4.11a). In HepG2 cells activities of both of these constructs differed significantly to pGL3-BM (1.5-fold higher for BM134C and 3-fold higher for BM134I) (Figure 4.11b). Constructs P134C and P134I had significantly higher activity than pGL3-P (1.5- and 2-fold higher respectively) in B103 cells (Figure 4.11a). In contrast, activity of P134C was significantly lower than activity of pGL3-P (approximately 50% less active) in HepG2 cells (Figure 4.11b); P134I activity did not differ from pGL3-P activity significantly (Figure 4.11b).

4.3.8 Dexamethasone regulates P2 activity in a cell-type specific manner

To investigate autoregulation of the GR promoter P2 was transfected into C6 and HepG2 cells, which were treated with the synthetic glucocorticoid dexamethasone (at 10^{-7}M, a concentration previously shown to maximally downregulate GR mRNA *in vitro* (Rosewicz, S. et al. 1988) 24h post-transfection; cells were harvested 24h later. Dexamethasone had no significant effect on P2 activity in HepG2 cells (Figure 4.12a) but resulted in a 34% decrease in P2 activity in C6 cells (Figure 4.12b).

(a) $P1_7$



(a) P2

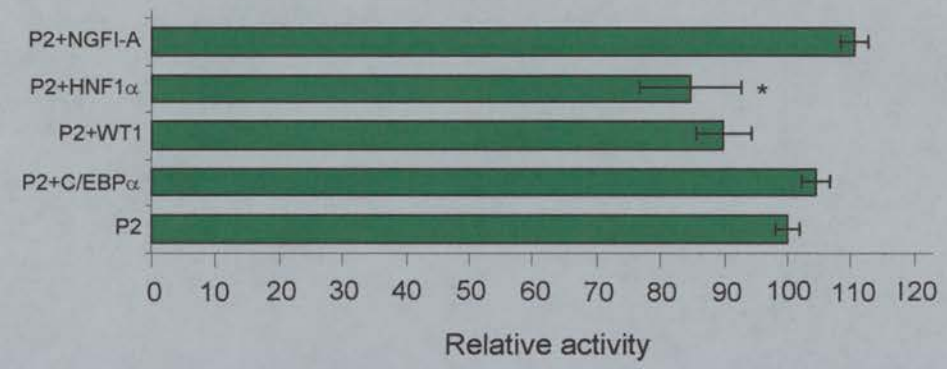


Figure 4.8: *Overexpression of NGFI-A, HNF-1α, WT-1 or C/EBPα does not increase $P1_7$ activity in B103 cells but HNF-1α overexpression leads to a small decrease in P2 activity*

The effects of overexpression of NGFI-A, HNF-1α, WT-1 and C/EBPα on (a) $P1_7$ and (b) P2 activities were determined by transient transfection analysis in B103 cells. Activity of P2 was nominally set at 100% for each cell line and activity of P2 cotransfected with transcription factors expressed relative to this value. n=3, means ± SEM. One-way ANOVA analysis revealed significant differences between groups in (b) ($F_{4,10}$=5.9, p=0.01) but not in (a). * $p<0.05$, *post-hoc* Fisher PLSD test.

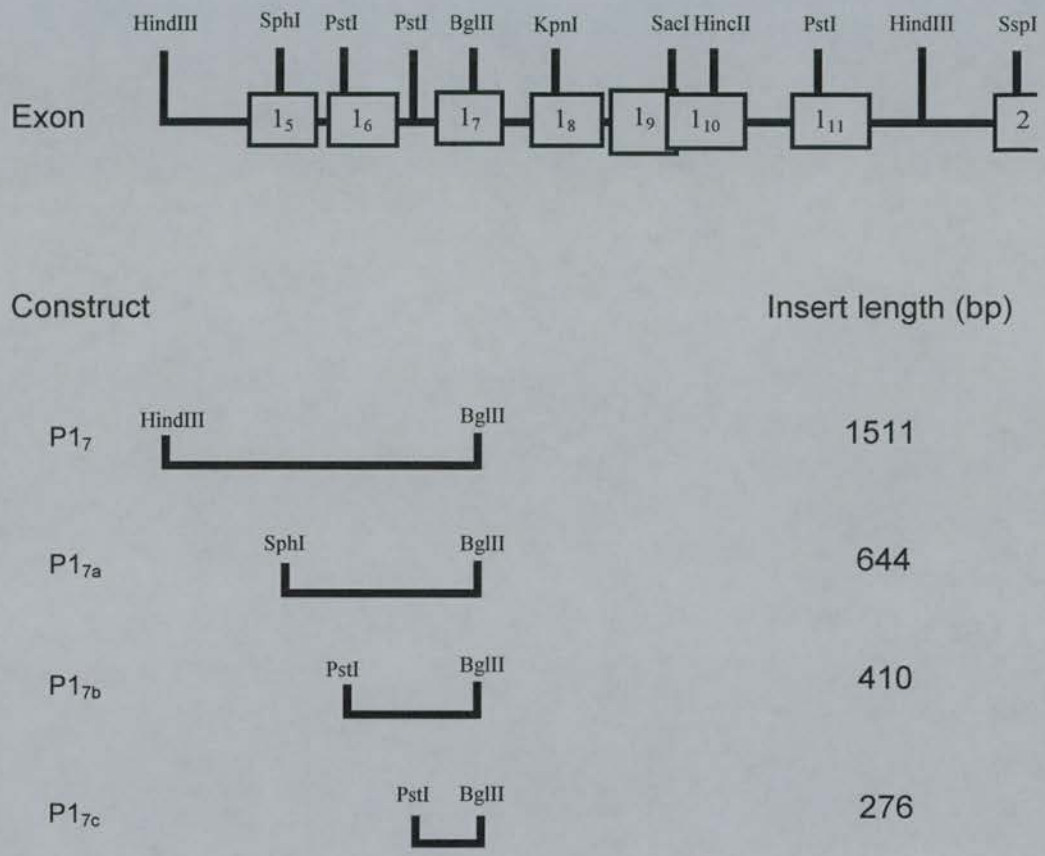


Figure 4.9: *Design of P1 7 subclones*

Construct P1 7, fused to luciferase within exon 1 7, was used as the parent plasmid to generate a 5' deletion series for use in transient transfection assays, to identify regions important for the high promoter activity of P1 7 in B103 and C6 cells. Relevant restriction sites are shown; fragments were fused to luciferase at a common *Bgl*II site.

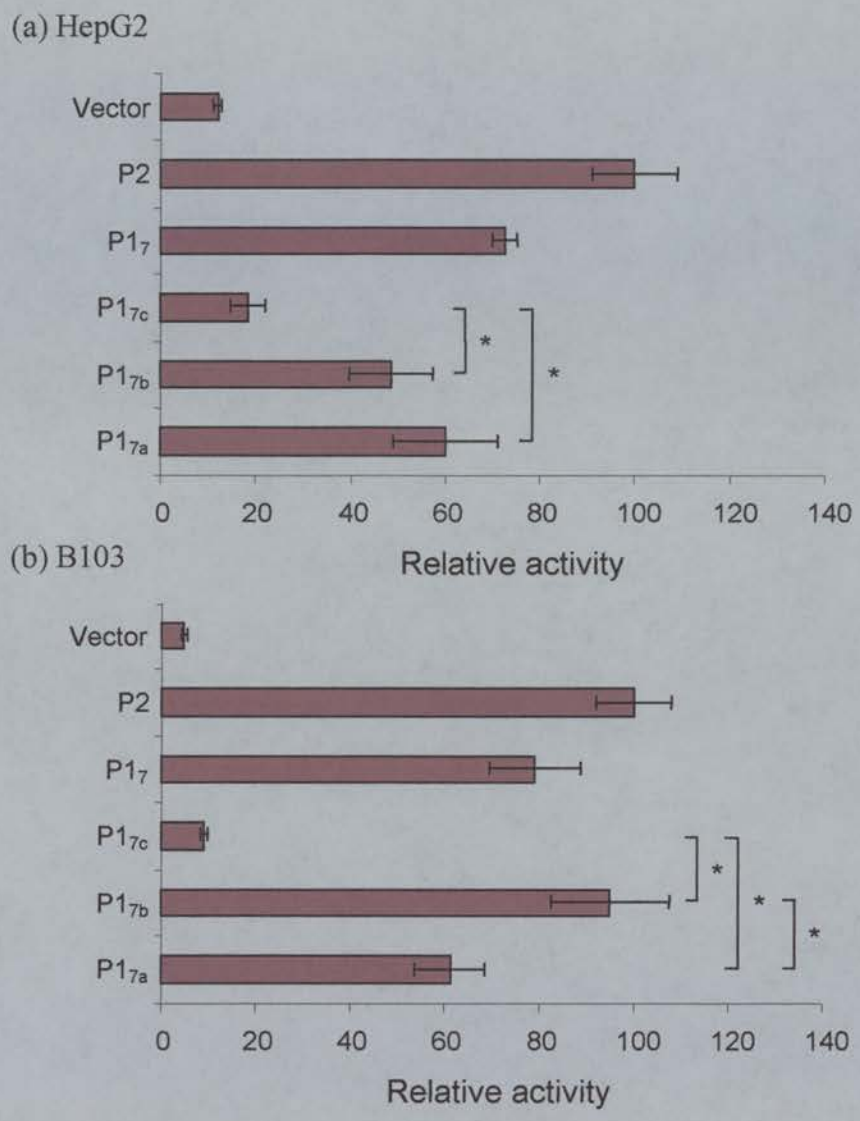
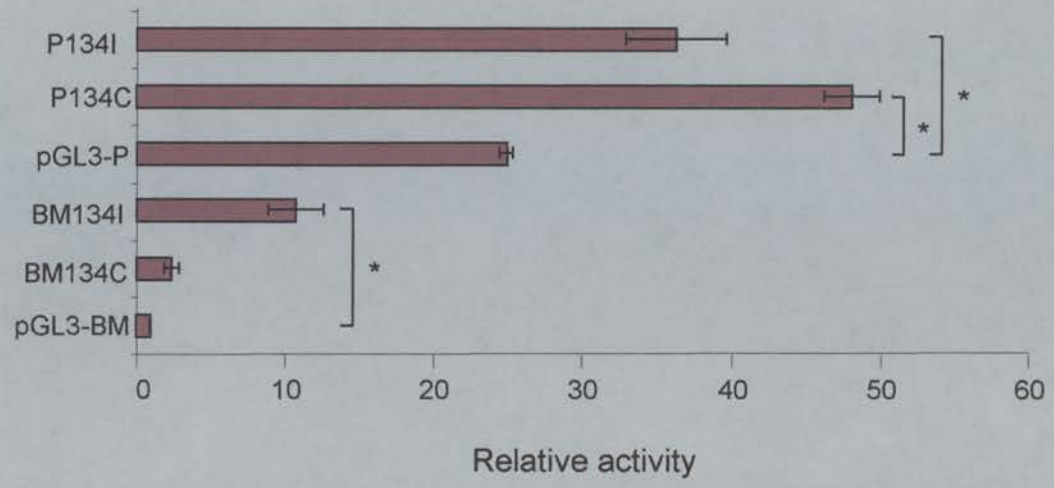


Figure 4.10: *A 134bp region of $P1_7$ is responsible for its cell line-specific activity*

To define regions important in conferring cell line-specific activity of $P1_7$, three subclones: $P1_{7a}$ (-3575/-2931), $P1_{7b}$ (-3341/-2931), and $P1_{7c}$ (-3207/-2931), in addition to P2 and $P1_7$ were transiently transfected into (a) HepG2 cells and (b) B103 cells. Activity of P2 was nominally set at 100% and activity of the other constructs expressed relative to this value. n=5-6 (using 2 independent plasmid DNA preparations), means ± SEM. One-way ANOVA analysis revealed significant differences between groups in B103 ($F_{3,19}$=19.5, p=0.0001 between $P1_7$ and its subclones) and in HepG2 ($F_{3,20}$=9.8, p=0.03 between $P1_7$ and its subclones) cells. * $p<0.05$, *post-hoc* Fisher PLSD test.

(a) B103



(b) HepG2

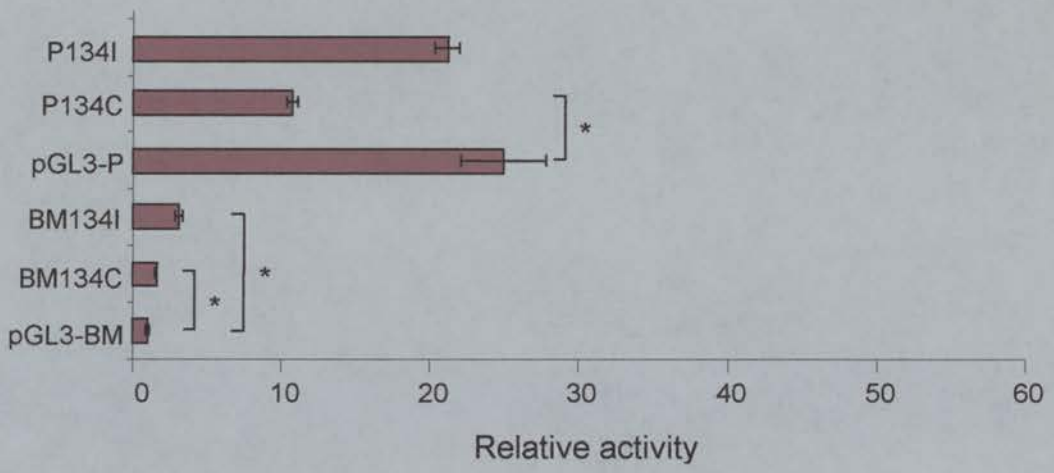


Figure 4.11: *A 134bp region of $P1_7$ acts as an orientation-independent enhancer in neuroblastoma cells but not in hepatoma cells*

To examine the properties of the 134bp region conferring high activity to $P1_7$, this region was subcloned into both pGL3-BM and pGL3-P, in both orientations to give the constructs BM134C (pGL3-BM, correct orientation), BM134I (pGL3-BM, incorrect orientation), P134C (pGL3-P, correct orientation) and P134I (pGL3-P, incorrect orientation). Transient transfection analysis of these constructs, along with P2 and $P1_7$ was performed in (a) B103 cells and (b) HepG2 cells. Activities of constructs are expressed relative to pGL3-BM, nominally set to 1. n=3, means ± SEM. One-way ANOVA analysis revealed significant differences between groups in B103 ($F_{2,5}$=181.5, p=0.0001 for pGL3-BM and $F_{2,5}$=18.1, p=0.05 for pGL3-P constructs) and in HepG2 ($F_{2,6}$=57.8, p=0.0001 for pGL3-BM and $F_{2,6}$=18.0, $p<0.05$ for pGL3-P constructs) cells. * $p<0.05$, *post-hoc* Fisher PLSD test.

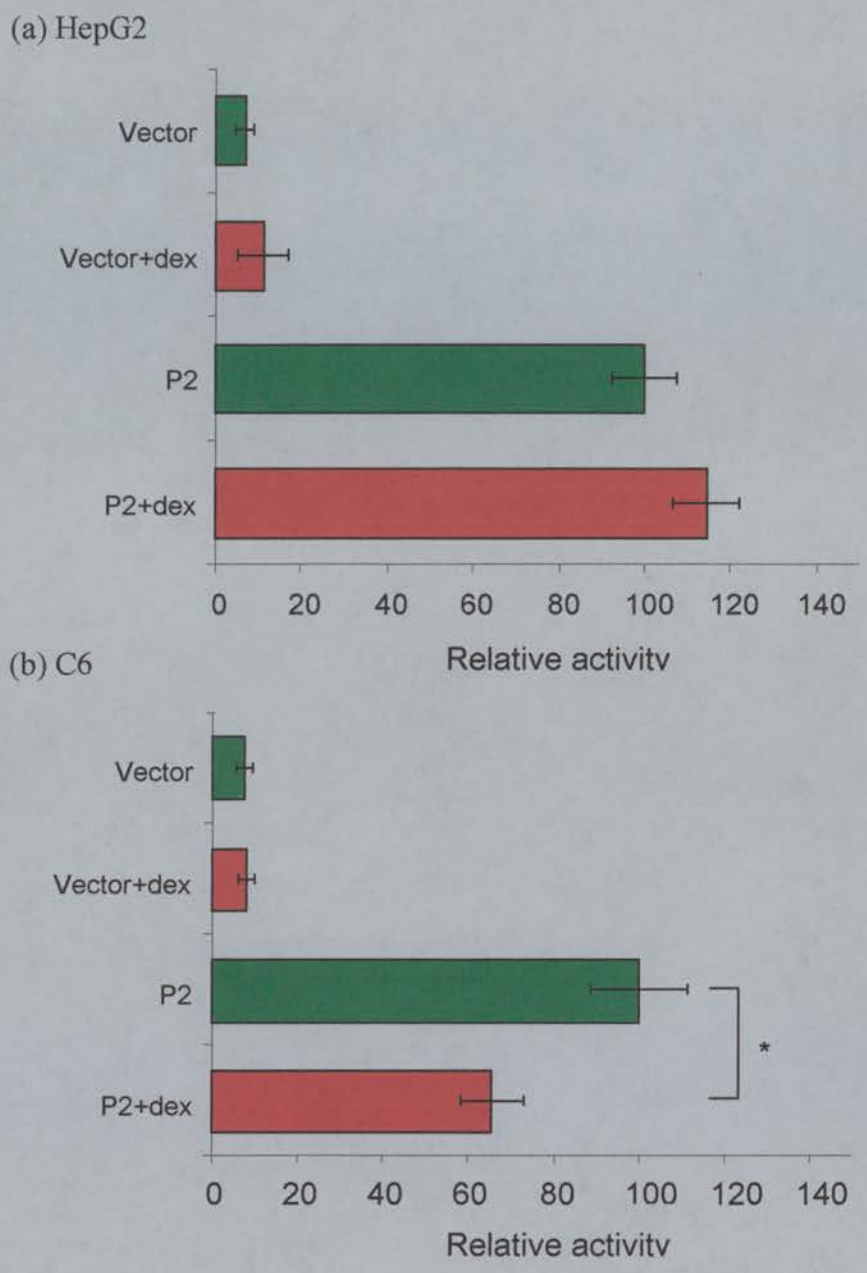


Figure 4.12: *Dexamethasone represses P2 activity in a cell-line specific manner*

To investigate potential autoregulation of the GR gene CpG island region, the effect of dexamethasone on P2 activity was assessed. (a) HepG2 cells and (b) C6 cells were transfected with pGL3-BM (vector) or P2 then treated 24h later with 10^{-7}M dexamethasone (red bars) or vehicle (ethanol) (green bars) for 24h. Activity of P2 in the absence of dexamethasone was nominally set at 100% and activity of vector and P2 in the presence of dexamethasone expressed relative to this value. n=6 to 9 ± SEM. * $p<0.05$, Student t test.

