Transcriptional control of human Glucocorticoid Receptor

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde
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List of Abbreviations

ABN	Arched-back nursing
ACTH	Adrenocorticotrophic hormone
ALL	Acute lymphoblastic leukemia
Amg	Amygdala
ANOVA	One way analysis of variance
AVP	Vasopressin
CAS	Calcarine sulcus
Cb	Cerebellum
CBG	Corticosteroid binding globulin
cDNA	Complementary DNA
CG	Cingulate gyrus
ChIP	Chromatin immunoprecipitation
CN	Caudate nucleus
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
Ct	Threshold cycle
DG	Dentate gyrus
DNase I	Deoxyribonuclease I
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphates
GC	Glucocorticoid
GP	Globus pallidus
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response element
GWAS	Genome-wide association studies
Hi	Hippocampus
HPA	Hypothalamus-pituitary-adrenocortical
IFG	Inferior frontal gyrus
ISPF	*In silico* phylogenetic footprinting
ITG	Inferior temporal gyrus
LBD	Ligand binding domain
LC	Locus coeruleus
LD	Linkage Disequilibrium
LG	Licking-grooming
MDD	Major depressive disorder
MFG	Middle frontal gyrus
MR	Mineralocorticoid receptor
MTG	Middle temporal gyrus
NA	Nucleus accumbens
NGFI-A	Nerve Growth Factor-Inducible A

ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
PG	Pineal gland
PHG	Parahippocampal gyrus
Pit	Pituitary
Pol	DNA polymerase
POMC	Pro-opiomelanocortin
Put	Putamen
PVN	Paraventricular nucleus
SEAP	Secreted alkaline phosphatase
SFG	Superior frontal gyrus
SN	Substantia nigra
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SOG	Superior occipital gyrus
SPG	Superior parietal gyrus
SUBv-sp	Ventral subiculum pyramidal layer
TF	Transcription factor
TFBS	Transcription factor binding site
UTR	Untranslated region
VMHvl	Ventromedial hypothalamus (ventrolateral)
WM	White matter
YY1	Yin Yang 1

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General Abstract

The hypothalamus-pituitary-adrenocortical (HPA) axis is one of the major stress response systems in human body, it maintains homeostasis and adaptation during challenges. Cortisol, the key stress mediators in human, exerts profound effects on a wide range of physiological and developmental processes that are crucial for the adaptation to stress. Cortisol action is mediated by two corticosteroid receptors including the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). In the human brain, the co-expression of GR and MR in the hippocampus plays an important role in regulating the HPA negative feedback loop and the balance between both receptors may be involved in vulnerability to disease. GR is ubiquitously distributed, but expression levels vary widely between tissues. The complicated promoter region of the *GR* plays a pivotal role in the regulation of GR levels. Among the multiple promoters, in particular, promoter 1F, is susceptible to methylation by adverse early life events. CpG methylation in promoter 1F is thought to interfere with transcription factor binding, subsequently inhibiting transcription and consequently lowering mRNA and protein levels.

The research presented in this thesis explored several layers of complexity in GR transcriptional regulation. The promoter activity of multiple alternative *GR* first exons which are located in an upstream CpG island was investigated. Subsequently, the susceptibility of *GR* promoters to epigenetic modification was examined. Furthermore, the new SNPs occurring in upstream promoter regions and their functions were tested by genotyping 221 donors, revealing a new promoter-specific haplotype and 5 new SNPs lowering promoter activity. The role of NGFI-A and several other transcription factors on exon 1F regulation and the epigenetic sensitivity of promoter 1F by performing the single CpG dinucleotide methylation was studied. In addition, the distribution patterns of *GR* first exon transcripts, 5' splice variants, GR/MR ratio and methylation status in *GR* promoters in different human health brain regions was determined.

The findings in this thesis showed that *GR* first exons are independently controlled by a unique promoter located directly upstream and promoter activities were cell type-specific, and varied considerably between cell types. These promoters were susceptible to silencing by methylation and the activity of the individual promoters was also modulated by sequence variants (SNPs). We provide evidence that E2F1 is a major element in the transcriptional complex capable of driving the expression of *GR* 1F transcripts and that single CpG dinucleotide methylation can not mediate the inhibition of transcription *in vitro*. We showed that the *GR* first exons distribution is expressed throughout the

human brain with no region specific alternative first exon usage. *GR* 3' splice variants (GRα and GR-P) were equally distributed in all the brain regions. These data mirrored the consistently low levels of methylation in the brain, and the observed homogeneity throughout the studied regions.

CHAPTER 1

General Introduction

Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more

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1.1 Introduction

Stress represents a significant problem for Western societies. The economic burden of stress already induces costs as high as 3-4% of the European gross national products, a burden that is continually increasing. The unfavourable effects of stress are visible as changes in social behaviour, mood, learning and memory, effects that are difficult to quantify in economic terms. In the classical stress concept the dynamic equilibrium, so called homeostasis, in all living organisms is threatened by physical and psychological events known as stressors (de Kloet et al. 2005). To cope with the stress and to reconstitute the initial homeostasis or allostasis (McEwen 2003), the stress response rapidly activates the sympathetic nervous system (SNS) followed by the stimulation of the hypothalamus-pituitary-adrenocortical (HPA) axis. The HPA axis constitutes the key connection between central nervous system (CNS), the endocrine and the immune systems. It is one of the major stress response systems in the body, it maintains homeostasis and adaptation during challenges (de Kloet et al. 2005).