4.4 Discussion

Mapping of transcription starts by RNase protection analysis revealed the existence of multiple transcription initiation sites for exon 1_{10} (summarised in Figure 4.13). This was consistent with sequence analysis of 5'-RACE PCR clones suggested considerable heterogeneity in the 5' end of exons 1 in hippocampus, primary hippocampal cultures and thymus (Figure 4.13). The longest hippocampal 5'-RACE PCR clone containing exon 1_{10} corresponds to one of the transcription starts mapped for human GR mRNA (Zong, J. et al. 1990). Interestingly, there were many differences between the locations of 5' ends mapped in liver by RNase protection analysis and those identified by 5'-RACE PCR. This could reflect the different experimental techniques. Alternatively, this could reflect the different tissues used; RNase protection was carried out on liver RNA whereas the other starts were mapped using RNA from hippocampus, primary hippocampal cultures and thymus. The population of exon 1-containing GR mRNA is more heterogeneous in hippocampus (Table 3.3), so differences between the populations of transcription factors in liver and hippocampus may result in the use of alternative transcription starts. The RNase protection analysis was performed before detailed analysis of the 5'-RACE PCR clones. It was anticipated that a predominant transcription start would be identified near the 5' end of the exon 1_{10}, with a few other minor initiation points located in a cluster around this predominant start. This is clearly not the case since starts appear to be spread over approximately 230 nucleotides (i.e. almost all of exon 1_{10}). Since the cRNA probe used in the RNase protection analysis has a 5' end 98 nucleotides upstream of the 3' end of exon 1_{10}, a large number of starts may have been overlooked by using this probe. A probe extending to the very 3' end of exon 1_{10} would, with hindsight, have been more appropriate for this experiment.

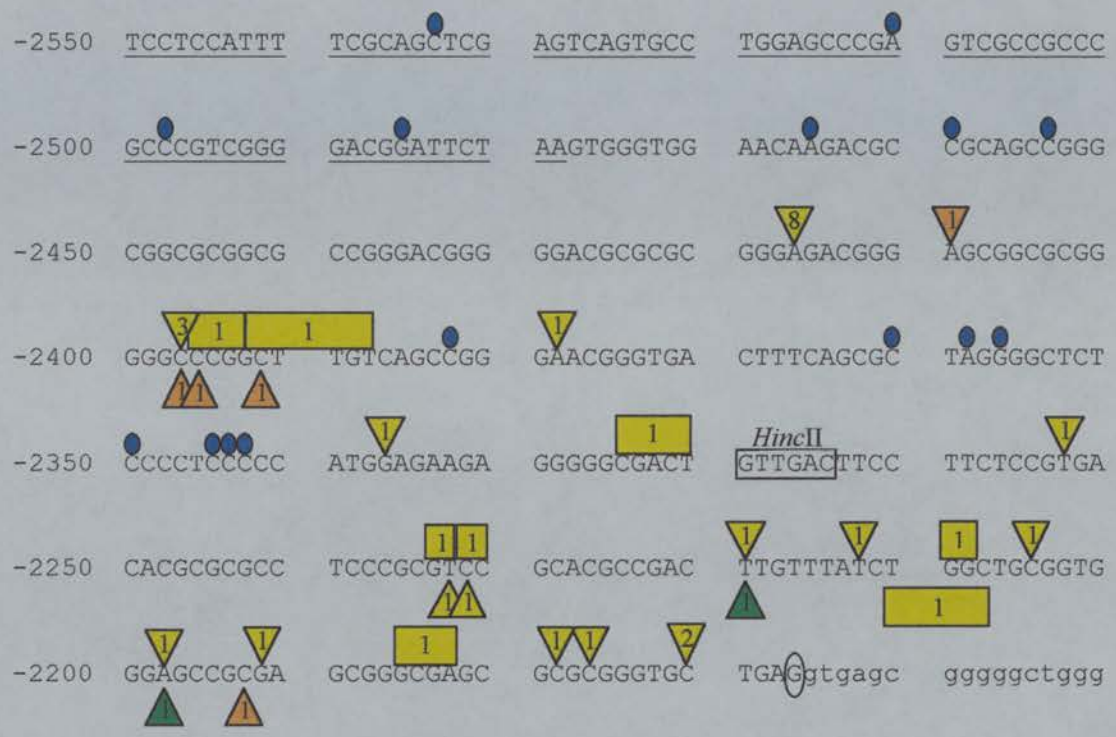


Figure 4.13: *Summary of data from RNase protection analysis and 5'-RACE PCR showing multiple transcription starts exist for exon 1_{10}-containing GR mRNA*

Transcription starts of exon 1_{10} in liver (Figures 4.2 and 4.3) and 5'-RACE PCR on hippocampus, primary hippocampal cultures and thymus were mapped onto exon 1_{10}. The *Hinc*II site used to determine starts mapped by RNase protection analysis is boxed. The oval at –2167 indicates the 3' end of exon 1_{10}. The region underlined represents sequence present in exon 1_9.△ hippocampal 5'-RACE PCR (carried out by V. Lyons) (where the site was only mapped approximately, this is shown by▭);▲ thymus 5'-RACE PCR (carried out by V. Lyons);△ primary hippocampal culture 5'-RACE PCR (Chapter 5)and● summary of data presented in Figures 4.2 and 4.3. Numbers in triangles and boxes indicated the number of independent 5'-RACE PCR clones mapping to a given nucleotide.

It is possible that some protected fragments result from protection of the probe by exon 1_9-containing GR mRNA, since the 3' end and longest known 5' end of this exon lie within the region to which the cRNA probe is complementary. Furthermore, fragments below 68 nucleotides could have arisen from protection of the probe by exon 1_8-containing GR mRNAs; the 3' end of the probe lies at a *Kpn*I site within exon 1_8, 61 nucleotides upstream of the 3' end of exon 1_8. Both of these possibilities are unlikely, however, since these exons 1 are unlikely to comprise a large (if any) proportion of variant GR mRNAs in liver (most have been accounted for (Chapter 3)).

Multiple transcription initiation sites are typical of TATA-less GC-rich promoters, so it is unsurprising that this is the case for exon 1_{10}-containing transcripts, initiating as they do in a CpG island. Indeed, data from 5'-RACE PCR suggests multiple 5' ends exist for all exon 1-containing GR mRNAs originating within the CpG island (data not shown; Val Lyons personal communication).

Transient transfection assays showed that a genomic construct (P2) spanning the entire GR CpG island and encoding the 5' end of exon 2 exhibited substantial promoter activity in a variety of cell lines and had the highest activity of any construct tested. P2 activity most likely reflects the activities of all promoters in the CpG island since a splice acceptor site exists 5' of exon 2 for all initiated exon 1 transcripts to be spliced to. In contrast, activities of constructs fused to luciferase within exon 1 represent activities of promoters immediately 5' of the fused exon 1. Although the broad pattern of activity for these constructs was similar between cell lines, there were one or two exceptions. Promoter activities of constructs $P1_{9/10}$ and $P1_{10}$ probably represent overall activity of $P1_{10}$, with little of the activity due to the $P1_9$ promoter, despite the $P1_{9/10}$ construct being fused within exon $P1_9$. RPAs showed that exon 1_{10}-containing transcripts accounted for the majority of GR mRNAs in all tissues studied (Table 3.3), and since the $P1_{9/10}$ construct had considerable activity in all cell lines (B103, C6, GH_3 and HepG2), this probably reflects predominantly $P1_{10}$ activity. Interestingly, the $P1_7$ construct had the highest activity of any construct other than P2 in B103 and C6 cells, both CNS-derived cell lines (rat neuroblastoma and rat glioma cells respectively); in HepG2 cells (human hepatoma) activity was considerably lower. Exon 1_7-containing GR mRNAs are

present at significant levels in hippocampus, but are absent from liver (Chapter 3). Taken together, these data suggest that regions of $P1_7$ may important in tissue-specific regulation of the GR gene, and transcription factors present in B103 and C6 cells result in the high activity of $P1_7$ in these cells compared with HepG2 cells. $P1_6$ exhibited significant activity in all cell lines tested, possibly reflecting the ubiquitous tissue distribution of exon 1_6-containing GR mRNAs shown by RPAs (Table 3.3). $P1_8$ activity was significant in all cell lines except GH_3 cells but was highest in B103 cells; exon 1_8 was originally identified by 5'-RACE PCR carried out on rat hippocampal RNA. Activity of $P1_{11}$ was only significantly above basal levels in HepG2 cells, whereas *in vivo* exon 1_{11}-containing GR mRNAs are present at significant levels in hippocampus as well as liver (Chapter 3). Data obtained from *in vitro* studies can not always be extrapolated to *in vivo* studies, and this might explain the discrepancy between $P1_{11}$ activity in CNS-derived cell lines and exon 1_{11}-containing mRNA levels determined by RPA on hippocampal RNA.

H3, fused to luciferase 5' of exon 2 but not within an identified exon 1 (-4572/-797), exhibited substantial activity in B103 cells, low but significant activity in HepG2 cells and no significant activity in C6 cells. Furthermore, a fragment (P0, -872/-9) fusing to luciferase within exon 2 with no known exons 1 present on the fragment (and therefore no known promoters) had a similar pattern of activity: considerable activity in B103 cells but relatively low activity in HepG2 and C6 cells. Taken together, these data that there may be at least 1 more as yet unidentified exon 1 3' of exon 1_{11}, reflecting a level of transcription initiation throughout and even downstream of the CpG island. It is worth noting that the 5' end of P0 and the 3' end of H3 overlap, so sequences in this specific region of overlap may be acting as a promoter in B103 cells. This putative promoter may be tissue-specific, reflected in the differences in activity observed between cell lines. RT-PCR or Northern analysis would confirm the existence of a novel exon 1 derived from this region which lies 3' of the CpG island.

A search for putative transcription factor binding sites revealed a number of putative binding sites including 25 CCAAT/enhancer-binding protein (C/EBP) sites, two NGFI-A sites (-2292 and -3316) and a single HNF-1 site (-13). C/EBPα is highly expressed in

liver and is an important determinant of terminal cell differentiation and function: ablation of C/EBPα expression in mice results in gross abnormalities of liver structure and function (Wang, N. D. et al. 1995; Flodby, P. et al. 1996). In addition, dexamethasone indirectly represses C/EBPα in adipocytes by rapidly inducing C/EBPδ (MacDougald, O. A. et al. 1994). C/EBPβ was originally identified as a mediator of Interleukin 6 (Il-6) signalling, binding to IL-6-responsive elements in the promoters of acute phase response genes (Poli, V et al. 1990; Akira, S. et al. 1990). Lack of C/EBPβ is also associated with neonatal lethality and decreased hepatic gluconeogenesis (Croniger, C. et al. 1997). Furthermore mice lacking C/EBPβ are deficient in glucocorticoid signalling (Arizmendi, C. et al. 1999). PEPCK expression is induced by GR *in vitro* (Friedman, J. E. et al. 1993). Finally, HNF-1α is implicated in glucose homeostasis (Yamagata, K. et al. 1996) and is involved in the regulation of numerous hepatic genes (reviewed in Hanson, R.W. 1994). Since these three transcription factors are closely linked to the actions of GR in liver, their effects on P2 activity (which spans the entire CpG island of the GR promoter region) were studied in HepG2 cells. Surprisingly, none of the cotransfections resulted in any induction of P2 activity, though this does not mean that these factors play no role in regulation of the GR promoter. It is possible that the putative binding sites actually binding these factors are fully occupied under basal conditions thus preventing any further induction of activity. HepG2 cells express C/EBPβ and HNF-1α, but only low levels of C/EBPα, which induced activity of pVL108. Furthermore, the *in vitro* setting of the transfection is rather artificial, and the lack of induction may simply reflect the particular cell line used or a requirement for a chromatin template (Struhl, K. 1996; Smith, C. L. et al. 1997). Band shift experiments would help to determine whether any of these factors are potentially able to bind to the putative binding sites present in P2 and footprint analysis would identify important sequence elements involved in protein-DNA binding.

Neonatal handling specifically increases the level of exon 1_7-containing GR mRNAs in hippocampus (McCormick, J. A. et al. 2000). 5-HT activation of adenylyl cyclase may be the crucial link between handling and induction of hippocampal GR (section *1.3.1*). cAMP, in turn, may regulate the activity or expression of transcription factors, which then act on the GR promoter region to increase GR gene transcription. Neonatal

handling and 5-HT increases NGFI-A and AP2 mRNA and protein in hippocampus (Meaney, M. J. et al. 1996). Handling also substantially reduces cyclic nucleotide response element binding modulators (CREMs) (Meaney, M. J. et al. 1996): CREMs antagonize the actions of cyclic nucleotide response element binding proteins (CREBs) which are activated by cAMP, so the reduction in CREMs levels may increase the promoter binding activity of CREBs.

The presence of two putative NGFI-A binding sites immediately upstream of exon 1_7 is strongly suggestive as to the mechanism of hippocampal GR programming in the neonatal handling paradigm, since neonatal handling induces both NGFI-A and exon 1_7-containing GR mRNA in the hippocampus. Furthermore, placing adult rats in an "enriched" environment leads to increased expression of NGFI-A and GR mRNA in the CA2 subregion of the hippocampus (Olsson, T. et al. 1994). However, a more recent study did not link NGFI-A and GR expression (Dahlqvist, P. et al. 1999), but interactions with other transcription factors may be important. To examine possible regulation of $P1_7$ by NGFI-A, a plasmid encoding NGFI-A was cotransfected with $P1_7$ in B103 cells. WT-1, which binds to the same DNA sequence as NGFI-A (Crosby, S. D. et al. 1991) was also tested, as were C/EBPα and HNF-1α; the effects of these factors on P2 activity were also determined. No large effect on $P1_7$ activity was seen with any of the transcription factors used, although HNF-1α cotransfection resulted in a very small but significant decrease in P2 activity, while the other factors had no effect.

It should be noted, however, that these cotransfection data are only from one experiment performed in triplicate. Furthermore, in both cell lines, effects may have been seen had a GR promoter construct with low basal activity been used (e.g. $P1_7$, has relatively low activity in HepG2 cells). Alternatively, other transcription factors may be binding to these sites, and not any of those tested.

Transfections using a 5' deletion series of $P1_7$ identified a 134bp region important for conferring high promoter activity to $P1_7$. $P1_{7c}$, the shortest construct, had no significant activity in either B103 cells or HepG2 cells. $P1_{7a}$ and $P1_{7b}$ exhibited significant activity in both cell lines and this activity was significantly higher than $P1_{7c}$ activity. At first

glance this data seems to contradict that presented in Figure 4.5, which showed that $P1_7$ activity was significantly higher in B103 cells than in HepG2 cells. When the data for $P1_7$ shown in Figure 4.10 is compared with vector, which had higher basal activity in HepG2 cells, this cell-line specificity is clear. The activity of $P1_7$ in B103 cells is 16-fold higher than vector; in HepG2 it is only 6-fold higher. A similar analysis reveals the extent of the importance of the 134bp fragment in conferring cell line-specificity to $P1_7$ activity. $P1_{7a}$, $P1_{7b}$ and $P1_{7c}$ have 12-, 19-, and 2-fold higher activity than vector in B103 cells (though the differences are only significant for $P1_{7a}$ and $P1_{7b}$). In contrast the corresponding values in HepG2 cells are 5-, 4-, and 1.5-fold respectively. Thus, the 134bp region seems to be inducing promoter activity to a much greater degree in B103 cells than in HepG2 cells. Interestingly, addition of a region from -3341 to –3575 led to a small but significant reduction in activity in B103 cells ($P1_{7b}$ compared with $P1_{7a}$). $P1_{7a}$ contains a region extending upstream of the longest known 5' end of exon 1_6. Activity of $P1_6$, reflecting activity associated with exon 1_6, is substantial in B103 cells (Figure 4.5). Thus, the drop in activity resulting from addition of DNA to $P1_{7b}$ to create $P1_{7a}$ could be due to competition for transcription factors between the promoters associated with exon 1_6 and exon 1_7. Alternatively, a binding site for a transcriptional repressor may be contained in the added region. In further support of these conclusions, the activities of $P1_{7a}$ and $P1_{7b}$ did not differ significantly from $P1_7$ in B103 cells, whereas in HepG2 cells $P1_{7b}$ activity was significantly lower than $P1_7$ activity while $P1_{7a}$ activity was not.

$P1_{7c}$ contains the putative NGFI-A site at -2992; $P1_{7b}$ contains both this site and the one at -3316. The massive increase in activity seen when the -3316 NGFI-A site was added raises the possibility that this site may contribute to cell line-specific activity of $P1_7$. The site at -2992 may not bind NGFI-A, or may require co-operative binding of a factor at –3316. When placed in front of a heterologous promoter (SV40 promoter, in pGL3-P), the 134bp region was able to act as an orientation-independent enhancer (albeit a relatively weak one) in B103 cells. This enhancer activity did not require the presence of any elements (e.g. the other putative NGFI-A site at –2992) in the downstream region, though they may be required for maximal promoter activity. In HepG2 cells the 134bp region was unable to act as an enhancer. Indeed, when subcloned into pGL3-P in

the correct orientation, it appeared to act as a repressor. Thus, the cell line-specific activity of $P1_7$ may involve binding of repressors or activators depending on the cell type. When subcloned into pGL3-BM in the correct orientation, the 134bp region acted as a weak promoter. When subcloned into pGL3-BM in the incorrect orientation, the 134bp region acted as a promoter in both B103 and HepG2 cells. This activity was probably an artifact, however, since when the entire $P1_7$ insert was subcloned into pGL3-BM in the incorrect manner, activity higher than that of P2 was observed in some experiments but not others (data not shown). Future experiments involving DNase I footprinting analysis and gel mobility shift assays would identify transcription factors binding the 134bp that may regulate $P1_7$ activity.

Interestingly, an Sp1 binding site has been identified in the human GR which corresponds to the putative NGFI-A site at -2992 in the rat gene (Nobukuni, Y. et al. 1995), so the possibility that this site acts co-operatively with the more 5' putative NGFI-A site is strong. Other putative transcription factor sites exist within the 134bp region, including one for AP2; it remains possible that activity is increased by the binding of a factor to one of these rather than to the putative NGFI-A site. Indeed, AP2 has also been implicated in the induction of GR in hippocampus following neonatal handling or 5-HT treatment of primary hippocampal cultures (Meaney, M. J. et al. 2000). Furthermore, a sequence in the human gene that binds AP2 *in vitro* (Nobukuni, Y. et al. 1995) is completely conserved in the rat GR gene (at -2718). Another possible candidate activator of $P1_7$ activity is Sp4, which binds to GC and GT boxes with affinity identical to that of Sp1 (Hagen, G. et al. 1992). *In vivo*, Sp4 transcripts are abundant in brain but barely detectable in other organs (Hagen, G. et al. 1992) so Sp4 may contribute to the cell-specific activity of $P1_7$.

Glucocorticoids are involved in the homologous downregulation of GR, with regulation occurring at the level of GR gene transcription (Kalinyak, J. E. et al. 1987; Rosewicz, S. et al. 1988; Dong, Y. et al. 1988; Meyer, T. et al. 1997). Dexamethasone treatment of C6 glioma cells transfected with P2 resulted in a substantial (37%) decrease in P2 activity; no effect was observed in HepG2 cells. Since dexamethasone is a poor ligand for MR, this effect is likely to be mediated by GR. These data suggest that

autoregulation of GR occurs on the GR gene promoter and is cell line-specific. Autoregulation of GR mRNA has been shown to occur in a tissue-specific manner with relatively more downregulation of GR mRNA in brain compared with liver (Kalinyak, J. E. et al. 1987). The lack of effect in HepG2 cells compared with C6 cells may reflect different pools of transcription factors within the cell lines: autoregulation may depend on interactions between ligand-activated GR and other transcription factors. There are several means by which activation of GR may lead to repression of GR gene transcription (summarised in Figure 1.3). Firstly, there may be direct competition between GR and other transcription factors for binding sites on the promoter (Akerblom, I. E. et al. 1988). Secondly, transcriptional repression by glucocorticoids may be mediated by direct protein-protein interaction with a positive regulator, e.g. AP1 (Mordacq, J. C. et al. 1989; Schule, R. et al. 1990; Yangyen, H. F. et al. 1990; Pfahl, M. 1993). In this case, repression does not require actual binding to a GRE, but depends on a protein-protein interaction between GR and either Jun or Fos, the components of AP1 (Jonat, C. et al. 1990; Touray, M. et al. 1991). Indeed, it has been suggested that autoregulation of the human GR gene occurs via interactions between ligand-bound monomeric GR and Jun, leading to a reduction in the amount of AP1 available to induce GR gene expression by binding a putative AP1 site in the GR promoter (Vig, E. et al. 1994). Glucocorticoids can repress gene activation mediated by NF-κB in a similar manner (Mukaida, N. et al. 1994; Ray, A. et al. 1994; Scheinman, R. I. et al. 1995a). Indeed, sequence analysis revealed the presence of a number of putative binding sites for both AP1 and NF-κB. However, it is unlikely that AP1 or NF-κB act as activators of the GR gene *in vitro* since they generally act as antagonists of GR (reviewed in McKay, L. I. et al. 1999). Thirdly, repression may occur via glucocorticoid induction of a repressor of the GR gene, analogous to the GR induction of I-κB, which acts to retain NF-κB in the cytoplasm and hence prevent activation of NF-κB target genes (Scheinman, R. I. et al. 1995a). Finally, the effect of dexamethasone on P2 activity may be the result of activated GR binding directly to a nGRE. Evidence has been presented for the existence of a negative GRE (with consensus sequence 5 '-CGTCCA) at nucleotide -63 in the pro-opiomelanocortin gene (Drouin, J. et al. 1990; Drouin, J. et al. 1993). It has been suggested that an exact match for the consensus of this site is present in the human GR gene promoter region at –1786 (Zong, J. et al. 1990). However, the

published human GR sequence (Zong, J. et al. 1990) differs to that in the Genbank (accession number U10403) database (5'-CCTCCA). The reverse antisense sequence 5'-TGGACT at –1482 in the human GR sequence corresponds to the sequence 5'-TGGCAG at –3381 in the rat sequence, and is thus a poor match for a negative GRE in rat. It is therefore questionable that this sequence does indeed resemble a nGRE.

A region from –250 to –750 relative to the transcription start of the exon 1 in the published human GR cDNA is implicated in autoregulation of the human GR gene, and an unidentified protein binds to this region (Leclerc, S. et al. 1991). This region corresponds to –2539 to –3013 in the rat GR gene, i.e. near the most 5' transcription start known for exon 1_{10}-containing GR mRNA, and 5' of the most 5' end of exon 1_7-containing GR mRNA so far identified. Therefore, constructs $P1_7$, $P1_8$, $P1_{9/10}$ and $P1_{10}$ would be the best constructs to use initially to define the regions repressed by glucocorticoids in C6 cells.

Several GRE-like elements have been identified in human GR cDNA (Webster, J. C. et al. 1994). Transfection studies in COS1 and CHO cells using human GR cDNA have suggested that the GR cDNA contains sufficient information to downregulate both mRNA and protein by binding to these GRE-like elements (Bellingham, D. L. et al. 1992). The sequences responsible for this effect are located towards the 3' end of the GR cDNA, and binding of GR leads to subsequent down-regulation of the human GR cDNA. Since the human GR cDNA used lacks any of the putative promoters it is unlikely that down-regulation is via an effect on the GR promoter (Webster, J. C. et al. 1994). The effect might, however, be due to ligand-activated GR bound to the GR cDNA blocking transcription by RNAP-II, though this is unlikely, since RNAP-II can transcribe through regions bound by transcription factors. It should be noted that since no GR promoter elements were present in the constructs used in these experiments, the intragenic GREs are unlikely to be of relevance to the effect of dexamethasone on P2 in C6 cells. There is evidence that GR participates in chromatin remodeling of glucocorticoid-responsive promoters (Fryer, C. J. et al. 1998) and alterations in chromatin structure may be involved in dexamethasone-induced repression of the GR

promoter. The data presented here indicate that autoregulation of the GR gene is at least partly chromatin-independent.

Experimental demonstration of promoter activity of the putative GR promoter region has only previously been shown for the human GR gene; for the mouse gene, only the existence of alternate exons 1 has been described with the mapping of their transcription starts. Activity of the human GR promoter region was tested using a 5' deletion series (Nobukuni, Y. et al. 1995), transfected into several cell lines including HepG2 cells: in those experiments, promoter activity therefore reflected activity of all promoters contained on the genomic DNA fragments and hence it was not possible to detect any cell line-specific promoter activities in these experiments. The longest construct spanned -857/+38 (+1 refers to the transcription start for the human exon 1 (homologous to the rat exon 1_{10}) in IM9 cells (Encio, I. J. et al. 1991). A construct spanning -374/+38 contained sufficient information to confer maximum luciferase activity in these experiments. Furthermore, in this laboratory, RNase protection analysis has shown that the published human exon 1 is present in approximately 50% of GR mRNA in human liver (Nelson, R., personal communication). The data presented in this chapter show that a 3' deletion series fused to luciferase within specific exons 1 drive luciferase expression in a cell line-specific manner, and activity of each construct most likely represents activity of an individual promoter. Rather surprisingly, no functional studies (i.e. transient transfection assays) have previously been performed using the rat or mouse GR gene promoter regions, though the existence of multiple exons 1 proposed to derive from alternate promoter usage has been reported (Gearing, K. L. et al. 1993; Chen, F. H. et al. 1999a).

In a similar manner to the mouse and human GR genes, the rat GR gene contains multiple exons 1. In rat, three of these untranslated exons 1 lie 5' of -15000; the remainder lie within a CpG island that extends from -1620 to -4520 relative to the translation start at +1. Data presented here indicate that the entire region extending from -9 to -4572 has high promoter activity and individual exons 1 are associated with promoter activity that varies in a cell line-specific manner. A region 5' of exon 1_7 appears to possess enhancer activity, which shows a degree of specificity for CNS-

derived cell lines. Multiple transcription initiation sites have been mapped for exon 1_{10}, as is common for mRNAs containing untranslated 5' ends initiated in GC-rich regions. For simplicity, such mRNAs can be said to be derived from an individual "promoter". The pool of transcription factors within a given cell type, which can vary depending on environmental factors (e.g. induction of NGFI-A in hippocampal neurons by environmental enrichment), will therefore determine which alternate exons 1 are expressed, though some appear to be constitutively expressed (Chapter 3). Cotransfections of promoter constructs with putative activators failed to reveal any large increases in promoter activity, though the roles of these transcription factors in regulation of the GR gene cannot be entirely dismissed. Dexamethasone treatment of cells transfected with a construct spanning the whole CpG island resulted in a significant reduction in the promoter activity of this construct, demonstrating autoregulation of the GR gene at the promoter level. The role of alternate promoters in programming of GR levels during development is examined in the following chapter.

Chapter 5: Differential expression of alternate exon 1-containing GR mRNAs following early life manipulations

5.1 Introduction

Environmental conditions in early life can have profound consequences on physiology and pathology in adult life. Such early life events permanently program or imprint the development of tissues and organs, with individual organ systems differing in vulnerability according to the nature of the manipulation and the timing of exposure (section *1.3*). Steroid hormones are involved in the development and maturation of foetal and postnatal organ systems and perinatal exposure produces permanent programming effects (Levine, S. et al. 1966; Levitt, N. S. et al. 1996; Nyirenda, M. J. et al. 1998).

In rats, *in utero* exposure to dexamethasone permanently increases hepatic PEPCK and induces glucose intolerance and hypertension in adulthood; the critical window of exposure is week 3 of gestation (Nyirenda, M. J. et al. 1998). Hepatic GR itself is permanently programmed by prenatal exposure to dexamethasone (Figure 5.1) (Nyirenda, M. J. et al. 1998), providing a possible mechanism for permanent PEPCK induction. Conversely, GR levels are permanently *reduced* in the hippocampi of these animals, with permanent effects on HPA axis reactivity to stress (Levitt, N. S. et al. 1996).

Hippocampal GR levels are also permanently programmed by neonatal handling (Figure 5.2). This manipulation permanently *increases* hippocampal GR, probably via a specific increase in the level of exon 1_7-containing GR mRNAs (McCormick, J. A. et al. 2000). The neonatal handling effect appears to be mediated by increased serotonergic neurotransmission in the hippocampus (Mitchell, J. B. et al. 1990b). Primary hippocampal cultures have been used to study the effects of 5-HT on GR expression (Mitchell, J. B. et al. 1990a; Mitchell, J. B. et al. 1992). 5-HT maximally

induces GR mRNA and protein levels in primary hippocampal cultures after 4d of exposure (Mitchell, J. B. et al. 1992).

The aims of the experiments described in this chapter were to determine whether alterations in GR following *in utero* dexamethasone exposure or 5-HT treatment of primary hippocampal cultures were reflected by changes in alternate exon 1-containing GR mRNAs. Any changes observed would suggest differences in promoter usage, which could then help to explain how levels of GR are programmed by perinatal manipulations.

5.2 Methods

5.2.1 Effect of in utero dexamethasone on abundances of alternate exon 1-containing GR mRNAs

To determine if prenatal dexamethasone exposure led to any changes in levels of alternate exon 1-containing GR mRNAs, RNase protection assays (section *2.2.7.1*) were performed on total RNA isolated from the livers of treated and control animals. Tissues were provided by Moffat Nyirenda, and were removed from 8m old male Wistar rats (Nyirenda, M. J. et al. 1998) (section *2.2.1*). Following preliminary experiments, in which no increase in the level of a specific exon 1-containing GR mRNA was detected, 5'-RACE PCR was performed by Val Lyons on liver RNA from the animals previously shown to express the lowest or highest level of GR mRNA (Nyirenda, M. J. et al. 1998). This was done to identify any novel exons 1 induced by prenatal dexamethasone exposure, or detect any increase in representation of a known exon 1 in clones obtained (data not shown). Exon $1_{4.5}$ was a novel candidate exon 1 identified by 5'-RACE PCR on liver from rats exposed to dexamethasone *in utero* and was therefore screened for in the assays. The 3'-boundary of exon $1_{4.5}$ is AGGAGTTTGGgt and 21 bp was isolated (resulting in a specific exon $1_{4.5}$-containing GR mRNA protected fragment of 207 nucleotides in RNase protection analysis). The position (determined so far) of exon $1_{4.5}$ in the GR gene is -3734 to -3714.

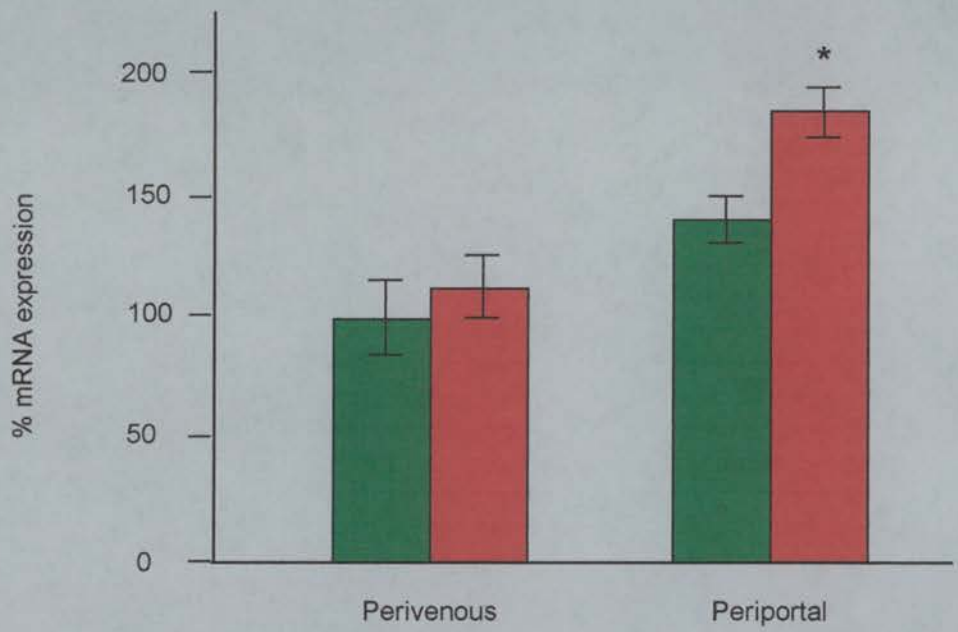


Figure 5.1: *Effect of prenatal dexamethasone on hepatic GR mRNA expression in 8m old offspring*

Hepatic GR expression in fed offspring of dams that received vehicle alone (green bars) or dexamethasone in week 3 of pregnancy (red bars) was measured by *in situ* hybridisation at the ages of 8m. Results are expressed as a percentage of mRNA in control animals for each region. Values represent mean ± SEM; n=9-11. *, $p<0.05$. Taken from Nyirenda M. J. et al. 1998.

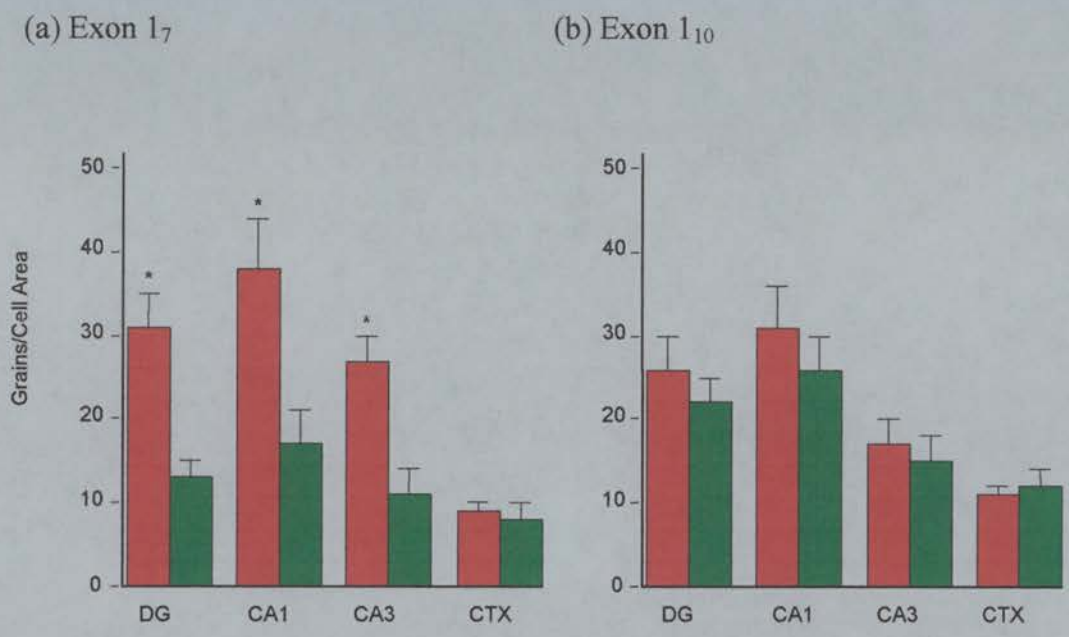


Figure 5.2: *Exon 1$_7$-containing GR mRNA is induced in hippocampus by neonatal handling*

In situ mRNA hybridisation analysis of GR mRNA containing exon 1$_7$ (a) or exon 1$_{10}$ (b) within the dentate gyrus (DG), the CA1 and CA3 pyramidal cell fields of the hippocampus and the cortex (CTX). Expression was measured in handled animals (red bars) and non-handled animals (green bars), and is expressed as the number of grains over an area equivalent to a CA1 neuron. Values represent mean ± SEM; n=5. *, $P<0.05$. Adapted from McCormick, J. A. et al. 2000.

5.2.2 Effects of 5-HT on alternate exon 1-containing GR mRNAs in primary hippocampal cultures

To determine whether 5-HT affects alternate exon 1-containing GR mRNAs, 5'-RACE PCR was performed on total RNA extracted from primary hippocampal cultures exposed to 100nM 5-HT for 4d and control cultures. The cell-type composition of the primary hippocampal cultures was assessed by immunocytochemistry, to verify that cultures consisted predominantly of neurons. 5'-RACE PCR was used to determine whether any previously unidentified exons 1 were induced by 5-HT treatment, as well as to determine if any known alternate exon 1-containing GR mRNAs became more abundant post-treatment. RT-PCR was used to determine whether exon 1_7-containing GR mRNAs were present in primary hippocampal cultures.

5.3 Results

5.3.1 Prenatal dexamethasone exposure reduces the proportion of exon 1_{10}-containing GR mRNA levels in the livers of adult rats

RPAs performed on total RNA from the livers of animals exposed to dexamethasone *in utero* revealed significantly lower levels of hepatic exon 1_{10}-containing GR mRNA (73% of total GR mRNA transcripts) compared with control animals (82% of total GR transcripts) ($p<0.05$) (Figure. 5.3 and Table 5.1).

5.3.2 Prenatal dexamethasone exposure has no effect on the levels of exons 1_1, 1_6, $1_{4.5}$, 1_5, 1_7, or 1_{11}-containing GR mRNAs in the livers of 8m old rats

No effect on the relative proportion of GR mRNA containing exons 1_1, $1_{4.5}$, 1_5, 1_6, 1_7, or 1_{11}-containing GR mRNAs in liver was detected following prenatal dexamethasone exposure (Figure 5.4 and Table 5.1). Exon 1_6-containing GR mRNAs accounted for 9% of total GR mRNA transcripts in both groups. Other exon

1-containing GR mRNA remained very low or were undetectable (Figure 5.4 and Table 5.1).

5.3.3 Exon 1_{10}-containing GR mRNAs probably account for the majority of GR mRNA transcripts in primary hippocampal cultures

5'-RACE PCR was performed using several independent RNA preparations from primary hippocampal cultures established on different dates. Immunocytochemistry confirmed that the cultures consisted predominantly of neuronal cells (Figure 5.5), with approximately 80% of cells being positive for neuron-specific enolase and 20% positive for glial acidic fibrillary protein. 5'-RACE PCR only yielded a limited number of independent clones. The majority of clones obtained contained exon 1_{10}; the only other exon 1 identified was exon 1_{11} (Table 5.2). There was no detectable effect of 5-HT treatment upon the relative proportions of clones containing exon 1_{10} and 1_{11} in primary hippocampal cultures.

5.3.4 RT-PCR reveals the presence of exon 1_7-containing GR mRNAs in primary hippocampal neurons

RT-PCR performed on total RNA extracted from primary hippocampal cultures not treated with exogenous 5-HT showed that primary hippocampal cultures expressed exon 1_7-containing GR mRNAs, as well as exon 1_{10} (used as a positive control) (Figure 5.6). As described in Chapter 3, hippocampus also expressed both of these variant GR mRNAs (Figures 3.6 and 3.10 and Table 3.3).

	% of total GR mRNAs	
Exon 1	**Control**	**Dexamethasone**
1_1	1 (5)	1 (5)
$1_{4.5}$	0 (4)	0 (5)
1_5	0 (5)	0 (5)
1_6	9 (5)	9 (4)
1_7	0 (5)	0 (5)
1_{10}	82 (9)	73* (10)
1_{11}	0 (5)	0 (5)

Table 5.1: *Summary of the effects of prenatal dexamethasone on variant GR mRNA levels in liver*

RNase protection analysis was performed on 50μg total liver RNA isolated from animal exposed to dexamethasone *in utero*. Data are presented as a proportion of total GR mRNA transcripts and are mean values; the number of independent RNA samples (i.e. animals) is presented in brackets. * denotes a significant difference in the level of a given variant GR mRNA between control animals and animal exposed to dexamethasone *in utero*, $p<0.05$. (n) = number of animals.