1.2 Regulation of the HPA

Neuroendocrine neurons of the paraventricular nucleus (PVN) of the hypothalamus synthesize and secrete the corticotrophin releasing hormone (CRH) and the arginine vasopressin (AVP) in response to a variety of stressors under the regulation originating from hippocampus, amygdala and *locus coeruleus (LC)*. CRH and VAP release leads to adrenocorticotrophic hormone (ATCH) secretion by the cleavage of pro-opiomelanocortin (POMC) from the corticotropic cells of the anterior pituitary. ACTH is transported by the peripheral circulation to the adrenal gland and stimulates the secretion of glucocorticoids (GCs) (cortisol in humans and corticosterone in rat and mice) from cells within the *zona fasciculata* of the adrenal cortex (Buijs et al. 2003; Nemeroff 1996; Webster and Sternberg 2004). GCs negatively feed back to inhibit the secretion of CRH and VAP at the level of the PVN and ACTH at the level of the anterior pituitary (Jacobson and Sapolsky 1991; Mendel 1989; Mendel et al. 1989) (Figure 1).

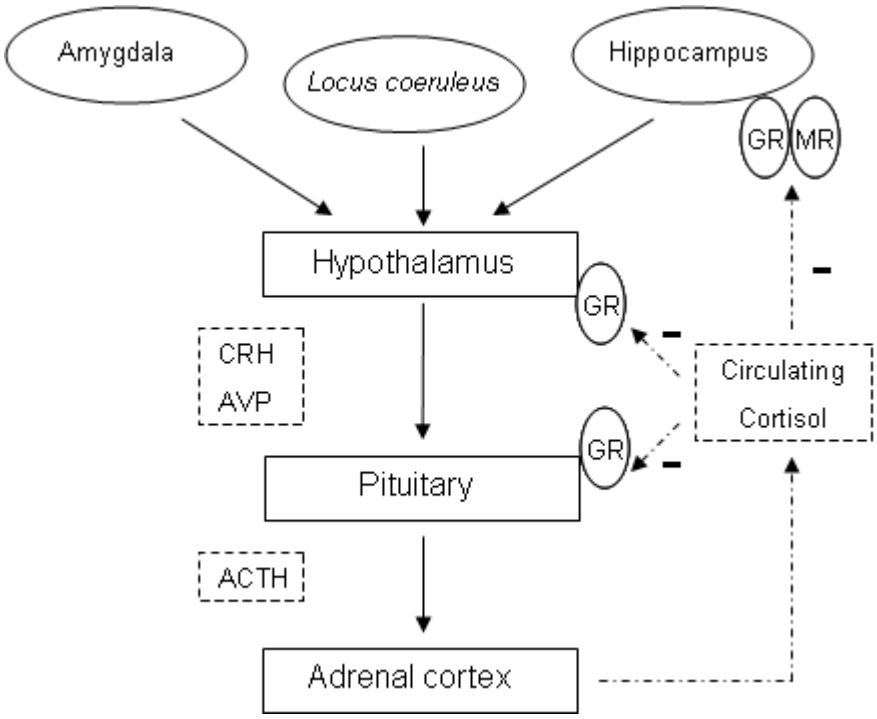

Figure 1. Schematic diagram of the HPA axis, describing regulation and negative feedback (-) of cortisol via glucocorticoid receptors (Adapted from (Juruena et al. 2004)).

1.3 Cortisol and its receptors

Cortisol, the key stress mediators in human, exerts profound effects on a wide range of physiological and developmental processes that are crucial for the adaptation to stress. Psychosocial stress has also been implicated in the development of mental disorders, including schizophrenia, anxiety disorders, and depression.

Cortisol was secreted with an ultradian rhythm of about one pulse per hour (Veldhuis et al. 1989). Cortisol is highly lipophilic and is transported in the bloodstream predominantly bound (~80%) to corticosteroid binding globulin (CBG) (Mendel et al. 1989; Torpy and Ho 2007). The action of free cortisol is mediated by two corticosteroid receptors including the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). In the brain, the MR is abundantly expressed in the limbic system and GR is widely expressed in the brain but are most abundant in hypothalamic CRH neurons and pituitary corticotropes (Reul and de Kloet 1985). MR has a ten fold higher affinity than the GR and it is shown to be activated at very low concentrations of GC (Conway-Campbell et al. 2007). GR has a high affinity for exogenous corticosteroids (eg. dexamethasone) and a low affinity for endogenous corticosteroid. GR is

believed to be more important at the ultradian peaks and after a stressful stimulus, when endogenous levels of GC are high (de Kloet et al. 2005).

1.4 The GR activations

Unliganded GR locates in the cytoplasm where it exists as receptor-chaperone complexes (Pratt et al. 2006; Ratajczak et al. 2003). Hsp40, Hsp70 and Hsp90 are essential chaperones which are involved in GR assembly and activity. In addition, other co-chaperones have been identified in the GR, such as the immunophilins FKBP51, FKBP52 (Pratt and Toft 2003) (Figure 2). After ligand binding, the GR dissociates from the Hsp90-chaperone complex. The dimerized GR translocates to the nucleus where it acts as a transcription factor that regulates the transcription of target genes via several mechanisms. Firstly, by binding to glucocorticoid-response element (GREs), liganded GR mediates transactivation and transrepression. Secondly, it may repress transcription by interfering with other transcription factors such as nuclear factor κB (NFκB), AP-1 (Gross and Cidlowski 2008; Hayashi et al. 2004). GR regulation depends on the specific cellular mechanisms of the promoter or cell type, which in turn alters the activity of the general transcription machinery.