	Number of independent clones	
Exon	**Control**	**+5-HT**
1_{10}	11	11
1_{11}	3	2

Table 5.2: *Summary Table of 5'-RACE PCR clones obtained from control or 5-HT-treated primary hippocampal cultures*

Four independent 5'-RACE PCR reactions were each performed on 5μg total RNA isolated from 3 independent primary hippocampal cultures treated with 100nM 5-HT for 4d (+5-HT) or vehicle (Control). Cultures were maintained for 4d after treatment before harvesting and isolation of RNA; RNA samples were pooled for individual animals from each group (section *2.2.2*).

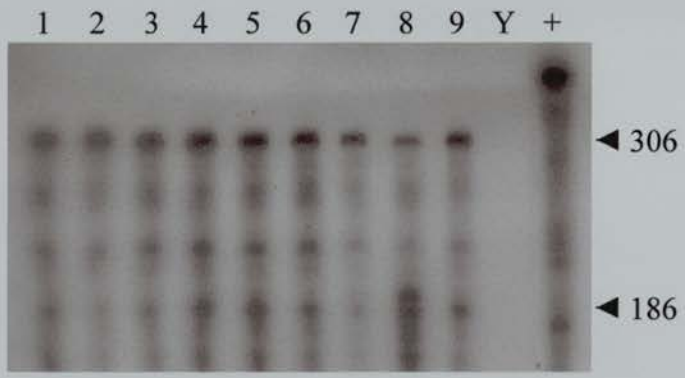


Figure 5.3: *RNase protection analysis of the effect of prenatal dexamethasone exposure on the abundance of exon 1_{10}-containing GR mRNA in rat liver*

RNase protection assays were carried out on 50μg total RNA from the livers of adult male rats exposed to dexamethasone *in utero*. Lanes 1-4 contained RNA from control animals and lanes 5-9 contained RNA from animals exposed to dexamethasone *in utero*. Lanes marked Y contained yeast RNA and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{10}, and the 306 nucleotide fragment protected by transcripts containing exon 1_{10} and exon 2.

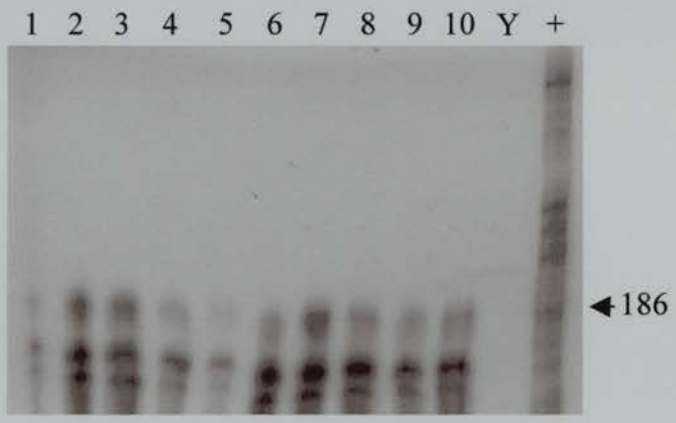
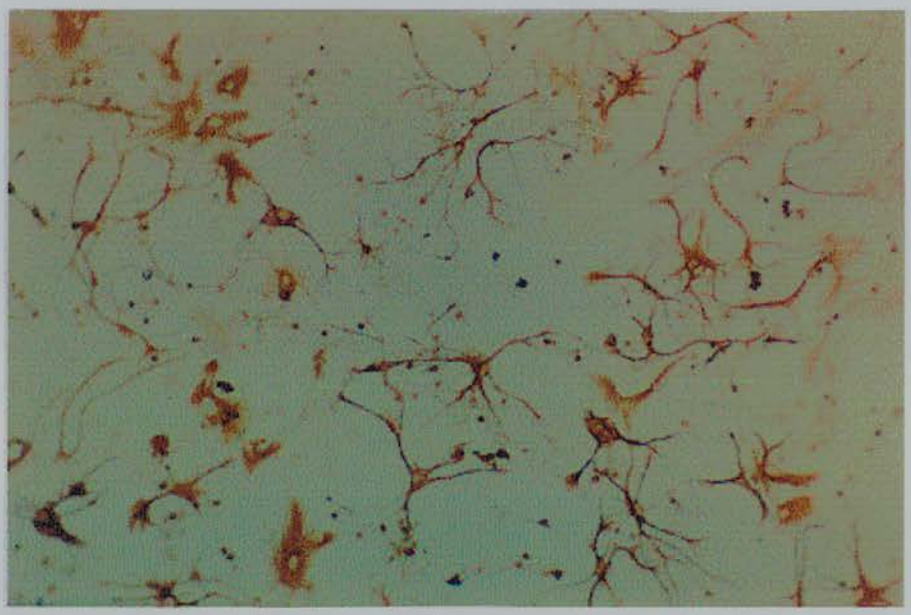


Figure 5.4: *RNase protection analysis of the effect of prenatal dexamethasone exposure on the abundance of exon $1_{4.5}$-containing GR mRNA in rat liver*

RNase protection assays were carried out on 50μg total RNA from the livers of adult male rats exposed to dexamethasone *in utero*. Odd-numbered lanes marked contained RNA from control animals and even-numbered lanes contained RNA from animals exposed to dexamethasone *in utero*. Lanes marked Y contained yeast RNA and +, undigested probe. Arrowheads indicate the positions of the 186 nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon $1_{4.5}$.

(a) GFAP



(b) NSE

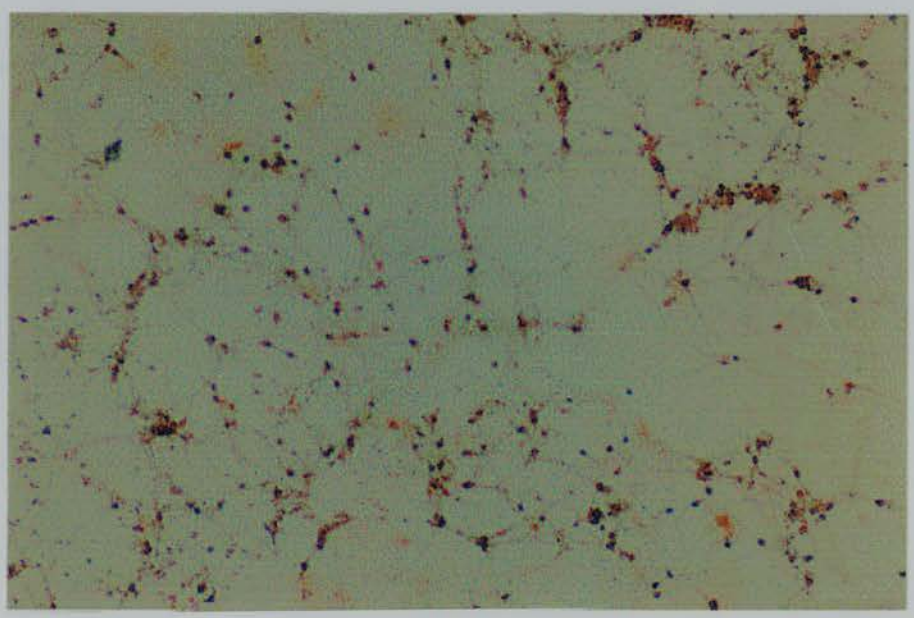


Figure 5.5: *Immunocytochemistry on primary hippocampal cultures confirms a predominantly neuronal cell composition*

Immunocytochemistry on primary hippocampal cultures using (a) anti-glial fibrillary acidic protein (GFAP) and (b) anti-neuron-specific enolase (NSE) primary antibodies. The chromagen used to localise peroxidase was 3-amino-9-ethyl carbazole; x10 magnification.

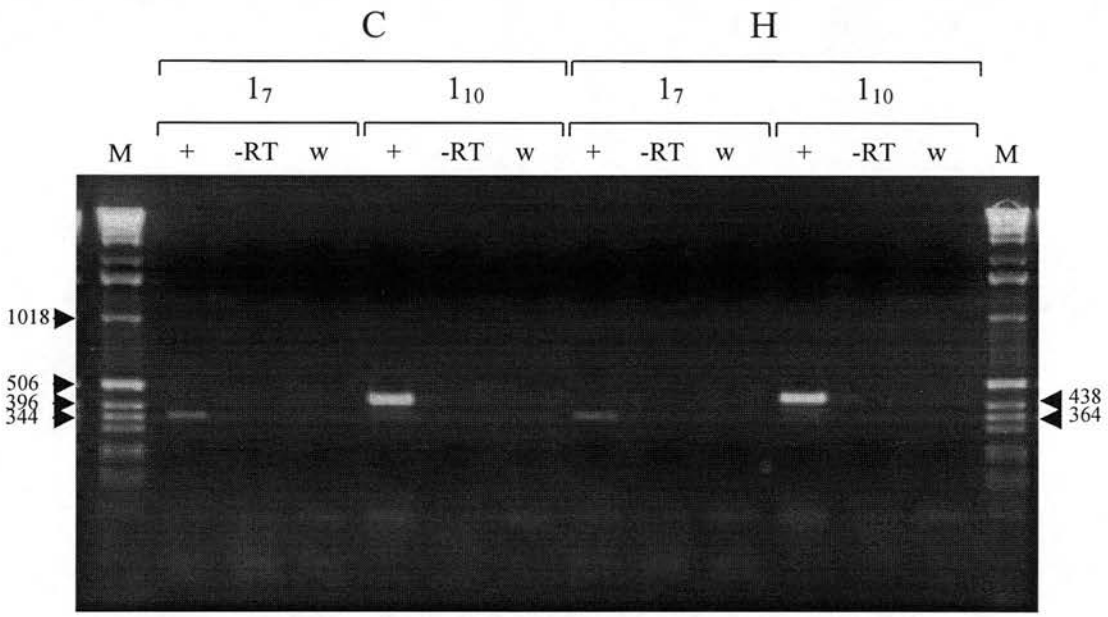


Figure 5.6: *Primary hippocampal cultures express exon 1_7-containing GR mRNA*

2μg of total RNA isolated from primary hippocampal cultures (C) and hippocampus (H) was reverse transcribed and subjected to PCR as described (section *2.2.2.8.2*). 5' primers were specific to either exon 1_7 or exon 1_{10}; the 3' primer to exon 2. 10μl of product was electrophoresed on a 1% agarose gel (+); control from which reverse transcriptase was omitted (-RT) or which contained water instead of RNA (w) were run in parallel. The predicted sizes of the PCR products were 364bp for exon 1_7 and 438bp for exon 1_{10}.

5.4 Discussion

RNase protection analysis has demonstrated that prenatal exposure to dexamethasone significantly reduces the level of exon 1_{10}-containing GR mRNAs as a proportion of total GR mRNAs. As discussed in Chapter 3, it is likely that exon 1_{10}-containing transcripts initiate from a constitutively active promoter. In these animals, prenatal dexamethasone exposure in week 3 of gestation resulted in a 25% increase in GR mRNA expression in the periportal region of the livers when the rats reached 8m of age (Nyirenda, M. J. et al. 1998). If prenatal dexamethasone exposure does not affect expression of hepatic exon 1_{10}-containing GR mRNAs, one would expect *relative* levels of exon 1_{10}-containing GR mRNAs to decrease as a proportion of total GR mRNAs as *absolute* levels of GR mRNA increase following dexamethasone exposure. Presuming there is no change in the absolute level of exon 1_{10}-containing GR mRNAs, the 10% decrease as a proportion of GR mRNAs described here is not quite sufficient to account for the 25% increase in absolute GR mRNA levels observed in these animals. The 25% increase in hepatic GR mRNA levels is, however, restricted to the periportal region of the liver; the RPAs performed here were performed using RNA from whole liver. Therefore it is likely that the decrease in relative levels of exon 1_{10}-containing GR mRNAs has been somewhat "diluted". Thus, it seems likely that a minor variant exon 1 is induced by prenatal dexamethasone exposure, reflecting increased activity of an associated promoter. I was unable to identify an induced exon 1; this may also have been partly due to "dilution" of periportal GR mRNAs by using total RNA from whole liver. However, the experiments did reveal some interesting data pertaining to tissue distribution of alternate exon 1-containing GR mRNAs (the focus of Chapter 3). Firstly, exon 1_1-containing GR transcripts were detected in liver, though at a very low level (1% of total GR transcripts); previously, transcripts containing this alternate exon 1 were undetectable (Figure 3.8 and Table 3.8). There are two possible explanations for this difference. Firstly, the rats used in the set of experiments described in Chapter 3 were 4m old whereas those used in these experiments were 8m old. The difference observed may therefore be age-related. More likely, however, is the second possibility that the exon 1_1-containing GR mRNA is not derived from hepatocytes,

but is expressed in lymphocytes in the large volume of blood perfusing the liver. Secondly, 5'-RACE PCR performed by Val Lyons on total RNA isolated from the livers of animals exposed to dexamethasone *in utero* identified a novel exon 1 variant, exon $1_{4.5}$, not found by 5'-RACE PCR in hippocampus. This exon 1 variant was also present in livers from control animals. The tissue abundance of exon $1_{4.5}$-containing GR mRNAs was not determined in the experiments described in Chapter 3, and the sums of abundances already obtained suggested that the majority of (but not all) GR mRNA had already been accounted for by the exons 1 examined. The 5'-RACE experiments suggested that exon $1_{4.5}$ was expressed more highly in the livers of animals exposed to dexamethasone *in utero* than in control animals (data not shown). However, RNase protection analysis failed to detect the presence of exon $1_{4.5}$-containing GR mRNA in total RNA from the livers of either group, presumably because the expression level was below the sensitivity of the assay.

Although the magnitude of the increase in hepatic GR programmed by prenatal dexamethasone treatment may be small, the potential pathophysiological effects may be profound (Levitt, N. S. et al. 1996; Nyirenda, M. J. et al. 1998; Welberg, L. A. M et al. 2000). The association between glucocorticoids and growth retardation may be linked to the regulation of key growth factors by GR. Dexamethasone in late gestation induces expression of hepatic insulin-like growth factor binding protein-1 (IGFBP-1), which antagonises the actions of insulin-like growth factor (IGF), a key growth factor in late gestation (Price, W. A. et al. 1992; Heyner, S. et al. 1994). Interestingly, overexpression of IGFBP-1 in transgenic mice results in low birth weight and subsequent hyperglycaemia (Rajkumar, K. et al. 1995). A key hepatic enzyme involved in the control of plasma glucose is PEPCK (Friedman, J. E. et al. 1993), the rate-limiting enzyme in gluconeogenesis. As well as increased hepatic GR, prenatal dexamethasone permanently increases PEPCK mRNA in the periportal region of the liver (Nyirenda, M. J. et al. 1998), the major site of hepatic gluconeogenesis. PEPCK overexpression in a rat hepatoma cell line impairs suppression of gluconeogenesis (Rosella, G. et al. 1993) and overexpression *in vivo* causes glucose intolerance (Valera, A. et al. 1994). Regulation of PEPCK by GR may therefore be critical in the development of glucose intolerance, and ultimately

NIDDM. The link between low birth weight and subsequent adult pathology may pivot critically on hepatic GR levels. The data presented here show that prenatal dexamethasone reduces the proportion of GR mRNA containing the major variant exon 1 (exon 1_{10}) in liver. The clear implication is that another, minor, variant GR mRNA is induced by prenatal dexamethasone, though I was unable to identify one. Interestingly, prenatal dexamethasone leads to a permanent *reduction* in hippocampal GR (Levitt, N. S. et al. 1996). Thus, the same treatment results in the opposite effect in different tissues, namely hippocampus and liver. Differences in transcriptional regulation of the GR gene might explain how GR increases in liver but decreases in hippocampus. Elucidation of the precise mechanism of hepatic GR regulation by prenatal dexamethasone will help explain the link between low birth weight and adult disease.

In rats, hippocampal GR levels are programmed by neonatal handling (Sarrieau, A. et al. 1988; Meaney, M. J. et al. 1988); the critical time window for the handling effect is the first week of life. Handled rats have lower basal plasma corticosterone levels, improved suppression of the HPA axis in response to stress and improved cognitive function in old age (Sarrieau, A. et al. 1988; Meaney, M. J. et al. 1988). Given the proposed role of GR activation in feedback regulation of the HPA axis, HPA axis suppression would be predicted to improve with elevation of hippocampal GR levels. Improved cognitive function in old age may be related to a reduction in exposure of hippocampal neurons to damaging levels of elevated glucocorticoids (section *1.2.6*). The mechanism of the handling-induced increase in hippocampal GR is related to altered 5-HT neurotransmission (Mitchell, J. B. et al. 1990b) and it has been shown that the effects of 5-HT expression can be recapitulated *in vitro*. Administration of 5-HT to primary hippocampal cultures results in increased GR expression, which is maintained after removal of 5-HT from the culture medium (Mitchell, J. B. et al. 1990a; Mitchell, J. B. et al. 1992). The data presented here showed no effect of 5-HT treatment on the distribution of known variant exons 1 in primary hippocampal culture, and no induction of novel exons 1. It should be noted, however, that very few independent clones were obtained, so no quantitative interpretation can be made of these data. The majority of clones obtained from both groups contained exon 1_{10};

a small number of exon 1_{11}-containing clones were also isolated. These data are similar to those describing abundances of alternative exon 1-containing GR mRNAs in hippocampus (Table 3.3). In hippocampus, the majority of GR mRNAs contain exon 1_{10}, a small proportion exon 1_{11}, and the remainder other variant exons 1, including the hippocampus-specific exon 1_7. Thus, the 5'-RACE PCR data described here suggests that primary hippocampal neurons in culture resemble adult hippocampus in their exon 1-containing GR mRNA composition.

Neonatal handling permanently programmes hippocampal GR and might do this by inducing exon 1_7 variant GR mRNA. Handling leads to a 2.5-3-fold increase in exon 1_7-containing GR mRNAs across all subfields of the hippocampus while exon 1_{10}, 1_5 and 1_{11}-containing GR mRNAs are unaffected (McCormick, J. A. et al. 2000). In cortex, where neonatal handling has no effect on GR expression, exon 1_7-containing GR mRNA levels are unchanged by handling (McCormick, J. A. et al. 2000). No exon 1_7-containing clones were obtained by 5'-RACE PCR on RNA from primary hippocampal cultures. One explanation for this is that primary hippocampal foetal cultures do not express exon 1_7-containing GR mRNA. Another is simply that clones containing exon 1_7 were missed: clones containing inserts that appeared to be identical in size were not sequenced to ensure sequenced clones were independent. Some of these discarded clones may have differed in size slightly and thus have been independent. Furthermore, clones with inserts close to the cut-off size of approximately 220bp (the size of inserts containing exon 2 and polylinker sequence after *EcoR*I digestion in all clones) were not sequenced since it was thought they would not contain a reasonable length of exon 1 sequence. Since neonatal handling increases exon 1_7-containing GR mRNA levels in hippocampus, and 5-HT treatment of primary hippocampal cultures increases GR expression (Mitchell, J. B. et al. 1990a; Mitchell, J. B. et al. 1992), RT-PCR was used to determine whether primary hippocampal cultures express exon 1_7-containing GR mRNA. The data described here show that primary hippocampal cultures *do* express exon 1_7-containing GR mRNA. Primary hippocampal cultures may therefore provide an appropriate *in vitro* system to further dissect the molecular mechanisms of GR upregulation by neonatal handling. Indeed, the transcription factors AP2 and NGFI-A have been implicated in

the induction of GR in the hippocampus following handling or with 5-HT (Meaney, M. J. et al. 2000). As described in detail in Chapter 4, a region upstream of exon 1_7 conferring CNS-derived cell line-specific promoter activity contains putative binding sites for AP2 and NGFI-A. Hence, there is the intriguing possibility that the handling effect on GR expression is mediated by the actions of one of these transcription factors on activity of the promoter associated with exon 1_7.

Chapter 6: Discussion

GR has many functions in many tissues, and levels of GR can vary greatly between tissues. Levels can also vary within tissues both perinatally and in adulthood in response to environmental stimuli (sections *1.1.4.2* and *1.3*). Thus, the transcriptional regulation of the GR gene is likely to be complex in order to allow effective control of GR levels. Transcription of the rat GR gene results in the production of at least 12 alternate exon 1-containing GR mRNAs (McCormick, J. A. et al. 2000) which may reflect transcription controlled by alternate promoters. The aims of this thesis were to determine whether variant GR mRNAs differ in their tissue distribution, if promoter activity is associated with alternate exons 1, and whether perinatal programming of GR is established by differential regulation of GR promoters.

The data described in Chapter 3 reveal that exon 1_{10}-containing GR mRNA is ubiquitous and is present in the majority of GR mRNAs in heart, hippocampus, kidney, liver, lung and thymus. Other alternate exons 1 mRNAs exhibit tissue-specific distributions, though exon 1_6-containing GR mRNA may also be expressed ubiquitously. Notably, exon 1_1-containing GR mRNA is only detectable in thymus while exon 1_7-containing GR mRNA is only detectable in hippocampus. Ubiquitous expression of exon 1_{10}-containing GR mRNA (and possibly exon 1_6-containing GR mRNA) may reflect use of a constitutive promoter that maintains a basal level of GR expression. Tissue-specific distributions of other exons 1 may reflect regulated expression. There are several examples of genes containing a promoter with a ubiquitous expression and alternative promoters with a more restricted spatial or temporal expression pattern; the human porphobilinogen deaminase gene contains both a housekeeping and a tissue-specific promoter (Chretien, S. et al. 1988). There is evidence from *in vivo* studies that there is a minimum level of GR expression in hippocampus and amygdala. A 2-4 fold increase in endogenous corticosterone in chronically stressed rats results in specific decreases (not attributable to loss of neurons) in GR levels in these regions (Sapolsky, R. M. et al. 1984a). Increasing serum corticosterone with further injections led to no further reductions in GR levels.

Furthermore, aged rats display significant declines in GR levels in hippocampus and amygdala (Sapolsky, R. M. 1983; Sapolsky, R. M. 1985a), and chronic stress results in no further reductions (Sapolsky, R. M. et al. 1984b). These data suggest a "floor-effect", or minimal level of constitutive production of GR; it would be interesting to determine whether the GR mRNA populations in the hippocampi of such animals are purely exon 1_{10}-containing (and/or exon 1_6-containing). The lowest level of exon 1_{10} expression is in the CA3 subregion of hippocampus (McCormick, J. A. et al. 2000). Interestingly, there are differing effects of glucocorticoids on electrophysiological properties of neurons in the CA1 and CA3 subregions of the hippocampus (Okuhara, D. Y. et al. 1998). Thus, variations in hippocampal GR levels between hippocampal subregions may be critical in determining hippocampal functions. Chronic exposure to various stressors can down-regulate GR mRNA and/or protein in the CA1 and CA2 hippocampal subregions (Sapolsky, R. M. et al. 1984a; Sapolsky, R. M. et al. 1985b; Makino, S. et al. 1995; Kitraki, E. et al. 1999), while GR mRNA is not altered in the CA3 subregion (Kitraki, E. et al. 1999). However, CA3 neurons are the most susceptible to damage by chronic stress (Watanabe, Y. et al. 1992). Thus it is conceivable that in CA3, GR promoter activity is already minimal, with no possibility of further reduction. Reduction of GR in CA1 and CA2 may serve to protect neurons from the neurotoxic effects of GR, but since CA3 neurons project onto CA1 and CA2, loss of CA3 neurons will influence the functional integrity of the entire hippocampus. Further evidence for a minimal level of GR expression comes from the experiments in Chapter 4 in which C6 glioma cells transfected with P2 (activity of which reflects activity of all promoters in the CpG island) were treated with dexamethasone. Autoregulation of GR appears to occur at least partly at the promoter level but a concentration of dexamethasone known to decrease GR expression maximally (Rosewicz, S. et al. 1988) did not eliminate P2 activity completely. Future work will identify the precise regions associated with this autoregulatory effect and determine the mechanism of GR gene repression by glucocorticoids.

Transient transfection assays (Figure 4.5) revealed that promoter activity is associated with the CpG island that contains 10 of the alternate exons 1. Furthermore, promoter activity is associated with individual exons 1. GC-rich sequences are able to interact

with a large number of different transcription factors, including Sp1 and AP2 (Azizkhan, J. C. et al. 1993), and this may provide greater regulatory options via alternate signal transduction pathways. Exon 1_{10}-containing GR mRNA exhibits a large degree of heterogeneity in transcription start sites (Figure 4.13) as is typical of genes containing CpG islands in their promoter regions. Other GR exons 1 also have multiple transcription starts (data not shown; V. Lyons, personal communication). This may simply reflect the large size of the CpG island. The reason that certain exons 1 are more highly expressed in some tissues may be a result of differences in the populations of transcription factors between tissues, or differences between promoters in their affinities for transcription factors (e.g. the promoter associated with exon 1_{10} is likely to contain high affinity sites). In addition to merely increasing GR protein levels by increasing GR mRNA, increased activity of alternate GR promoters may increase GR expression by producing GR mRNAs with increased stability. Alternatively, mRNAs with a large degree of secondary structure are likely to be translated less efficiently (Kozak, M. 1991). The existence of so many alternate promoters may allow more effective fine-tuning of GR levels.

One intriguing possibility is that there is transcription of *all* the possible exons 1 within the CpG island. *In vivo*, differing affinities for transcription factors will lead to promoter competition for the limited pool of factors. Low affinity sites will still bind transcription factors to some degree, resulting in a very low expression of transcripts derived from associated promoters. As signal transduction pathways are activated and levels of transcription factors increase, promoters associated with low affinity binding sites will become more active. There is preliminary evidence from this laboratory that *all* the putative promoters lying within the CpG island may be "constitutively" active, although those active at very low levels are unlikely to be of significance *in vivo* since their contribution to total GR mRNA levels will be extremely small. Recent work has shown that most exons 1 located in the CpG island (exons $1_{4.5}$ to 1_{11}) are detectable by RT-PCR (which can theoretically detect a single transcript) in all tissues examined (Freeman, A., personal communication), including those in which expression is undetectable by RPA (with a lower limit detection of about 0.5% of total GR mRNAs). One would predict that GR transcripts containing exons 1_1, 1_2 and 1_3, which lie outside

the CpG island and are located 5' of –15000 exhibit true tissue-specific regulation, being present in some tissues but absent from others due to their controlling promoters being "on" or "off". One problem with testing this hypothesis, however, is that if they are expressed in lymphocytes (as has been demonstrated to a limited extent for exon 1_{10}), peripheral blood contamination of tissues may result in false positive results (Table 5.1). Indeed, RT-PCR is able to detect the presence of exon 1_1-containing GR mRNA in all tissues studied, including non-immune tissues (Freeman, A., personal communication). One solution to this problem would be to flush out peripheral blood from tissues by perfusing with cold saline prior to killing the animal or to look in cell lines. However, the use of cell lines would impose the difficulty of extrapolating to the *in vivo* situation.

The mechanism by which heterogeneous transcription starts results in alternate splicing to generate multiple exons 1, some of which are overlapping may hinge on how the donor splice site is determined. It may merely be a "default" mechanism that can sometimes be inaccurate (e.g. in hippocampus giving rise to a large number of alternate exons 1 present at significant levels). Alternatively, the process may be regulated by the splicing machinery, or simply splicing at the first suitable splice donor site.

The perinatal environment can permanently program levels of GR expression within tissues with profound consequences on adult physiology and pathophysiology (section *1.3*). Data presented within Chapter 5 begin to elucidate the mechanism of programming of GR.

When adult, rats exposed to dexamethasone *in utero* exhibit a reduction in the proportion of exon 1_{10} in their livers (Table 5.1). This suggests the level of a minor variant GR mRNA is increased, accounting for the increase in hepatic GR associated with this treatment. I was unable to identify a minor variant upregulated by prenatal dexamethasone which may account for the increased hepatic GR observed in these animals but this may have been due to a "dilution" effect, or induction of an untested or as yet unidentified exon 1-containing GR mRNA. Prenatal dexamethasone permanently programs hepatic GR specifically in the periportal region of the liver (Nyirenda, M. J. et

al. 1998), and this may occur via a classic hormonal imprinting mechanism (Levine, S. et al. 1966). Furthermore, prenatal dexamethasone treatment permanently increases periportal PEPCK mRNA expression, with consequences on glucose metabolism (Nyirenda, M. J. et al. 1998). Whether this induction of PEPCK is a direct result of the increased GR is unclear. The best way to assess precisely which alternate GR mRNA is increased by prenatal dexamethasone would be *in situ* mRNA hybridisation. This would allow precise localisation of variant GR mRNA levels (notably at the periportal region) without the dilution problem associated with using RNA prepared from whole liver. Interestingly, the increase in hepatic GR following prenatal dexamethasone is more pronounced 5d after birth. Examination of these tissues may provide a more sensitive means of elucidating the programming mechanism.

The hippocampus-specific expression of exon 1_7-containing GR mRNA is especially interesting. In rats, neonatal handling permanently increases hippocampal GR, leading to permanent alterations in HPA axis suppressibility post-stress and a reduction in age-related cognitive impairment (section *1.2.6*). Altered serotonergic neurotransmission is associated with the handling effect, and in primary hippocampal cultures 5-HT leads to a long-term increase in GR expression that persists after removal of 5-HT from the culture medium (Mitchell, J. B. et al. 1990a; Mitchell, J. B. et al. 1992). We have shown that neonatal handling specifically increases the expression level of exon 1_7-containing GR mRNA in hippocampus but not in other brain regions (McCormick, J. A. et al. 2000). Primary rat hippocampal cultures may be a useful model system to study the mechanism of GR induction by 5-HT (and by extrapolation in rat hippocampus by neonatal handling) since they express GR mRNA containing exon 1_7 (Figure 5.6). Interestingly, a construct in which transcription runs through exon 1_7 exhibits substantially higher promoter activity in C6 and B103 cells (both CNS-derived) than in HepG2 (hepatoma) cells (Figures 4.5 and 4.10). Further analysis of a region flanking exon 1_7 revealed that a 134bp fragment acts as an orientation-independent enhancer in B103 cells but not in HepG2 cells. This 134bp region contains putative binding sites for NGFI-A and AP2, transcription factors whose expression is increased in the hippocampus by neonatal handling (Meaney, M. J. et al. 2000). In contrast, stress is associated with a reduction in hippocampal GR (section *1.1.4.2*); intriguingly, NGF

binding is reduced in the hippocampus after cold stress (Taglialatela, G. et al. 1991). Further analysis is required to determine if these transcription factors do indeed bind to this region and play a role in regulation of an associated promoter.

Another interesting candidate transcription factor for regulation of the rat GR gene promoter is Sp1, which can bind to the same DNA binding site as NGFI-A. Sp1 can be permanently increased in the rat hippocampus by a single kainic acid treatment, and this programming of Sp1 parallels a permanent induction of the proenkephalin gene (Feng, Z. H. et al. 1999). Furthermore, Sp1 expression in the brain has been reported to decrease with age (Ammendola, R. et al. 1992), raising the possibility that the reduction in Sp1 is linked to HPA axis dysregulation in old age by reducing hippocampal GR expression. Sp1 has been linked to the maintenance of methylation-free CpG islands (Brandeis, M. 1994; MacLeod 1994, D.). Induction of Sp1 or related transcription factors (including NGFI-A) by neonatal handling may protect regulatory regions associated with exon 1_7 expression from methylation and hence silencing. Animals that have not been handled do not exhibit zero expression of exon 1_7-containing GR mRNA, so perhaps rather than being completely silenced by methylation, only a few key regulatory elements are affected, leading to the permanent reduction in expression. Though the majority of DNA methylation associated with gene silencing occurs early in development (section *1.4.3*), *de novo* DNA methylation can occur in later life (Issa, J. P. J. et al. 1994; Issa, J. P. J. et al. 1996).

Other interesting avenues to explore with regard to regulation of hippocampal GR include the effects of prenatal dexamethasone exposure, which permanently reduces hippocampal GR. The effects of this treatment on the cognitive function of aged animals is unknown, though younger animals exposed to dexamethasone *in utero* exhibit higher anxiety-related behaviours (Welberg, L. A. M et al. 2000). It would be interesting to see if this manipulation *reduces* exon 1_7-containing GR mRNA expression in the hippocampus and/or amygdala. In rats, hippocampal GR expression follows a diurnal rhythm, peaking in the morning and troughing in the evening (the nadir of plasma corticosterone is in the morning and the peak in the evening) (Holmes, M. C. et al. 1995). In contrast to other exon 1-containing GR mRNAs, exon 1_7-containing GR

mRNA levels appear to be highly variable (Chapter 3) though an attempt to determine whether this reflected the time of tissue sampling was unsuccessful (data not shown).

Little is known about programming of GR in the immune system, though HPA axis dysregulation is associated with alterations in immune responses (section *1.2.6*). GR levels can be permanently programmed in the thymus by various environmental manipulations but the effects of this programming on immune function are unclear. Exon 1_1-containing GR mRNA accounts for a substantial proportion of GR mRNA in thymus (Table 3.3). It has been suggested that expression of a homologous mouse exon 1 targets GR to the plasma membrane and may mediate the apoptosis of lymphocytes induced by glucocorticoids (Chen, F. H. et al. 1999a; Chen, F. H. et al. 1999b), though both of these hypotheses have not been conclusively proven. The location of exon 1_1 (and exon 1_2) upstream of the CpG island may reflect the evolutionary development of transcriptional regulation of the GR gene.

In summary, the data presented in this thesis show that alternative exon 1-containing GR mRNAs are expressed in a tissue-specific manner. Most of these alternate exons 1 lie within a large (approximately 3kb) CpG island; considerable heterogeneity in transcription initiation points for alternate GR mRNAs reflects this promoter region structure. Transient transfection assays revealed that promoter activity is associated with this CpG island, and also with individual exons 1. The promoter activity associated with the entire CpG island can be repressed by the GR agonist dexamethasone, confirming that GR autoregulation occurs at least partly at the promoter level. Perinatal manipulations can permanently alter the expression level of GR in a number of tissues with long-term effects on physiology and pathophysiology. I have shown that prenatal dexamethasone exposure, which permanently reduces hepatic GR, leads to a decrease in the proportion of hepatic GR mRNAs expressing exon 1_{10}, the ubiquitously expressed variant GR mRNA. This suggests that a minor variant GR mRNA is induced by prenatal dexamethasone that results in increased hepatic GR expression, though I was unable to determine the specific variant. Neonatal handling leads to a permanent increase in hippocampal GR probably via an induction of a promoter associated with the hippocampus-specific exon 1_7. Treatment of primary

hippocampal cultures with 5-HT mimics the handling effect, and I have shown that they express exon 1_7-containing GR mRNA. These cultures may therefore provide a useful model system to study the handling effect *in vitro*. A 134bp region associated with the 5' end of exon 1_7 acts as an orientation-independent enhancer in neuroblastoma cells but not in hepatoma cells and contains putative binding sites for NGFI-A and AP2, both of which are induced by neonatal handling. This adds weight to the hypothesis that the signal transduction pathway activated by neonatal handling increases hippocampal GR by inducing a promoter associated with exon 1_7. However, further experiments are required to confirm this.

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5′-Heterogeneity of Glucocorticoid Receptor Messenger RNA Is Tissue Specific: Differential Regulation of Variant Transcripts by Early-Life Events

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Glucocorticoid receptor (GR) gene expression is regulated in a complex tissue-specific manner, notably by early-life environmental events that program tissue GR levels. We have identified and characterized several new rat GR mRNAs. All encode a common protein, but differ in their 5′-leader sequences as a consequence of alternate splicing of, potentially, 11 different exon 1 sequences. Most are located in a 3-kb CpG island, upstream of exon 2, that exhibits substantial promoter activity in transfected cells. Ribonuclease (RNase) protection analysis demonstrated significant levels of six alternate exons 1 *in vivo* in rat, with differences between liver, hippocampus, and thymus reflecting tissue-specific differences in promoter activity. Two of the alternate exons 1 (exons 1_6 and 1_{10}) were expressed in all tissues examined, together present in 77–87% of total GR mRNA. The remaining GR transcripts contained tissue-specific alternate first exons. Importantly, tissue-specific first exon usage was altered by perinatal environmental manipulations. Postnatal handling, which permanently increases GR in the hippocampus, causing attenuation of stress responses, selectively elevated GR mRNA containing the hippocampus-specific exon 1_7. Prenatal glucocorticoid exposure, which increases hepatic GR expression and produces adult hyperglycemia, decreased the proportion of hepatic GR mRNA containing the predominant exon 1_{10}, suggesting an increase in a minor exon 1 variant. Such tissue specificity of promoter usage allows differential GR regulation and programming. (Molecular Endocrinology 14: 506–517, 2000)

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INTRODUCTION

Glucocorticoids maintain homeostasis after stress and play key roles in differentiation, nervous system function, and intermediary metabolism (1). The principal receptor for glucocorticoids, the type II or glucocorticoid receptor (GR), a member of the nuclear receptor family (reviewed in Ref. 2), is essential for life *ex utero* (3). Although GRs are expressed in almost all cells, the level of expression and receptor regulation vary considerably between tissues, and even within a tissue [*e.g.* hippocampus (4)]. The importance of maintaining an appropriate level of expression of GR for the functional effects of glucocorticoids has been demonstrated *in vitro* (5) and in transgenic mice a reduction of 30–50% in tissue levels of GR results in major neuroendocrine, metabolic, and immunological abnormalities (6, 7). The level of expression of GR is thus critical for the correct level of function of a cell. In some tissues, GRs are regulated by glucocorticoids them-

selves, but again the regulation is highly tissue specific, with GR down-regulated by glucocorticoids in some tissues, but unaltered or even induced in others (8–10). This regulation occurs chiefly at the level of transcription (9, 11). GR gene transcription therefore must be tightly regulated with appropriately high expression for the function of any particular cell.

Much evidence suggests that GR gene transcription is, in part, permanently determined or programmed by perinatal events, again in a cell-specific manner. Thus, animals exposed to short periods of infantile stimulation (handling) have, as adults, permanently elevated GR expression selectively in hippocampal neurons (12). The hippocampus is a site of glucocorticoid feedback inhibition upon the hypothalamic-pituitary-adrenal (HPA) axis, and adult rats handled as neonates are therefore more sensitive to glucocorticoid-negative feedback with decreased HPA responsivity to stress throughout life (12, 13). In contrast, prenatal treatment of rats with the synthetic glucocorticoid dexamethasone permanently reduces GR mRNA in the hippocampus (14), but increases GR mRNA in the liver (15). These animals have permanently elevated levels of plasma corticosterone, fasting hyperglycemia (attributable to elevated levels in liver of the glucocorticoid-inducible enzyme, phosphoenolpyruvate carboxykinase, the rate limiting step in gluconeogenesis), hyperinsulinemia (15), and hypertension (14). A key question therefore, is how can GR mRNA levels be regulated in a cell-specific and even opposite manner during adult life and particularly by prenatal manipulations?