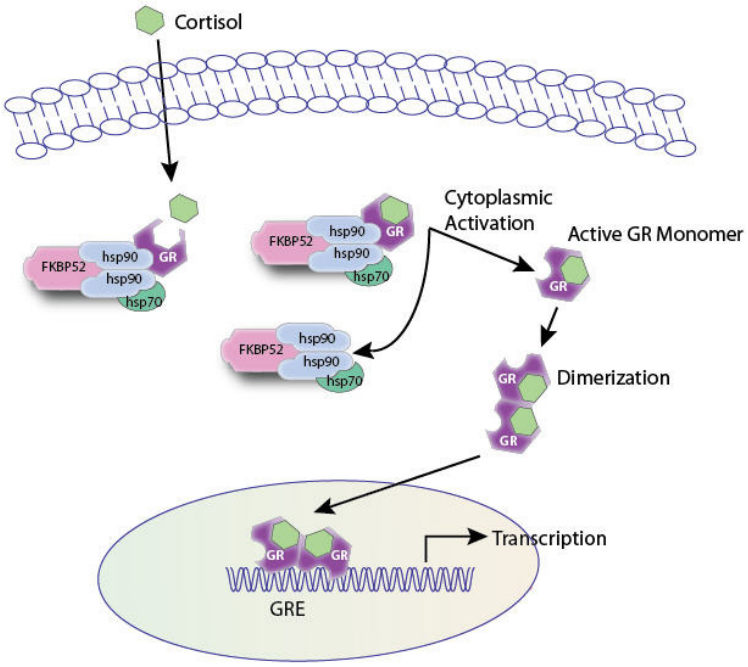

Figure 2. Molecular mechanism of GR activation upon cortisol binding. Taken from: http://www.panomics.com/index.php?id=product_94.

1.5 Structure of the NR3C1 gene

The human *GR* gene (OMIM + 138040; NR3C1) is located on chromosome 5q31-q32 (Hollenberg et al. 1985) and contains 8 translated exons (exon 2-9) and 9 untranslated alternative first exons. We and others have shown that GR levels are under the transcriptional control of a complex 5' structure of the gene, containing the untranslated first exons important for differential expression of the GR. All of the alternative first exons identified are located in one of the two promoter regions: the proximal or the distal promoter region, located approximately 5kb and 30kb upstream of the translation start site, respectively (Barrett et al. 1996; Breslin et al. 2001; Breslin and Vedeckis 1998; Geng and Vedeckis 2004; Nunez and Vedeckis 2002; Wei and Vedeckis 1997). Alternative first exons 1A, and 1I are under the control of promoters in the distal promoter region, whereas the promoters of exons 1D, 1J**,** 1E, 1B, 1F, 1C (1C1-3), 1H (Figure 3A) are located in the proximal promoter region (Presul et al. 2007; Turner and Muller 2005). Exons 1D to 1H are found in an upstream CpG island with a high sequence homology between rats and humans.

The region- or tissue-specific usage of alternative first exons leading to different GR mRNA transcripts (Presul et al. 2007; Turner and Muller 2005) (Figure 3B) provides a mechanism for the local fine-tuning of GR levels. Since the ATG start codon lies only in the common exon 2, this 5' mRNA heterogeneity remains untranslated, but is important for translational regulation (Pickering and Willis 2005).

Alternative mRNA transcript variants are generated by splicing of these alternative first exons to a common acceptor site in the second exon of the *GR*. Exon 2 contains an in-frame stop codon immediately upstream of the ATG start codon to ensure that this 5' heterogeneity remains untranslated, and that the sequence and structure of the GR is not affected.