Surprisingly little is known of the mechanisms that control GR gene transcription. The GR gene spans more than 80 kb and contains 8 coding exons (exons 2 to 9) (16, 17). The human (16, 18), mouse (17, 19), and rat (20) (M. D. Jacobson and K. R. Yamamoto, unpublished data) GR gene promoter regions have been cloned and partially characterized. A single promoter has been described for the human GR gene (16, 18). In mouse, expression of the GR gene is controlled by at least 3 promoters, resulting in GR transcripts with different 5′-untranslated exons designated exons 1A (restricted to T cell lines), 1B, and 1C (the latter is homologous to the exon 1 present in the human GR cDNA) (17), and very recent evidence suggests the existence of 2 more (21). It has been suggested that rat GR mRNA might also exhibit 5′-heterogeneity (20). Little is known about GR promoter usage in tissues *in vivo*. Here we demonstrate tissue-specific 5′-heterogeneity of rat GR mRNA and present compelling evidence for early-life environmental programming of specific GR gene promoters in the hippocampus.

RESULTS

DNA Sequence of the Rat GR Gene Flanking the 5′-End of Exon 2

We have determined the sequence of 4600 bp of the rat GR gene flanking the 5′- end of exon 2 (Fig. 1). The majority of this region corresponds to a CpG island (68% CG, with a CG/GC ratio of ≥0.8 between −1620 and −4520 relative to the translation start at +1, within exon 2) and contains the exon 1 sequence present in the published rat GR cDNA sequence (22) (−3269 to −3322; here designated exon 1_6). The sequence is highly conserved when compared with the mouse GR gene (19) (91% identity throughout the whole region), including the mouse exons 1B and 1C (17). The rat sequence shows moderate conservation with the corresponding human GR gene sequence (18, 23) (~70% identity over the CpG island; nucleotides −1600 to −4220, but only 40% identity over the region between −50 and −1600), including exon 1 present in the human GR cDNA (24).

5′-RACE (Rapid Amplification of cDNA Ends)-PCR Identifies at Least 11 Alternative 5′-Leader Sequences in GR mRNA

To investigate 5′-heterogeneity in rat GR mRNA, we carried out 5′-RACE-PCR on total RNA isolated from rat hippocampus or thymus. Eight separate 5′-RACE reactions were carried out on the products of 5 different dC-tailed cDNA reactions from hippocampal RNA; one 5′-RACE reaction was carried out on dC-tailed cDNA produced from thymus RNA. Subcloned products were sequenced with a primer complementary to exon 2 of GR. A total of 54 independent 5′-RACE products were obtained from hippocampal RNA, deriving from independent cDNAs generated in the initial reverse transcription reactions (the 5′-ends of the cDNAs terminate at different positions in exon 1, indicating they are unlikely to be generated by PCR from the same initial tailed cDNA). In addition, four independent 5′-RACE products were obtained from thymus RNA. The cDNA products fall into 10 classes based on the sequences immediately upstream of exon 2 (Table 1). The majority of clones (31) from rat hippocampal GR mRNA contained exon 1_{10} (corresponding to exon 1C of the mouse GR gene and the exon 1 sequence present in the human GR cDNA sequence). In addition, we found 7 novel exon 1 sequences present in rat hippocampal GR mRNA, exons 1_3, 1_4, 1_5, 1_7, 1_8, 1_9, and 1_{11} (Table 1); exons 1_5 and 1_{11} are likely to correspond to the recently described mouse exons 1D and 1E (21), respectively. Of the minor exon 1 species, exons 1_7 and 1_{11} represented the major variants, present in 8 (exon 1_7) and 5 (exon 1_{11}) of the independent clones. Exons 1_4, 1_5, and 1_9 were present in, respectively, 3, 2, and 3 of the independent 5′-RACE clones. Two of the classes (1_3 and 1_8) were represented each by a single independent clone. No clones were found corresponding to the exon 1 sequence present in the published rat GR cDNA sequence (22) (but see below). Of the 4 independent clones produced from 5′-RACE PCR of thymus RNA, 2 contained exon 1_{10}. In addition, a further 2 novel variants of rat GR mRNA were identified in thymus RNA, containing exons 1_1 and 1_2 (Table 1). Exon 1_1 is likely to

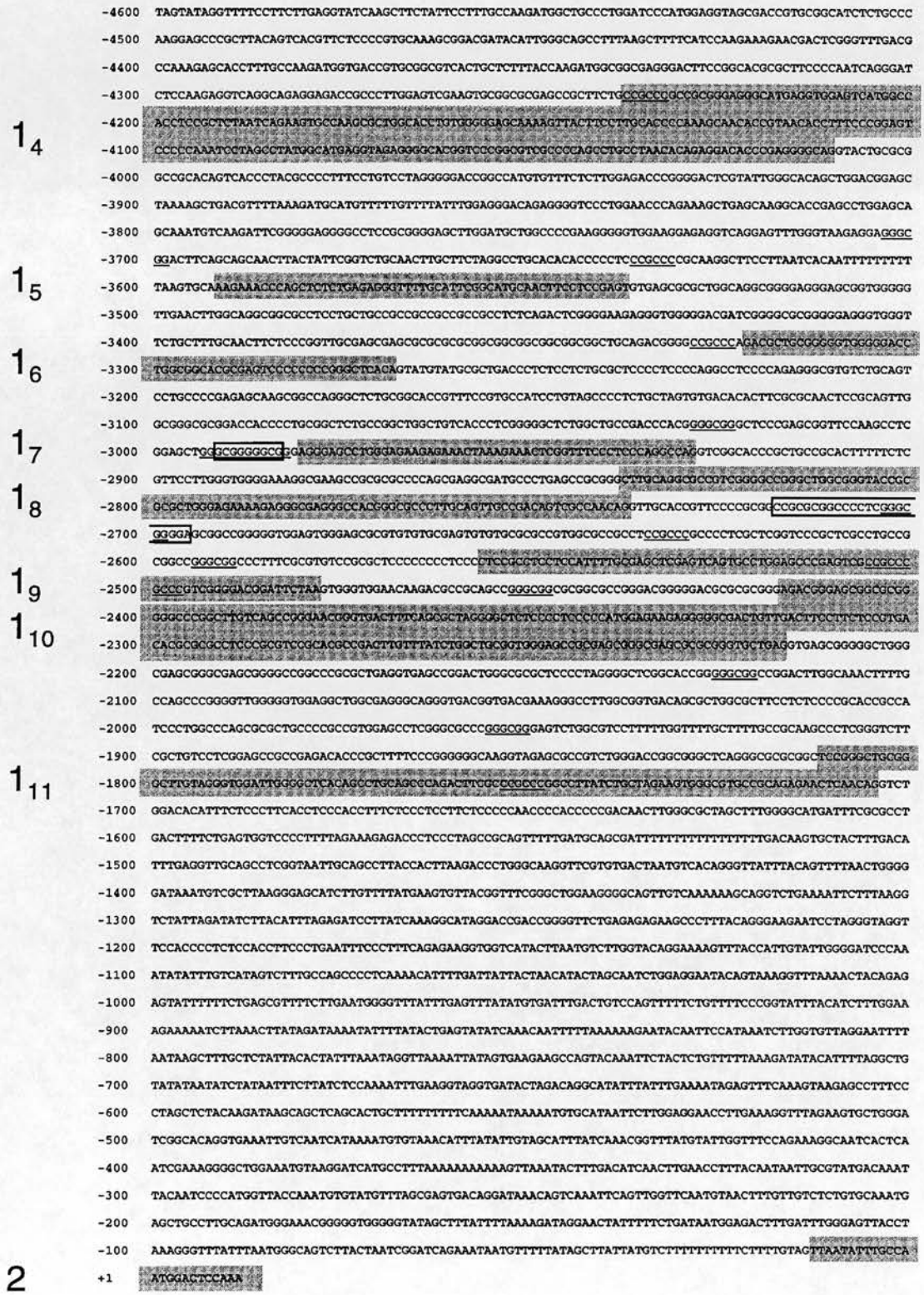
-4600 TAGTATAGGTTTTCCTTCTTGAGGTATCAAGCTTCTATTCCTTTGCCAAGATGGCTGCCCTGGATCCCATGGAGGTAGCGACCGTGCGGCATCTCTGCCC
-4500 AAGGAGCCCGCTTACAGTCACGTTCTCCCCGTGCAAAGCGGACGATACATTGGGCAGCCTTTAAGCTTTTCATCCAAGAAAGAACGACTCGGGTTTGACG
-4400 CCAAAGAGCACCTTTGCCAAGATGGTGACCGTGCGGCGTCACTGCTCTTTACCAAGATGGCGGCGAGGGACTTCCGGCACGCGCTTCCCCAATCAGGGAT
-4300 CTCCAAGAGGTCAGGCAGAGGAGACCGCCCTTGGAGTCGAAGTGCGGCGCGAGCCGCTTCTGCCGCCCGCCGCGGGAGGGCATGAGGTGGAGTCATGGCC
-4200 ACCTCCGCTCTAATCAGAAGTGCCAAGCGCTGGCACCTGTGGGGGAGCAAAGTTACTTCCTTGCACCCCAAAGCAACACCGTAACACCTTTCCCGGAGT
-4100 CCCCCAAATCCTAGCCTATGGCATGAGGTAGAGGGGCACGGTCCCGGCGTCGCCCCAGCCTGCCTAACACAGAGGACACCCGAGGGGCAGGTACTGCGCG
-4000 GCCGCACAGTCACCCTACGCCCCTTTCCTGTCCTAGGGGGACCGGCCATGTGTTTCTCTTGGAGACCCGGGGACTCGTATTGGGCACAGCTGGACGGAGC
-3900 TAAAAGCTGACGTTTTAAAGATGCATGTTTTTGTTTTATTTGGAGGGACAGAGGGGTCCCTGGAACCCAGAAAGCTGAGCAAGGCACCGAGCCTGGAGCA
-3800 GCAAATGTCAAGATTCGGGGGAGGGGCCTCCGCGGGGAGCTTGGATGCTGGCCCCGAAGGGGGTGGAAGGAGAGGTCAGGAGTTTGGGTAAGAGGAGGGC
-3700 GGACTTCAGCAGCAACTTACTATTCGGTCTGCAACTTGCTTCTAGGCCTGCACACACCCCCTCCCGCCCCGCAAGGCTTCCTTAATCACAATTTTTTTTT
-3600 TAAGTGCAAAGAAACCCAGCTCTCTGAGAGGGTTTTGCATTCGGCATGCAACTTCCTCCGAGTGTGAGCGCGCTGGCAGGCGGGGAGGGAGCGGTGGGGG
-3500 TTGAACTTGGCAGGCGGCGCCTCCTGCTGCCGCCGCCGCCGCCGCCTCTCAGACTCGGGGAAGAGGGTGGGGGACGATCGGGGCGCGGGGGAGGGTGGGT
-3400 TCTGCTTTGCAACTTCTCCCGGTTGCGAGCGAGCGCGCGCGGCGGCGGCGGCGGCGGCTGCAGACGGGGCCGCCCCAGACGCTGCGGGGGTGGGGGACCC
-3300 TGGCGGCACGCGAGTCCCCCCCCGGGCTCACAGTATGTATGCGCTGACCCTCTCCTCTGCGCTCCCCTCCCCAGGCCTCCCCAGAGGGCGTGTCTGCAGT
-3200 CCTGCCCCGAGAGCAAGCGGCCAGGGCTCTGCGGCACCGTTTCCGTGCCATCCTGTAGCCCCTCTGCTAGTGTGACACACTTCGCGCAACTCCGCAGTTG
-3100 GCGGGCGCGGACCACCCCTGCGGCTCTGCCGGCTGGCTGTCACCCTCGGGGGCTCTGGCTGCCGACCCACGGGGCGGGCTCCCGAGCGGTTCCAAGCCTC
-3000 GGAGCTGGGCGGGGGCGGAGGGAGCCTGGGAGAAGAGAAACTAAAGAAACTCGGTTTCCCTCCCAGGCCAGGTCGGCACCCGCTGCCGCACTTTTTCTC
-2900 GTTCCTTGGGTGGGGAAAGGCGAAGCCGCGCGCCCCAGCGAGGCGATGCCCTGAGCCGCGGGCTTGCAGGCGCCGTCGGGGCCGGGCTGGCGGGTACCGC
-2800 GCGCTGGGAGAAAAGAGGGCGAGGGCCACGGCGCCCTTGCAGTTGCCGACAGTCGCCAACAGGTTGCACCGTTCCCCGCGCCCGCGGCGCCCTCGGGC
-2700 GGGGAGCGGCCGGGGGTGGAGTGGGAGCGCGTGTGTGCGAGTGTGTGCGCGCCGTGGCGCCGCCTCCGCCCGCCCCTCGCTCGGTCCCGCTCGCCTGCCG
-2600 CGGCCGGGCGGCCCTTTCGCGTGTCCGCGCTCCCCCCCCTCCCCTCCGCGTCCTCCATTTTGCGAGCTCGAGTCAGTGCCTGGAGCCCGAGTCGCGGCCC
-2500 GCCCGTCGGGGACGGATTCTAAGTGGGTGGAACAAGACGCCGCAGCCGGGCGGCGCGGCCGGGACGGGGGACGCGCGCGGGAGACGGGAGCGGCGCGG
-2400 GGGCCCGGCTTGTCAGCCGGGAACGGGTGACTTTCAGCGCTAGCGGCTCTCCCCTCCCCCATGGAGAAGAGGGGGCGACTGTTGACTTCCTTCTCCCTGA
-2300 CACGCGCGCCTCCCGCGTCCGCACGCCGACTTGTTTATCTGGCTGCGTGGGAGCCGCGAGCGGGCGAGCGCGCGGGTGCTGAGGTGAGCGGGGGCTGGG
-2200 CGAGCGGGCGAGCGGGGCCGGCCCGCGCTGAGGTGAGCCGGACTGGGCGCGCTCCCCTAGGGGCTCGGCACCGGGGGCGGCCGGACTTGGCAAACTTTTG
-2100 CCAGCCCGGGGTTGGGGGTGGAGGCTGGCGAGGGCAGGGTGACGGTGACGAAAGGGCCTTGGCGGTGACAGCGCTGGCGCTTCCTCTCCCCGCACCGCCA
-2000 TCCCTGGCCCAGCGCGCTGCCCCGCCGTGGAGCCTCGGGCGCCCGGGCGGGAGTCTGGCGTCCTTTTTGGTTTTGCTTTTGCCGCAAGCCCTCGGGTCTT
-1900 CGCTGTCCTCGGAGCCGCCGAGACACCCGCTTTTCCCGGGGGGCAAGGTAGAGCGCCGTCTGGGACCGGCGGGCTCAGGGCGCGCGGCTCCGGGCTGCGG
-1800 GCTTGTAGGGTGGATTGGGGCTCACAGCCTGCAGCCAGACTTCGCCCGCCCGGCCTTATCTGCTAGAAGTGGGCGTGCCGCAGAGAACTCAACAGGTCT
-1700 GGACACATTTCTCCCTTCACCTCCCACCTTTCTCCCTCCTTCTCCCCCAACCCCACCCCCGACAACTTGGGCGCTAGCTTTGGGGCATGATTTCGCGCCT
-1600 GACTTTTCTGAGTGGTCCCCTTTTAGAAAGAGACCCTCCCTAGCCGCAGTTTTTGATGCAGCGATTTTTTTTTTTTTTTTTGACAAGTGCTACTTTGACA
-1500 TTTGAGGTTGCAGCCTCGGTAATTGCAGCCTTACCACTTAAGACCCTGGGCAAGGTTCGTGTGACTAATGTCACAGGGTTATTTACAGTTTTAACTGGGG
-1400 GATAAATGTCGCTTAAGGGAGCATCTTGTTTTATGAAGTGTTACGGTTTCGGGCTGGAAGGGGCAGTTGTCAAAAAAGCAGGTCTGAAAATTCTTTAAGG
-1300 TCTATTAGATATCTTACATTTAGAGATCCTTATCAAAGGCATAGGACCGACCGGGGTTCTGAGAGAGAAGCCCTTTACAGGGAAGAATCCTAGGGTAGGT
-1200 TCCACCCCTCTCCACCTTCCCTGAATTTCCCTTTCAGAGAAGGTGGTCATACTTAATGTCTTGGTACAGGAAAAGTTTACCATTGTATTGGGGATCCCAA
-1100 ATATATTTGTCATAGTCTTTGCCAGCCCCTCAAAACATTTTGATTATTACTAACATACTAGCAATCTGGAGGAATACAGTAAAGGTTTAAAACTACAGAG
-1000 AGTATTTTTTCTGAGCGTTTTCTTGAATGGGGTTTATTTGAGTTTATATGTGATTTGACTGTCCAGTTTTTCTGTTTTCCCGGTATTTACATCTTTGGAA
-900 AGAAAAATCTTAAACTTATAGATAAAATATTTTATACTGAGTATATCAAACAATTTTTAAAAAAGAATACAATTCCATAAATCTTGGTGTTAGGAATTTT
-800 AATAAGCTTTGCTCTATTACACTATTTAAATAGGTTAAAATTATAGTGAAGAAGCCAGTACAAATTCTACTCTGTTTTTAAAGATATACATTTTAGGCTG
-700 TATATAATATCTATAATTTCTTATCTCCAAAATTTGAAGGTAGGTGATACTAGACAGGCATATTTATTTGAAAATAGAGTTTCAAAGTAAGAGCCTTTCC
-600 CTAGCTCTACAAGATAAGCAGCTCAGCACTGCTTTTTTTTTCAAAAATAAAAATGTGCATAATTCTTGGAGGAACCTTGAAAGGTTTAGAAGTGCTGGGA
-500 TCGGCACAGGTGAAATTGTCAATCATAAAATGTGTAAACATTTATATTGTAGCATTTATCAAACGGTTTATGTATTGGTTTCCAGAAAGGCAATCACTCA
-400 ATCGAAAGGGGCTGGAAATGTAAGGATCATGCCTTTAAAAAAAAAAAAGTTAAATACTTTGACATCAACTTGAACCTTTACAATAATTGCGTATGACAAAT
-300 TACAATCCCCATGGTTACCAAATGTGTATGTTTAGCGAGTGACAGGATAAACAGTCAAATTCAGTTGGTTCAATGTAACTTTGTTGTCTCTGTGCAAATG
-200 AGCTGCCTTGCAGATGGGAAACGGGGGTGGGGGTATAGCTTTATTTTAAAAGATAGGAACTATTTTTCTGATAATGGAGACTTTGATTTGGGAGTTACCT
-100 AAAGGGTTTATTTAATGGGCAGTCTTACTAATCGGATCAGAAATAATGTTTTTATAGCTTATTATGTCTTTTTTTTTTCTTTTGTAGTTAATATTTGCCA
+1 ATGGACTCCAAA
1 4
1 5
1 6
1 7
1 8
1 9
1 10
1 11
2

Table 1. 5′-RACE Reveals at Least 11 Alternative Exons 1 in GR mRNA

Exon	Source	3′-Boundary	Position
1_1	Thymus	. . . TGGGATGAAG. . . .	5′ of −15,000
1_2	Thymus	. . . GGGCGACCGG. . . .	5′ of −15,000
1_3	Hippocampus	. . . CAACAGCAAG. . . .	5′ of −15,000
1_4	Hippocampus	. . . CGAGGGGCAGgt. . .	−4238 to −4011
1_5	Hippocampus	. . . TCCTCCGAGTgt. . .	−3592 to −3538
1_6	Liver[a]	. . . CGGGCTCACAgt. . .	−3322 to −3269
1_7	Hippocampus	. . . CCCAGGCCAGgt. . .	−2981 to −2929
1_8	Hippocampus	. . . TCGCCGACAGgt. . .	−2838 to −2738
1_9	Hippocampus	. . . CGGATTCTAAgt. . .	−2557 to −2479
1_{10}	Hippocampus, thymus	. . . GGGTGCTGAGgt. . .	−2417 to −2217
1_{11}	Hippocampus	. . . AACTCAACAGgt. . .	−1812 to −1705

A total of 10 different alternative exons 1 were found in GR mRNA from hippocampus or thymus. 54 independent 5′-RACE clones were isolated from a total of 8 different 5′-RACE reactions carried out on hippocampal RNA and a further 4 clones were isolated from thymus RNA. Sequences shown in *uppercase* are those at the 3′-end of each exon 1; sequences shown in *lowercase* (gt) represent the consensus splice site at the beginning of the following intron.

[a] Exon 1_6 [present in the published rat cDNA sequence (22)] was not detected in 5′-RACE, but is included for completeness.

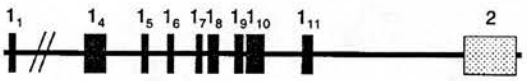

Fig. 2. Relative Positions of Alternative Exons 1 of the Rat GR Gene

Shown are the relative positions of exons 1_4, 1_5, 1_6, 1_7, 1_8, 1_9, 1_{10}, and 1_{11}, all located within the CpG island 5′ of exon 2. Exon 1_1 is located at least 15 kb 5′ of exon 2. Note, the size of exon 2 is not to scale.

correspond to mouse exon 1A (76% identity) (25), reportedly specific to T lymphocytes (17).

Sequence comparisons located 8 of the alternate exons 1 to the CpG island upstream of exon 2 (Table 1; summarized in Fig. 2). Each of the exons 1 that mapped to this region was flanked, at the 3′ end, by the conserved GT dinucleotide splice donor site (Table 1). Sequences corresponding to exons 1_1, 1_2, and 1_3 were not found within the sequenced region, and Southern blot hybridization showed they were not located within λ208 (see *Materials and Methods* for details of λ208), indicating that they lie at least 15 kb 5′ of exon 2 (data not shown).

Exon 1_{10} Is Present in the Majority of GR mRNA Transcripts in Hippocampus, Liver, and Thymus, but Some Other Promoters Are Tissue Specific

To investigate the relative abundance of each of the variant GR mRNA transcripts, RNase protection analysis was carried out with cRNA probes generated using 5′-RACE clones as templates. Each probe was complementary to a specific exon 1, and also to 186 nucleotides of exon 2 (common to all GR mRNA transcripts), thus giving 2 protected products; a fragment of 186 nucleotides representing GR transcripts containing exon 2 but lacking the target exon 1 sequence, and a larger fragment complementary to transcripts containing both exons 1 and 2. The sum of both fragments equates to total GR mRNA, allowing the amount of exon 1-exon 2 containing mRNA to be calculated as a percentage of total GR mRNA transcripts, after correction for differences in specific activity. In hippocampus, liver, and thymus, as well as heart, kidney, lung, and testis, transcripts containing exon 1_{10} predominate (Fig. 3A, Table 2, and data not shown). Interestingly, although exons 1_5 and 1_7 were both present in hippocampal GR mRNA (together accounting for ~17% of GR mRNA in hippocampus), in liver and thymus they were below the limit of detection of the RNase protection assay (*i.e.* <1% of the total) (Table 2). Furthermore, exon 1_{11}-containing transcripts were relatively more abundant in hippocampus than in liver RNA but were below the limit of detection of the assay in thymus (Table 2). Exon 1_1, identified in a 5′-RACE clone from thymus, was detectable only in thymus RNA by RNase protection analysis (Fig. 3B and Table 2). Exon 1_6 was not detected among the products of 5′-RACE PCR on hippocampal RNA, possibly as a consequence of the hybridization of the

Fig. 1. Sequence of 5′-DNA Flanking Exon 2 of the Rat GR Gene

Numbering is with respect to the translation start, at +1. *Shaded regions* indicate exon 1 sequences found in 5′-RACE clones amplified from hippocampal GR mRNA. In the case of exon 1_6 (which was not represented among the RACE clones) the *shaded* nucleotides are those present in the cDNA sequence (22). The start of exon 2 is at −13. GC boxes referred to in the text are *underlined*; a putative NGFI-A site and a sequence identical to a footprinted region in the human GR gene which binds AP2 (referred to in the text) are *boxed*.

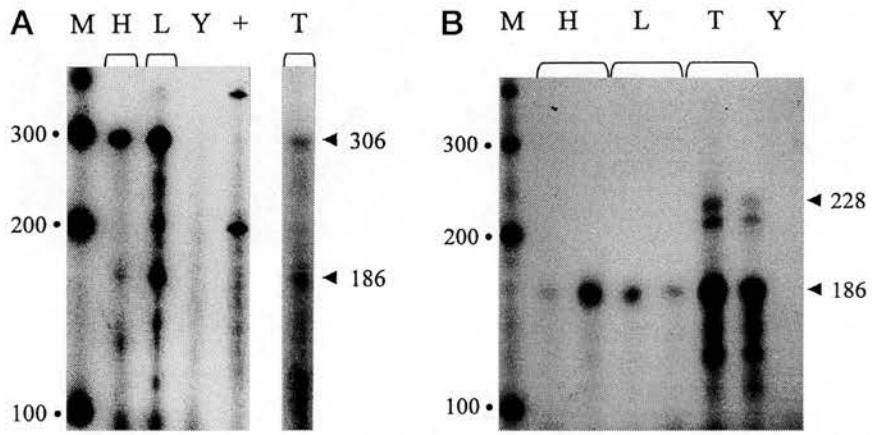

Fig. 3. RNase Protection Analysis of the Abundance of Alternative Exon 1-Containing GR mRNAs in Rat Hippocampus, Liver, and Thymus

RNase protection assays were carried out on 50 μg total RNA from adult male rat hippocampus (H), liver (L), or thymus (T). Lanes marked Y contained yeast RNA. Lanes marked + contained undigested probe; M, markers. A, RNase protection of exon 1_{10}-containing GR mRNA. *Arrowheads* indicate the positions of the 186-nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_{10} and the 306-nucleotide fragment protected by transcripts containing exon 1_{10} and exon 2. The lane containing thymus RNA was from an adjacent gel run in parallel under identical conditions. B, RNase protection of exon 1_1-containing GR mRNA. *Arrowheads* mark the 186-nucleotide fragment protected by GR mRNA transcripts containing exon 2 but not exon 1_1 and the 228-nucleotide fragment containing exon 1_1 and exon 2. Note that, for quantitation, the 215-nucleotide band (which also contains exon 1_1 spliced to exon 2) was included.

Table 2. Relative Abundance of Alternative Exon 1-Containing GR mRNA in Rat Hippocampus, Liver, and Thymus

Exon	Hippocampus	Liver	Thymus
1_1	0	0	25.6%, 27.1%
1_2	0	0	0
1_4	0	0	0
1_5	8.1%, 7.4%	0	0
1_6	17.0%, 20.7%	10.0 ± 1.3% (6)	21.9%, 19.9%
1_7	8.0 ± 3.7% (3)	0	0
1_{10}	61.3%, 63.8%	74.8%, 77.5%	52.7%, 58.9%
1_{11}	12.5%, 7.9%	2.2%, 1.7%	0

Summary of RNase protection assays carried out as described in the text. In each case, the amount of exon 1 is expressed as a percentage of the total amount of GR mRNA in that particular tissue. Where individual values are shown, the results for each of two independent experiments are given. Where the experiment was carried out more than twice, values shown represent the mean ± SEM (n). Levels of exon 1_4- and exon 1_2-containing mRNA were below the limits of detection (~1%) in the three tissues examined in each of two experiments.

5′-RACE UAP-anchor primer [which contains a $G(GGIIG)_3$ sequence] to a C_8 sequence close to the 3′-end of exon 1_6; 5′-RACE products likely to contain only very short amounts of exon 1 sequence were not analyzed. However, exon 1_6 mRNA transcripts were expressed in all three tissues, representing around 10–20% of total GR mRNA (Table 2). Levels of exon 1_4- and exon 1_2-containing GR mRNA were below the limit of detection of the RNase protection assay (Table 2).

Distribution of Alternate Exon 1-Containing GR mRNA Transcripts within the Hippocampus

Using *in situ* mRNA hybridization, we mapped the distribution of transcripts containing the major alternate first exons of the GR gene expressed in the hippocampus. A similar sized cRNA probe complementary to the 5′-end of the common exon 2 hybridized in a pattern equivalent to that documented for GR mRNA in previous studies (26) (Fig. 4A). Using cRNA probes specific to the major alternate first exons employed in the hippocampus, we found that the predominant exon 1_{10}-containing transcript was distributed very similarly to total GR mRNA, with high expression in the dentate gyrus and CA1 and lower expression in CA3 and CA4 (Fig. 4E). In contrast, GR mRNA transcripts containing exons 1_5, 1_7, or 1_{11} showed a more homogeneous distribution, although in each case expression was highest in the dentate gyrus and CA1 region of hippocampus (Fig. 4F, C, and G, respectively).

Promoter 1_7 Activity Is Highest in Central Nervous System (CNS)-Derived Cells

To investigate whether the alternate exons 1 are associated with promoter activity, regions of the rat GR gene were joined, within each of the alternate exons 1, directly to a luciferase reporter gene (Fig. 5A). Lucif-

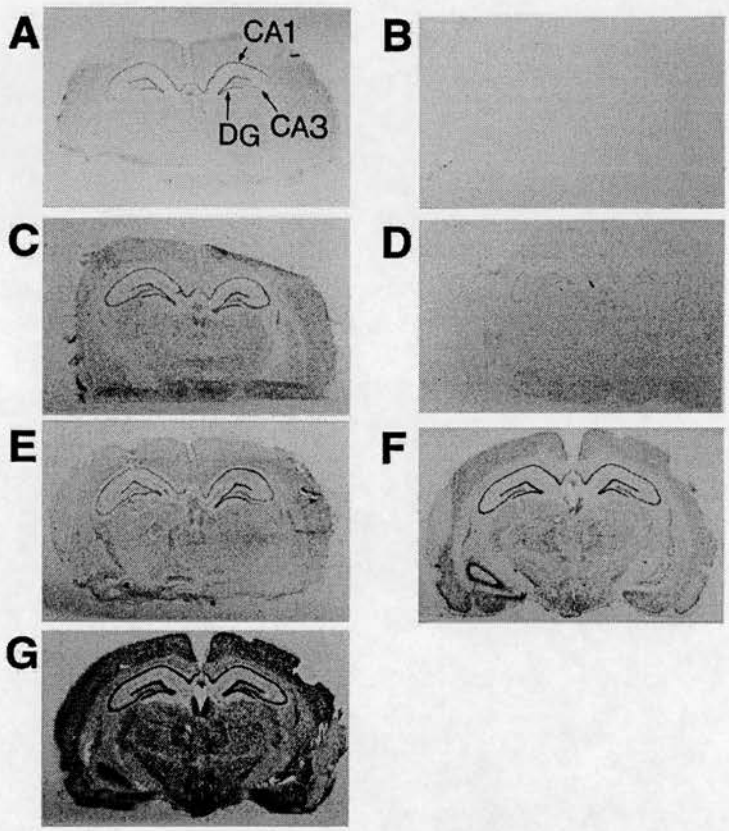

Fig. 4. *In Situ* Hybridization Analysis of the Distribution of GR mRNA within Hippocampus

In situ mRNA hybridization was carried out on rat hippocampus using exon 1-specific cRNA probes. Distribution of GR mRNA containing (A) exon 2 (total GR mRNA) and alternative exons 1 as follows: (C) exon 1_7; (E) exon 1_{10}; (F) exon 1_5; and (G) exon 1_{11}. Representative sense controls are shown in (B) exon 2 (sense control) and (D) exon 1_7 (sense control). *Arrows* indicate dentate gyrus (DG), CA1, and CA3. Exposure times were 5 days (A, B, and G), 6 days (C, D, and E), and 12 days (F).

erase activity therefore arises from chimeric RNA transcripts encoding part of an alternate exon 1 of the GR gene at the 5′-end and represents the activity of the promoter that directs transcription through that individual exon 1. Although transcription may additionally originate from alternate promoters present on the same genomic DNA fragment, these transcripts will not be transcriptional fusions to luciferase and will not, therefore, result in luciferase activity (there is no splice acceptor site upstream of the luciferase gene in these constructs). To measure promoter activity of the whole CpG island, including exons 1_4-1_{11}, plasmid P2 was constructed in which the GR gene is joined to luciferase within exon 2 (just before the translation start) (Fig. 5A). In P2, RNA initiating at any of the transcription start sites will be spliced, from the donor site at the 3′-end of the respective exon 1 onto the acceptor site at the 5′-end of the common exon 2; luciferase reporter activity therefore reflects the total promoter activity of the DNA fragment inserted into P2.

Promoter activity was assayed in transiently transfected HepG2 (human hepatoma), C6 (rat glioma), and B103 (rat neuroblastoma) cells. P2 had the highest promoter activity in all three cell lines examined, whereas the same fragment in the reverse orientation with respect to luciferase had no significant activity (Fig. 5B). Activity of all constructs was similar in all three cell lines with the exception of $P1_7$. $P1_7$ was, apart from P2, the most active construct in B103 and C6 cells but was relatively less active in HepG2 cells (Fig. 5B). Promoter activity of $P1_{10}$ was high and $P1_{11}$ activity low in all three cell lines (Fig. 5B).

Differential Regulation of Variant GR mRNA Transcripts by Early-Life Events

Neonatal handling causes marked and permanent increases in GR mRNA expression in hippocampus (27). Strikingly, neonatal handling induced expression of GR mRNA containing the hippocampus-specific exon 1_7 by 2.5- to 3-fold selectively across all hippocampal

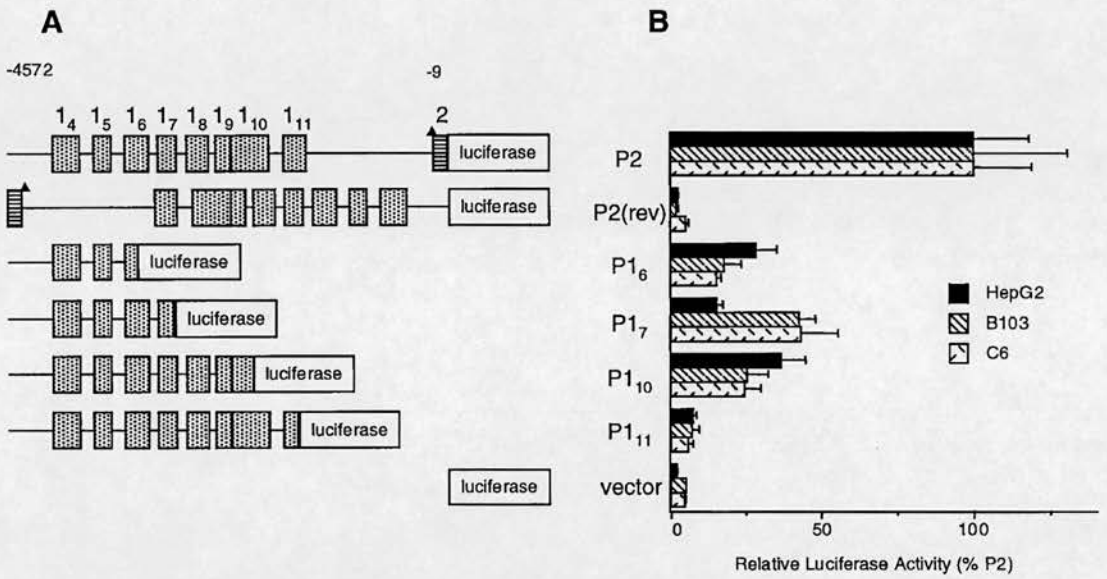

Fig. 5. Transfection Analysis of Promoter Activity Associated with the Alternate Exons 1 of the Rat GR Gene

A, Diagrammatic representation of constructs used in transfection analysis. Restriction fragments containing regions of the rat GR gene were fused, within specific exons, to the luciferase reporter gene in a modified pGL3-Basic vector. P2(rev) contains the identical fragment to P2, in the reverse orientation with respect to luciferase. ▲ indicates the splice acceptor site in the intron 5′ of exon 2. B, Promoter activity of regions of the GR gene in three cell lines, HepG2 hepatoma cells, C6 glioma cells, and B103 neuroblastoma cells. Activity of P2 (spanning the whole CpG island, fused to luciferase within exon 2) was nominally set to 100% for each cell line, and activity of the other constructs was expressed relative to this value. Values represent means ± SEM.

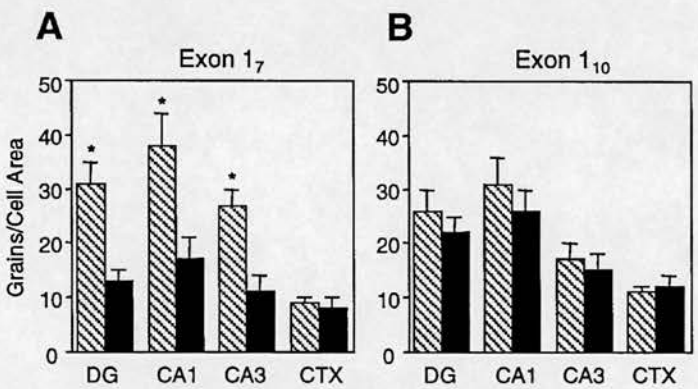

Fig. 6. Exon 1_7-containing GR mRNA Is Induced in Hippocampus by Neonatal Handling

In situ mRNA hybridization analysis of GR mRNA containing exon 1_7 (A) or exon 1_{10} (B) within the dentate gyrus (DG), the CA1 and CA3 pyramidal cell fields of the hippocampus and the cortex (CTX). Expression was measured in handled animals (*hatched columns*) and nonhandled animals (*black columns*) and is expressed as the number of grains over an area equivalent to a CA1 neuron. Values represent mean ± SEM; n = 5. *, $P < 0.05$.

subfields (Fig. 6A), whereas expression of exon 1_7-containing GR mRNA in cortex, where neonatal handling has no effect on expression of GR, was unchanged by the manipulation (Fig. 6A). In contrast, the level of expression and distribution of the major exon 1_{10}, and the other hippocampus-specific exons 1_5- and 1_{11}-containing GR mRNAs were unaffected by neonatal handling (Fig. 6B and data not shown). To start to examine whether this effect was confined to the hippocampus, we examined rats exposed prenatally to dexamethasone (dexamethasone administered during week 3 of gestation). This manipulation selectively and permanently increases hepatic GR mRNA levels by 25% (15). In these animals RNase protection assays show a significant decrease in the relative amount of exon 1_{10}-containing GR mRNA in the liver in

the dexamethasone-treated group (73 ± 3%; n = 10) compared with controls (82 ± 2%; n = 9, $P < 0.05$) (an increase in one of the minor GR mRNA species would reduce the level of exon 1_{10}-containing RNA as a percentage of the total). These data suggest that prenatal dexamethasone treatment induces one of the minor mRNA variants. However, RNase protection assays demonstrated that the level of exon 1_6-containing GR mRNA was unchanged in livers from adult rats treated with dexamethasone prenatally (9 ± 1%; n = 4), compared with controls (9 ± 1%; n = 5). Exon 1_1, 1_5, 1_7, and 1_{11}-containing GR mRNAs remained very low or were undetectable, suggesting that an as yet unidentified exon 1-containing GR mRNA is induced in these animals.