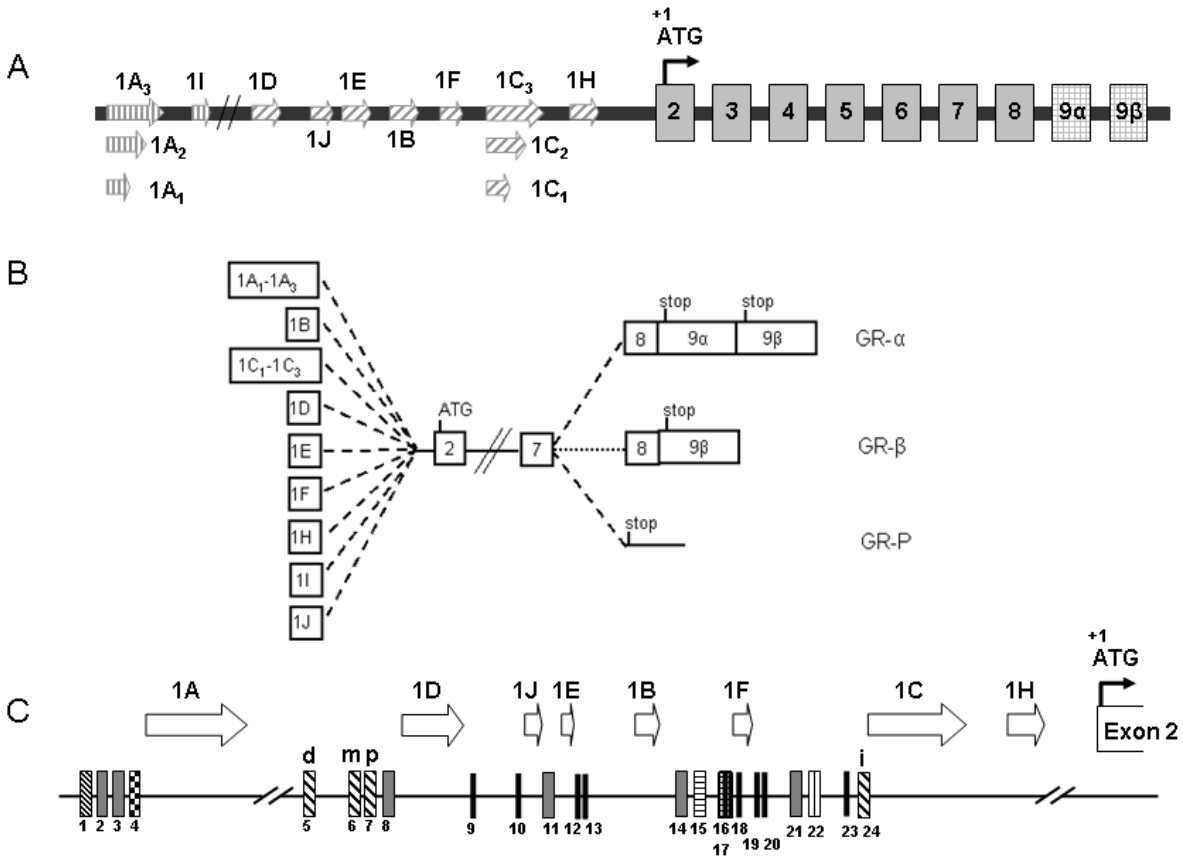

Figure 3. Structure of the *GR* gene, the potential mRNA transcripts and the binding sites within the CpG island.

Panel A The genomic structure of the *GR*. ▥ 5' untranslated distal exons; ▨ 5'untranslated CpG island exons; ■ Common exons; ▦ 3' alternatively spliced exons.

Panel B shows the potential mRNA transcripts encoding the three GR isoforms: GRα, GRβ and GR-P and the location of the known transcription factor binding sites. ▧ IRF 1 and IRF2 (position 1); ▩ c-Myb, c-Ets ½ and PU1 (position 4); ▨ Yin Yang 1 (positions 5,6,7 and 24); ■ Glucocorticoid response elements (GRE, positions 2, 3, 8, 11, 14 and 21); ■ Sp1 binding sites (positions 9, 10, 12, 13, 18, 19, 20 and 23); □ NGFIA binding site (position 16); ▦ Glucocorticoid response factor-1 (GRF1, position 17); ▤ Ap-1 (position 15); and ▥ Ap-2 (position 22).

The *GR* also has a variable 3' region. Unlike the 5' region, the 3' variability encodes splice variants with different functions. The three main 3' splice variants of the GR are GRα, GRβ, and GR-P (Figure 3B). GRα and GRβ are generated by two alternatively spliced 3' exons, 9α and 9β. GR-P lacks both exons 8 and 9 and is translated into a protein with a truncated ligand binding domain (LBD) which is thought to enhance GRα activity. The GRα exists as several N-terminal truncated isoforms designated as GRα-A, B, C1-3 and D1-3. GRα-A isoform has the 777 amino acids full-length. These isoforms are all functional receptors and display a tissue specific distribution as well as a gene-specific regulation (Lu and Cidlowski 2005). GRα is by far the most active form of the receptor; GRβ is thought to be a dominant negative regulator of the receptor. The relatively high ratio between GRβ and GRα has been involved in GC resistance (Lu and Cidlowski 2005). Little is known about the function of GR-P.

1.6 Alternative first exon usage and 3' splice variants

The recent observation that transcription factors binding to the pol II promoter modulate alternative splicing supports a physical and functional link between transcription and splicing (Kornblihtt 2005). Several factors were identified that were critical for the recruitment of a specific set of co-regulators to the target gene promoter and the production of a specific splice variant. The splice variant produced depends on the structural organisation of the gene and the nature of the co-regulators involved (Auboeuf et al. 2004). A link between transcription initiation sites and the resulting splice variant was suggested since it was shown that promoters controlled alternative splicing also via the regulation of pol II elongation rates or processivity. Slow pol II elongation paired with internal elongation pauses favoured the inclusion of alternative exons governed by an exon skipping mechanism, whereas high elongation rates of pol II, without internal pauses favoured the exclusion of such exons.

Many eukaryotic genes contain multiple promoters that are alternatively used for the production of different protein isoforms, with important physiological consequences. However, the *GR* with its variable 5' untranslated region (UTR), and alternative splicing in the 3' coding region is unique. Little is known about the association between the promoter usage and the resulting GR protein isoform. The 5´ UTR has tight control over local GR expression levels.

There seems to be also a poorly understood statistical link between the 5'UTR and 3' splice variants produced. One of the first studies to address this question showed that exon 1A3, and to a lesser extend 1B and 1C contribute most to the expression of GRα isoform (Pedersen and Vedeckis 2003). By comparing the most abundant exon 1 containing transcripts (1A, 1B 1C) with GRα, GRβ, and GR-P containing transcripts in different tissues and cell lines, Russcher *et al*. found a correlation between promoter usage and alternative splicing of the *GR* gene (Russcher et al. 2007). More specifically they found that the expression of GRα is preferentially regulated by promoter 1C, whereas 1B usage favours the expression of GR-P isoform. No association was found with transcripts including exon 9β or with those transcribed from 1A, suggesting that GRβ splicing may be associated with one of the recently identified exon 1 variants such as 1D to 1F and 1H that were not included in the above study (Russcher et al. 2007).

We also confirmed that in post-mortem brain tissues of patients with major depressive disorder (MDD) altered promoter usage influenced the resulting 3' GR isoform, with a negative correlation between GR-P expression and promoter 1B usage in all brain areas of MDD patients but not in normal control brains. A negative correlation was also found between the 1C promoter usage and GR-P expression in MDD brains. These results suggest that the promoters 1B or 1C do not play a significant role in GR-P expression in MDD, and that they were rather linked to other forms with lower expression (Alt et al. 2010). Thus, current data suggest a link between the two ends of the mRNA transcript, but there is no consensus as to the nature of this link.

1.7 Transcription factors and transcriptional control within the CpG island

The human *GR* was initially described as a housekeeping gene or constitutively expressed gene with promoters that contain multiple GC boxes and no TATA or TATA-like box (Zong et al. 1990). A wide variety of transcription factors have been identified that bind in the CpG island upstream of the gene. The description of the transcription factors active within this region is complicated by their tissue-specific usage. These transcription factors were not assigned to the different first exon promoters since most of this work was performed before our detailed description of the first exons in this region. The transcription factors so far identified are summarised (Table 1) and their location within the CpG island shown in Figure 1C. Initially, 11 DNase I footprints representing unique transcription factor binding sites were found in the 1C to 1F region of the CpG island (-3259 to -2522 from the ATG start codon) including, one AP-2 and 5 Sp-1 binding sites were identified (Nobukuni et al. 1995). It was initially thought that the latter transcription factors played an essential role in the basal expression of the human GR, although this is now less clear. Further studies identified one of the footprints in promoter 1C as a binding site for the transcription factor Yin Yang 1 (Breslin and Vedeckis 1998). YY1, expressed in a wide variety of mammalian cell types, is a zinc-finger transcription factor that can act as an activator, a repressor, or an initiator of transcription (Nunez and Vedeckis 2002; Shrivastava and Calame 1994). The same authors also revealed three other YY1 sites and another Sp1 site, initially assigned to promoter 1B. The later identification of promoter 1D suggested that these YY1 sites are probably associated with this promoter (Turner et al. 2006). Similarly, the Sp1 sites correspond to a region that was later identified as promoter 1J (Presul et al. 2007).

Table 1. Transcription factor binding sites in the human *GR* proximal promoter region.

Promoter	TF [a]	N°	Sequences	Cell lines / Tissues	Technique [b]	Location [c]		reference
Distal Promoter								
	IRF-1		GTAGAGGCGAATCACTTTCACTTCTGCTGGG	CEM-C7	FP, EMSA, RG	-34574	-34544	Breslin et al. 2001; Nunez et al. 2005
	IRF-2		GTAGAGGCGAATCACTTTCACTTCTGCTGGG	CEM-C7, Jurkat	FP, EMSA, RG	-34574	-34544	Breslin et al. 2001; Nunez et al. 2005
	GR-α		TCTGATACCAAATCACTGGACCTTA	CEM-C7	FP, EMSA	-34490	-34466	Geng et al. 2004
	GR-α		GACCGTAAAATGCGCATG	CEM-C7, IM-9	FP, EMSA, ChIP	-34436	-34419	Geng et al. 2004, 2005, 2008
	GR-β		GAGAAGGAGAAAACTTAGATCTTCTGATACCAA	CEM-C7	FP, EMSA	-34512	-34480	Breslin et al. 2001
	c-Myb		ATGTGTCCAACGGAAGCACT	CEM-C7	FP, EMSA, ChIP	-34421	-34402	Geng et al. 2004, 2005
	c-Ets 1/2		ATGTGTCCAACGGAAGCACT	CEM-C7	FP, EMSA	-34421	-34402	Geng et al. 2004
	PU.1		ATGTGTCCAACGGAAGCACT	IM-9	EMSA, ChIP	-34421	-34402	Geng et al. 2005, 2008
Proximal promoter								
1D	dYY1	1	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4807	-4799	Breslin et al. 1998
	mYY1	2	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4635	-4627	Breslin et al. 1998
	pYY1	3	CCAAGATGG	NIH 3T3, Hela	FP, D, E	-4591	-4583	Breslin et al. 1998
	GRE	4	GGCTTCCGGGACGCGCTTCCCCAATCGTCTTCAAG	Jurkat, IM-9, CEM-C7	ChIP, E	-4574	-4540	Geng et al. 2008
1J	Sp1	5	GCTGGGGCGGGGGCTT	NIH 3T3, Hela	FP, E	-4250	-4235	Breslin et al. 1998
	Sp1	6	TTCGGGGGTGGGG	Jurkat, HepG2, Hela	RG, FP, E	-4011	-3999	Nunez et al. 2002
1E	GRE	7	GTGGAAGAAGAGGTCAGGAGTTTC	Jurkat, IM-9, CEM-C7	ChIP, E	-3962	-3939	Geng et al. 2008
1B	Sp1	8	CACATTGGGCGGGAGGGG	Jurkat, HepG2, Hela	RG, FP, E	-3774	-3757	Nunez et al. 2002
	Sp1	9	TTGAACTTGGCAGGCGGCGCC	Jurkat, HepG2, Hela	RG, FP, E	-3750	-3730	Nunez et al. 2002
1F	GRE	10	GCACCGTTTCCGTGCAACCCCGTAGCCCCTTTCGAAGTGACACACT	Jurkat, IM-9, CEM-C7	ChIP, E	-3438	-3393	Geng et al. 2008
	AP-1	11	TGACACA (consensus TGAC/GTCA)	AtT-20,NIH3T3	EMSA	-3401	-3395	Breslin et al. 1996; Wei et al. 1997
	Sp1	12	TGGGCGGGGGCGGGAA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3228	-3213	Nobukuni et al. 1995
	NGFIA	13	GGGCGGGGGCGG	Rat Hippocampi / HEK293	ChIP	-3227	-3216	Weaver et al. 2004; McGowan et al. 2009
	GRF-1	14	GAAGGAGGTAGCGAGAAAAGAAACTGGAGAAACTCGGTGG	MCF7, CV-1	EMSA	-3215	-3176	LeClerc et al. 1991a, b
	Sp1	15	TCTTAACGCCGCCCCAGAGA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3172	-3153	Nobukuni et al. 1995
1C	Sp1	16	GGAGTTGGGGGCGGGGGGCG	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3107	-3088	Nobukuni et al. 1995
	Sp1	17	GCGCACCGGGCGGGGCGGCC	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-3080	-3061	Nobukuni et al. 1995
	GRE	18	CTGCAGTTGCCAAGCGTCACCAACAGGTTGCATCGTTCCCC	Jurkat, IM-9, CEM-C7	ChIP, E	-2971	-2931	Geng et al. 2008
	AP-2	19	CCGCGCGGCCCCTCGGGCGGGGA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-2923	-2901	Nobukuni et al. 1995
	Sp1	20	CGCCGTGGCGCCGCCTCCA	Hela, NIH3T3, CV1, HepG2	RG, FP, EMSA	-2856	-2838	Nobukuni et al. 1995
	iYY1	21	CTCCTCCATTTTG	NIH 3T3, Hela	FP, D, E	-2755	-2743	Nobukuni et al. 1995