DISCUSSION

The organization of the 5′-end of the rat GR gene is complex. Here we show the gene encodes at least 11 alternate first exons, some of which are tissue-specific, and one of which is differentially and permanently induced by early-life manipulation. None of the alternate exons 1 is predicted to alter the amino acid sequence of the GR itself; there is an in-frame stop codon present immediately 5′ to the translation initiation site in exon 2, common to all the mRNA variants. Of the 10 alternate exons 1 we identified by 5′-RACE, 4 correspond to alternative exons 1 previously identified in mouse, exons 1_1, 1_5, 1_9, and 1_{10} (17, 21). Rat exon 1_1 lies at least 15 kb upstream of exon 2 (it is not present on λ208 containing 15 kb of DNA 5′ of exon 2) and is probably in a similar position to the corresponding mouse exon. All the other alternate exons 1 identified here are novel. Sequence analysis of DNA flanking the 5′-end of exon 2 revealed that most of the novel exons 1 lie within a CpG island highly conserved between rat and mouse. The 3-kb CG-rich region, therefore, contains at least 8 alternate exons 1 (including exon 1_6, present in the published rat GR cDNA sequence), at least 5 of which are conserved in the mouse. The CpG island is also conserved in the human GR gene, indicating that the use of alternate exons 1 in GR gene expression may also occur in humans.

At least 6 of the alternate exons 1 are present *in vivo* in rat GR mRNA. In all adult rat tissues examined, GR mRNA containing exon 1_{10} predominated, accounting for at least half of total GR transcripts. Exon 1_6 was also present ubiquitously, in a substantial minority of total GR mRNA. All other alternate exons 1 were, to varying extents, expressed in a tissue-specific manner. Exon 1_1 was well represented in GR mRNA in thymus, but was absent from hippocampus and liver. Preliminary data suggest that exon 1_1 is not restricted to a specific subset of cells in thymus, but is expressed similarly in thymocytes and thymic epithelium (A. Dammermann, C. Blackburn, and K.E. Chapman, unpublished observations). Hippocampal RNA contained significant levels of the minor exon 1_5-, 1_7-, and 1_{11}-containing GR mRNA variants that were expressed at either low or undetectable levels in liver and thymus. Five other exon 1 variants (1_2, 1_3, 1_4, 1_8, and 1_9) are unlikely to be of significance as they were poorly represented in the 5′-RACE PCR or were undetectable by RNase protection assays. It is unlikely that any further GR mRNA variants are present at significant levels in hippocampus as the sum of the exon 1 variants examined was close to 100% of total mRNA. Interestingly, exon 1_{10}-, 1_6-, and 1_{11}-containing transcripts accounted for only 90% of the GR mRNA in liver, suggesting that additional novel exon 1 sequences may be present.

In transient transfection experiments, a construct encoding the whole CpG island of the GR gene, including 8 of the alternate exons 1 and the splice acceptor site within the intron 5′ of exon 2, fused to a luciferase reporter gene within exon 2 (P2), exhibited substantial promoter activity in all cell lines tested. This activity results from transcripts originating at any point within the CpG island that are spliced from an appropriate donor site onto the splice acceptor site 5′ to exon 2, and represents the sum of the activity of individual promoters on the genomic DNA fragment. Promoter activity was also associated with particular regions of the CpG island, where the fusion to luciferase was made within specific exon 1 sequences. In these cases, no splice acceptor site is available within the luciferase gene, and a transcriptional fusion is generated between the specific exon 1 and the luciferase reporter; luciferase activity therefore reflects transcription through the specific exon 1. Relative activity of these constructs in different cell types was similar with one notable exception, $P1_7$ (see below). The low activity of $P1_{11}$, compared with the shorter constructs or to P2, probably results from promoter competition by the stronger promoters directing transcription of exon 1_6 and exon 1_{10}, neither of which will generate productive RNA transcripts encoding luciferase, due to the lack of a splice acceptor site. Interestingly, $P1_7$, fused to luciferase within exon 1_7, had the highest activity of any single promoter construct (P2 activity reflecting activity of the whole region) in B103 and C6 cells, both CNS derived. The activity of this construct was low in hepatic cells, in which $P1_6$ and $P1_{10}$ had the highest activity. *In vivo,* GR mRNA transcripts containing exon 1_7 were present at significant levels in hippocampus, but absent from liver, suggesting that factors present in cells of CNS origin are responsible for transcription initiation at the promoter upstream of exon 1_7 in rat hippocampus.

Neonatal handling induces an increase of approximately 50% in total GR mRNA levels in all subfields of the hippocampus, but not in cortex (27). Only the 1_7 variant GR mRNA was induced in the hippocampus by handling, with a 2- to 3-fold increase, also across all fields of the hippocampus. RNase protection assays, carried out on RNA extracted from the whole hip-

pocampus (which will include glia and interneurons, as well as pyramidal cells and the granule cells of the dentate gyrus) showed that exon 1_7-containing GR mRNA is normally present in approximately 10% of total GR mRNA in hippocampus. The observed induction of 1_7 may not appear sufficient to account for the overall increase of approximately 50% in steady state GR mRNA levels after handling (27). However, the heterogeneity of the hippocampus as a whole may have resulted in a dilution of exon 1_7-containing GR mRNA if it is expressed predominantly in the pyramidal cell layers of the hippocampus and granule cells of the dentate gyrus, thereby lowering the estimate of the amount of exon 1_7-containing GR mRNA present in these hippocampal neuronal layers obtained by RNase protection assays of whole hippocampus. Indeed, we have previously noted a similar discrepancy between the magnitude of change in mRNA encoding the type I inositol 1,4,5-triphosphate receptor during human pregnancy measured by RNase protection assays and *in situ* mRNA hybridization (28). Although we cannot exclude the possibility that an additional minor variant of GR mRNA is induced by neonatal handling, none of the other main variant GR transcripts were altered by handling. These data suggest that neonatal handling programs increased hippocampal GR via increased transcription from a novel promoter, 1_7, active predominantly in CNS-derived cells. A similar permanent induction of a minor promoter of the GR gene appears likely in the liver after prenatal dexamethasone exposure. Within the overall increase in GR mRNA in liver of prenatally treated rats, the proportion containing the predominant exon 1_{10} fell, although we were unable to identify a specific transcript induced. Nevertheless, the clear implication is that early life programming events selectively alter otherwise minor tissue-specific GR gene transcripts, whereas the major and ubiquitous promoters are unaffected, thus programming GR levels for the lifetime of the animal in a tissue-specific manner. Conversely, it is possible that manipulations that decrease GR levels may decrease the levels of the minor GR mRNA variants.

5HT appears crucial in mediating the effects of neonatal handling upon GR expression in hippocampus (29, 30), with subsequent effects upon HPA axis activity (31, 32). The transcription factors NGFI-A and AP2 have been implicated in the induction of GR in the hippocampus after handling or with 5HT (33). Intriguingly, a sequence in the human GR gene that binds AP2 *in vitro* (34) is completely conserved in the rat GR gene (at −2718). Additionally, within the CpG island, the GR gene contains 16 GC boxes (GGGCGG), which form the core consensus Sp1 site (35) and which may also bind NGFI-A; indeed, there is a sequence exactly matching the consensus binding site for the family of zinc finger proteins that includes NGFI-A (36) immediately upstream of exon 1_7. We speculate that the increases in AP2 and NGFI-A induced by neonatal handling cause increased transcription from a promoter adjacent to exon 1_7, leading to increased total GR mRNA.

It remains possible that transcription may originate at a common promoter further upstream, resulting in a common exon 0, which is then spliced upstream of the alternate exons 1. We consider this to be extremely unlikely for the following reasons. First, sequence analysis of 58 independent 5′-RACE clones neither provided evidence for a common 5′-leader sequence nor revealed any lack of colinearity with the genomic sequence. Second, the predominant rat exon 1_{10} is homologous to exon 1 of the human GR gene (16, 18) for which a transcription start site has been mapped. A number of transcription start sites exist (typically for a TATA-less CG-rich promoter), but all are located within the region corresponding to rat exon 1_{10}, and all appear to extend to the same 3′-splice site (16, 18). Similarly, sequencing of our 5′-RACE clones and RNase protection analysis (to map transcription start sites) suggests that a number of transcription starts exist at least for exon 1_{10} and probably for other exons 1 also (J. A. McCormick, V. Lyons, and K. E. Chapman, unpublished observations). Indeed, the 5′-end of the longest of our 5′-RACE clones containing exon 1_{10} corresponds exactly to one of the transcription starts mapped for human GR mRNA (18) (Fig. 1). Finally, our transfection data suggest that promoter activity is associated with the 5′-flanking regions of specific exons 1. Thus, it is most probable that alternate exon 1 usage results from transcription initiation at a number of predominant transcription start sites within the CpG island, associated with promoter activity. CpG islands are frequently associated with multiple transcription initiation sites, often spread over a distance of up to 1 kb, resulting in transcripts with differing exons 1, all of which, however, are spliced at the same 3′-splice donor site onto exon 2 (*e.g.* Refs. 37 and 38). Multiple 5′-ends are not usually associated with alternate splice donor sites, giving rise to discrete alternate exons 1, as we have observed for the rat GR gene. It is possible that, as the CpG island in the GR gene is very large, transcription initiates at a large number of initiation sites spread over the entire 3-kb region. The probability of splice donor sites occurring within such a large region is high, and the splicing machinery associated with the RNA polymerase complex may simply splice from the first appropriate splice donor site that occurs to the splice acceptor site before exon 2. This hypothesis is supported by the sequence of mouse exon 1E (corresponding to exon 1_{11}) which, at the 5′-end, includes a portion of exon 1C (corresponding to exon 1_{10}) as well as the intervening genomic DNA (21). Possibly, transcription originated too far 3′ within exon 1_{10} to utilize the exon 1_{10} splice donor site; splicing then occurred at the next available splice donor site, 3′ of exon 1_{11}. Certain sites within the CpG island will be favored for transcription initiation, and this will probably vary in a tissue-specific manner. Indeed, we see the highest number of variant exons 1 in hippocampus, a tissue exhibiting a high degree of

complexity. This hypothesis predicts that more alternative exons 1 may exist in the CpG island if more splice donor sites are predicted, and we have preliminary evidence that this is the case (V. Lyons and K. E. Chapman, unpublished observations).

The use of multiple and tissue-specific promoters provides a flexible mechanism for distinct tissue-specific regulation of individual promoters by hormonal signals and has been described for other members of the steroid receptor family (39–41). GRs are widely expressed in virtually all cell types, although expression levels and functions vary considerably. The complex organization of the 5′-end of the GR gene may reflect this need for diverse tissue-specific regulation. We speculate that exon 1_{10} is constitutively expressed in all tissues, providing a basal or minimal constitutive level of GR gene transcription (*e.g.* Ref. 8). The existence of tissue-specific promoters (*e.g.* 1_1 in thymus and 1_7 in hippocampus) permits differential regulation of GR in specific cell types and may explain the opposite regulatory effects of glucocorticoid hormones on the levels of GR in T lymphocytes and hippocampus (10, 42). In addition, the presence of several minor promoters clustered together may permit regulation of one or more by signal transduction pathways, resulting in moderate, but biologically significant, changes in total GR mRNA in a specific cell type and thus, ultimately, the glucocorticoid signal on the target genes. Our data illustrate the complexity of transcriptional regulation of GR and provide a basis to understand tissue-specific effects of early-life programming.

MATERIALS AND METHODS

Experimental Animals

Animals were maintained under controlled lighting (lights on 0700 to 1900 h) and temperature (22 C) with water and food available *ad libitum*. Tissues for RNA isolation and *in situ* hybridization were from adult (3–8 months) male Wistar rats (Charles River UK Ltd, Margate, Kent, UK). Animals treated *in utero* with dexamethasone were male offspring (8 months old) of female Wistar rats administered dexamethasone (100 μg/kg per day) during week 3 of pregnancy, as previously described (15). For neonatal handling, Long-Evans rats (Charles River Canada, St. Constant, Québec, Canada) were used as previously described (30). Handling was carried out daily for 2 weeks. Male animals were selected at random from a total of five litters and were used when they reached adult age (3–5 months).

All studies involving animals described herein were approved of by the UK Home Office and were performed in strict accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986.

Isolation of RNA

Total RNA was isolated using the guanidinium isothiocyanate method (43). Integrity was verified by electrophoresis on formaldehyde-agarose gels.

5′-RACE PCR

5′-RACE PCR was performed using a commercial kit (Life Technologies , Gaithersburg, MD) according to the manufacturer's instructions. First-strand synthesis of GR cDNA was carried out at 42 C for 30 min on 5 μg total RNA using 8 U/μl SuperScript II RT and 100 nM primer GSP1 (5′-AAGGGATGCTGTATTCA-3′) in a 25 μl reaction containing 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM deoxynucleoside triphosphates (dNTPs). After RNase H treatment, cDNA was added to a 24 μl reaction containing 20 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dCTP, and 0.4 U/μl terminal deoxynucleotidyl transferase. dC-tailed cDNA (5 μl) was used in a PCR reaction with 400 nM anchor primer (5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′), 440 nM primer GSP2 (5′-ACTCCAAATCCTTCAAGAGGTCA-3′), 20 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 2.5 U *Taq* DNA polymerase (Promega Corp., Madison, WI), with 35 cycles of PCR amplification (96 C, 45 sec; 45 C, 40 sec; 72 C, 1.5 min), followed by 10 min, 72 C. A nested PCR was carried out on the products of the first PCR reaction, under the same conditions with the following primers: UAP (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′) and GSP3 (5′-TTGGAATCT-GCCTGAGAAGC-3′). PCR products were cloned into pGEM-T or pGEM-T-easy (Promega Corp.) and sequenced using GSP3.

Subcloning and Sequence Analysis of the Rat GR Promoter

λ208 contains exon 2 and approximately 15 kb of the rat GR gene flanking the 5′-end of exon 2 (M. D. Jacobson, unpublished data). The sequence between −4600 and +500 (the translation start close to the 5′-end of exon 2 is designated +1) was determined from restriction fragments subcloned from λ208 on both strands using the Sequenase II system (Amersham International, Buckinghamshire, UK) or the Thermo Sequenase ^{33}P-radiolabeled terminator cycle sequencing system (Amersham International). Sequence analysis, including identification of putative transcription factor-binding sites was carried out using computer software available at the UK MRC Human Genome Mapping Project Resource Centre.

Accession Number

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number AJ271870.

Transfection Analysis Of GR Promoter Activity

Plasmids that fused the rat GR gene to a luciferase reporter gene were constructed from appropriate restriction fragments ligated into pGL3-Basic (Promega Corp.) containing a modified polylinker as follows: P2, a *Hin*dIII/*Ssp*I fragment encoding rat GR from −4572 to −9 (the ATG translation start is designated +1); P2(rev), the same fragment in the reverse orientation with respect to luciferase; $P1_6$, a *Hin*dIII/*Pst*I fragment encoding −4572 to −3336; $P1_7$, a *Hin*dIII/*Bgl*I fragment encoding −4572 to −2931; $P1_{10}$, a *Hin*dIII/*Hin*cII fragment encoding −4572 to −2318, and $P1_{11}$, a *Hin*dIII/*Pst*I fragment encoding −4572 to −1767. Plasmid DNAs used for transfections were purified by CsCl density gradient centrifugation.

HepG2 (human hepatoma), C6 (rat glioma), and B103 (rat neuroblastoma) cells were maintained in DMEM supplemented with 10% (vol/vol) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Twenty four hours prior to transfection, HepG2 and C6 cells were seeded at 5–7 × 10^5 cells per 60-mm dish and B103 cells at 2 × 10^5 cells per 60-mm dish.

Cells were transfected using the calcium phosphate procedure (44) with 1 μg modified pGL3-Basic or an equimolar amount of GR promoter-luciferase plasmid (plasmids varied markedly in size), 1 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia Biotech, Piscataway, NJ), and carrier pGEM-3 (Promega Corp.) to a total of 10 μg. Forty eight hours after transfection, cells were lysed and luciferase activity determined as previously described (44). β-Galactosidase activity was determined using the Tropix Galacto-Light kit (Cambridge Bioscience, Cambridge, UK), and luciferase activity/β-galactosidase activity was calculated. Transfections were carried out in triplicate; each experiment was repeated at least twice and two independently prepared plasmid DNAs were used for each promoter construct.

RNase Protection Assays

With the exception of exon 1_6 (see below), exon 1-specific cRNA probes were synthesized from corresponding 5′-RACE subclones, linearized, and transcribed with either T7 or SP6 phage polymerase, as appropriate, in the presence of either [α-^{32}P]-UTP or [α-^{32}P]-GTP (3000 Ci/mmol; Amersham International). The template used to synthesize an exon 1_6-specific cRNA probe was made by subcloning into pGEM-T-easy an RT-PCR product generated from total rat liver RNA using GSP3 (complementary to exon 2) and 5′-primer (5′-ACCTGGCGGCACGCGA-3′).

RNase protection assays were carried out using a HybSpeed RPA kit (Ambion, Inc., Austin, TX). Hybridization conditions were optimized in preliminary experiments using synthetic RNA templates. Total RNA (50 μg) was coprecipitated with 5–10 × 10^5 cpm cRNA probe, resuspended in 20 μl hybridization buffer (supplied with the kit) at 95 C, and incubated at 68 C for 1 h. Reactions were incubated with RNase A/T_1 (1:25 dilution) for 30 min, 37 C, and RNA products were separated on a 4% polyacrylamide gel containing 7 M urea and visualized using autoradiography or a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Data were analyzed using Student's *t* test. Significance was set at $P < 0.05$.

In Situ mRNA Hybridizations

[^{35}S]UTP-labeled RNA probes were synthesized as previously described (26). After DNase I treatment, unincorporated nucleotides were removed by passage over a Sephadex G-50 Nick column (Pharmacia Biotech, St Albans, UK). Exon 1_5-, 1_7-, 1_{11}-, and exon 2-specific templates were generated by PCR carried out on subclones of λ208 using the following oligonucleotides tagged with sequences encoding either a T3 promoter (to make sense RNA) or a T7 promoter (to make cRNA or antisense RNA): 1_5, 5′-primer (5′-TATTAACCCTCACTAAAGGGTAAGAGGAGGGCGGACT-3′), 3′-primer (5′-TTAAT-ACGACTCACTATAGGGCCAGCGCGCTCACACT-3′); 1_7, 5′-primer (5′-CATTAACC-CTCACTAAAGGGCACCGTTTCCGTGCAT-3′), 3′-primer (5′-TTAATACGACTCAC-CAC-TATAGGGCAGCGTGTGCCGACCT-3′); 1_{11}, 5′-primer (5′-TATTAACCCTCACTAAA-GGGAGCGGCGTCTGGACC-3′), 3′-primer (5′-TTAATACGACTCACTATAGGGCTAGCGCTCAAGTTGTC-3′) and exon 2, 5′-primer (5′-ATTAACCCTCACTAAAGGGCC-AATGGACTCCAAAGAA-3′) and 3′-primer (5′-ATAATACGACTCACTATAGGGAATCTGCCTGAGAAGC-3′). The template used to synthesize exon 1_{10}-specific cRNA was generated by PCR from an exon 1_{10} 5′-RACE clone using UAP and 3′-primer (5′-ATAATACGACTCACTATAGGGCTTTGGAGTCCATTGGCA-3′).

In situ-hybridization histochemistry was carried out as previously described (26, 45). Silver grains were counted under bright-field illumination using an image analysis system (MCID, Research Imaging, St. Catherine's, Ontario, Canada). Results were analyzed blind and background, counted over adjacent areas of neuropil, was subtracted. Data were analyzed using Student's *t* test. Significance was set at $P < 0.05$.

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