[a] TF: Transcription Factor
[b] RG: reporter gene. FP: DNAse protection / DNA footprinting. ChIP: chromation precipitation. D: deletion analysis. EMSA: electrophoretic mobility shift assay
[c] Locationswith repect to the ATG start codon in exon 2.

Similarly, several transcription factors initially assigned to promoter 1C should be reassigned to promoter 1F. AP-1, a transcription complex whose components are encoded by c-fos and c-jun proto-oncogenes binds to the AP-1 site within the human *GR* promoter 1F (Breslin and vedeckis 1996; Wei and Vedeckis 1997). This same region was also shown to bind Ku70 and Ku 80 in a tissue-specific manner (Warriar et al. 1996). Whilst most of the transcription factors identified upregulate GR expression, GRF-1 (glucocorticoid receptor DNA binding factor 1) has been identified as a repressor of GR transcription (LeClerc et al. 1991a; LeClerc et al. 1991b). At the 3' end of the rat 1_7 promoter a Factor Nerve Growth Factor-Inducible A (NGFI-A) binding site was identified only 2bp upstream of the transcription initiation site of this exon (Weaver et al. 2004). Recently, the homologous human NGFI-A binding site, together with numerous non-canonical NGFI-A sites were identified in promoter 1F of the human *GR* (McGowan et al. 2009).

As a transcription factor, GR also auto-regulates its own CpG island promoters. Several GREs half-sites, acting in concert with c-Myb, and c-Ets protein members have been identified in promoter 1D, 1E, 1F and 1C (Geng et al. 2008).

The currently known transcription factors provide only an incomplete picture of the complex regulatory mechanisms. For instance, little is known about the proximal elements in promoters 1B and 1H. Using an *in silico* phylogenetic footprinting (ISPF) technique we were able to find the majority of the experimentally identified transcription factors, and predicted a wide variety of factors that are conserved between many species (Turner et al. 2008). These are interesting candidate regulators of GR expression that warrant further investigations.

It has not yet been shown whether the transcription factors that bind immediately upstream of exons D, E, F, H, and I, activate the expression of these exons. Only site 13, one of 6 in the region immediately upstream of exon 1F (Figure 3C), has been shown to activate transcription of the downstream exon. Furthermore, the link between the transcription factors previously identified, or predicted and the transcription of the new CpG island first exons must be established.

1.8 Transcriptional control upstream of the CpG island: Exon 1A and 1I

Whilst the majority of the *GR* first exons and their promoters are located within the CpG island, exons 1A and 1I map 25 Kb upstream of the CpG island and 32 Kb upstream of the main GR open reading frame (ORF) in the distal promoter region (Breslin et al. 2001; Presul et al. 2007). Exon 1A has also been identified in the mouse, and three possible homologues 1_1, 1_2, and 1_3 have been found in the rat (McCormick et al. 2000; Strahle et al. 1992). The human promoter 1A generates three alternatively spliced transcripts, 1A1, 1A2 and 1A3 (Breslin et al. 2001). Expression of the 1A transcripts appears to be limited to the immune system in both humans and rodents. The human 1A3 transcript is widely expressed in both acute lymphoblastic leukaemia (ALL) cell lines and in children with this malignancy (Breslin et al. 2001; Chen et al. 1999, b; Presul et al. 2007; Russcher et al. 2007; Tissing et al. 2006). Similarly, exon 1I is used predominantly in T cells, although it is also present in HeLa cells (Presul et al. 2007).

Promoter 1A is regulated by the GR itself. The human 1A promoter contains a GC-responsive cassette containing a non-canonical GRE adjacent to overlapping binding sites for c-Myb and c-Ets protein family members. In the presence of c-Myb the ligand-bound GR is recruited to the promoter and up-regulates 1A transcripts, while the interaction with c-Ets family members leads to a repression of 1A promoter activity (Geng et al. 2005; Geng and Vedeckis 2004). This explains in part the opposite effects observed in different tissues: in most tissues GC decreases GR expression, but in and certain T cells GCs increase GR expression. The synergy between c-Ets and the GR has been shown to be responsible for the down regulation of 1A3 in the B lymphoblastoid cell line IM-9 after GC exposure. In contrast, the presence of c-Myb in T cells increased the activity of promoter 1A in human cell lines such as the ALL T cell line CEM-C7. As a result of the higher level of total GR T cells are more sensitive to GC induced apoptosis, and 1A3- transcripts were shown to be the most GC responsive of all first exons investigated (Breslin et al. 2001; Pedersen and Vedeckis 2003). Although 1A containing transcripts correspond to only about 10% of all *GR* transcripts (Tissing et al. 2006), their contribution to the tissue-specific response to GC treatment was considered essential (Breslin et al. 2001; Pedersen and Vedeckis 2003). The human promoter 1A also has a functional binding site for Interferon Regulatory Factors (IRF-1 and IRF-2), however IFNγ stimulation of CEM-C7 cells did not

increase 1A transcript levels, nor did it alter their susceptibility to GC-mediated apoptosis (Nunez et al. 2005).

Thus the evidence suggests that 1A transcript levels are critically involved in mechanisms of therapeutic induction of apoptosis in ALL T cells. However, GC resistance in pediatric T- and B- ALL cells obtained directly from patients did not correlate with either the basal or the stimulated expression of the 1A-, 1B- or 1C- transcripts. The relation between GC sensitivity and expression of *GR* transcripts may be complicated by the overlapping effects such as multi drug resistance genes that prevent GC concentrations from building up in the cells. In addition, ALL cells were shown to have an up-regulated GR expression upon prednisolone treatment regardless of their phenotype or sensitivity to GC-induced apoptosis (Tissing et al. 2006).

In mice the presence of the membrane-bound GR was a better correlate of GC-induced apoptosis than the intracellular GR level (Gametchu and Watson 2002; Gametchu et al. 1991). Exon 1A was found to be highly expressed in a T lymphoma cell line with elevated levels of membrane-bound GR and enhanced sensitivity to GC-dependent cytotoxicity. 1A transcripts appear to contain all the necessary information for both the synthesis and the subcellular trafficking of the membrane GR, although the exact mechanism remains unknown (Chen et al. 1999). It is interesting to hypothesise that the initially presumed link between GC induced apoptosis and 1A transcript levels is indirect, with 1A transcripts producing membrane GR the true correlate of GC sensitivity. Recently a similar membrane GR has been detected in human cells, but its sequence is not known (Bartholome et al. 2004; Spies et al. 2006). The membrane GR may eventually explain the discrepancy between the *in vivo* and *in vitro* observations in ALL. It is interesting to speculate that, as in the mouse, the different first exons play determine the cellular fate of the human GR.

Further sequence analysis of mouse exon 1A found 5 small upstream-ORFs preceding the major ORF of the *GR*. It has been shown that GR expression from the 1A transcript requires translation of the peptide encoded by uORF-2. The peptide was detectable in both cells with naturally 1A transcripts and cells transfected with 1A. The role of this peptide in the regulation of the membrane GR is still unclear (Diba et al. 2001).

1.9 Single nucleotide polymorphisms (SNPs) in *GR*

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variability. SNPs were defined to have a frequency of at least 1% of the population (Muller-Myhsok 2005). SNPs are scattered throughout the 23 pairs of chromosomes of the human genome, and roughly 11 million common polymorphisms are estimated to exist (Musunuru and Kathiresan 2008). A third generation human haplotype map catalogues from 1.3 to 4.1 million SNPs depending on ethnicity (Altshuler et al. 2010).

The variation of GR function has been associated with polymorphisms in the *GR* gene and the polymorphisms have the potential to explain individual specific differences in health, disease and response to glucocorticoid therapy (Gross and Cidlowski 2008). Several SNPs of *GR* have been characterized and well studied (Table 2 and Figure 4).

Table 2. Overview of the *GR* gene variants.

Name	dbSNP reference	Nucleotide change	Amino acid change	Locations	References
*TthIII*I	rs10052957	C/T	-	Promoter 1D	(Detera-Wadleigh et al. 1991)
NR3C1-1	rs10482605	T/C	-	Promoter 1C	(van West et al. 2006)
ER22/23EK	rs6189 rs6190	G/A G/A	- Arginine to lysine	Exon 2	(van Rossum and Lamberts 2004a) (Koper et al. 1997)
N363S	rs6195	A/G	Asparagine to Serine	Exon 2	(Koper et al. 1997)
*Bcl*I	rs41423247	C/G	-	Promoter exon 3	(Murray et al. 1987)
A3669G	rs6198	A/G	-	Exon 9β	(Derijk et al. 2001)

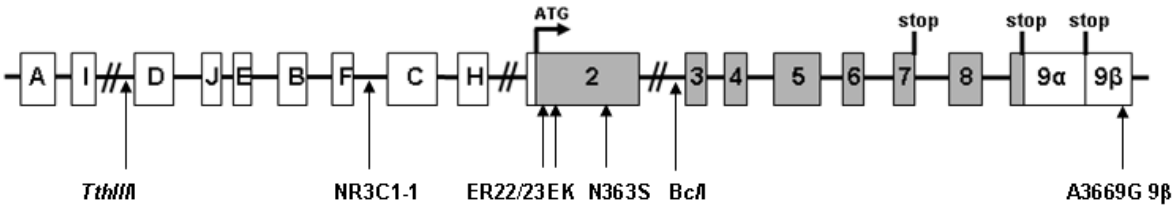

Figure 4. Schematic representation of the location of the reported polymorphisms in the human *GR* gene.

*TthIII*I is a restriction fragment length polymorphism which located in promoter 1D, a large intron of approximately 27kb downstream exon 1A. This polymorphism was reported to be associated with higher basal cortisol secretion in men (Rosmond et al. 2000)

NR3C1-1 minor C allele showed less transcriptional activity in two human brain turmor cell lines (Kumsta et al. 2009). Labuda et al. (Labuda et al. 2010) showed increased promoter activity in a human choriocarcinoma cell line. Also the minor C allele was observed to be significantly associated with reduced GR expression in the Mexican-American population (Niu et al. 2009).

In exon 2, between the first and second ATG, codons 22 and 23 which separated by 1bp, showed 100% linked polymorphisms. This ER22/23EK polymorphism increased expression of GRα-A, the change of ratio between GRα-A and GRα-B could lead to display decreased transactivation (Russcher et al. 2005a). This polymorphism reduced sensitivity to glucocorticoids and therefore seems to confer relative corticosteroid resistance (van Rossum and Lamberts 2004a). Also an association of the ER22/23EK allele with major depression was reported (van Rossum et al. 2006; van West et al. 2006).

The N363S was associated with increased sensitivity to glucocorticoids (Huizenga et al. 1998; Koper et al. 1997). 363S allele is more active in vitro than 363N allele (Russcher et al. 2005b). The 363S carriers showed increased saliva cortisol to stress (Wust et al. 2004). Similarly, 363S carriers in males showed highest responses compared to females (Kumsta et al. 2007).

The *Bcl*I was associated with increased sensitivity to glucocorticoid (van Rossum and Lamberts 2004a). *Bcl*I heterozygous was associated with an increased susceptibility to develop major depression (van Rossum et al. 2003) and the homozygous carrier had a lower cortisol response stress (Wust et al. 2004).

The A3669G increased stability of human GRβ mRNA in rheumatoid arthritis. This polymorphism could contribute to an alter glucocorticoid sensitivity since the human GRβ is thought to function as an inhibitor of human GRα activity (Derijk et al. 2001). This A3669G was associated with high level plasma cortisol and ACTH only in males (Kumsta et al. 2007).

SNPs often appear in combination named haplotype. Actually the haplotype are estimated based on the frequencies of the individual SNPs using statistical program. The most frequent haplotype so-called wild-type. Linkage Disequilibrium (LD) is a statistical measure of the non-random association of 2 alleles, it indicates that some SNPs occur more often together based on their frequency (Derijk et al. 2009).

The combinations of SNPs or alleles are named haplotype. Compared to individual SNPs, the combinations can have different effects (Derijk and de Kloet 2008). Haplotype is calculated on the statistical association of a set of SNPs on a chromosome pair.

The *GR* haplotype structure has been reported in several studies (Kumsta et al. 2008; Kumsta et al. 2009; van Rossum et al. 2004b) and similar five haplotypes have been shown such as in Otte et al (Otte et al. 2009). The highest frequency haplotype 1 contains the major alleles of the four SNPs (EK22/23EK, N363S, *Bcl*I C/G and 9beta A/G). Haplotype 2 and haplotype 3 was characterized by the minor G allele of the *Bcl*I and that of the 9beta A/G respectively. Haplotype 4 consisted of the minor A allele of the EK22/23EK and the *Bcl*I and that of the 9beta A/G. Haplotype 5 includes the minor G allele of N363S. *GR* haplotype 3 including the minor allele of the 9beta A/G was associated with depression in patients with stable coronary disease who were enrolled in the Heart and Soul Study (Otte et al. 2009). The 9beta A/G has been shown to be in complete LD with a promoter SNP NR3C1-1 (Kumsta et al. 2009). Also, the finding that NR3C1-1 and *Bcl*I associated with MDD has been shown (van Rossum et al. 2006; van West et al. 2006). Another study reported that *GR* polymorphisms and haplotype associated with chronic fatigue syndrome (Rajeevan et al. 2007). Moreover, the *GR* haplotype carrying *TthIII*I, ER22/23EK and 9beta A/G had a more aggressive disease course in multiple sclerosis patients (van Winsen et al. 2009). Recently, a common haplotype potentially covering a regulatory region of *GR* was associated with an increased risk of hospital admission for depressive disorders (Lahti et al. 2011). Together, these finding suggest that variation in the *GR* gene and *GR* haplotype may be involved in the pathogenesis of diseases.

1.10 Epigenetic programming of *GR* promoters

DNA methylation is the covalent addition of a methyl group to 5th position of cytosine of CpG dinucleotides via the DNA methyltransferases (DNMTs). CpG dinucleotides are the least frequent of all nucleotide pairs, and they can be scattered in clusters throughout the genome termed CpG islands (Robertson and Wolffe 2000).

Such epigenetic methylation of the 5'-cytosine of a CpG dinucleotide is associated with gene silencing either by inhibition of transcription factor binding (Figure 5) or by chromatin inactivation (Bird 2007; Bredy et al. 2007; Szyf 2009). For instance, prenatal epigenetic methylation governs genomic imprinting and inactivation of one X-chromosome (Bird 2002). The epigenetic chromatin status is sensitive to the host environment. Thus, epigenetic methylation represents a link between the environment and gene activity. In particular, early life events can have a long-lasting effect on epigenetic programming (Bredy et al. 2007; McGowan et al. 2009). In many instances, minor changes in GR levels can have a significant impact, for example on feedback regulation of the HPA axis, where hippocampal or pituitary GR levels determine the HPA axis set-points and the response to stress.

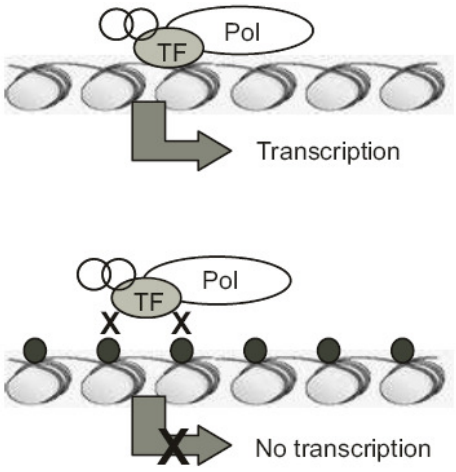

Figure 5. DNA methylation inhibits transcriptional regulation of genes.
A complex of transcription factors (TF), the DNA polymerase (Pol) and co-activators (open circles) normally binds to the *GR* promoter and thus regulates gene expression. Epigenetic modifications can lead to methylation of the transcription factor binding site in the *GR* promoter (full ovals). Therefore binding to the promoter is inhibited and expression of the GR is reduced.

Experimentally, maternal care such as licking-grooming (LG) and arched-back nursing (ABN) has been shown to translate into epigenetic methylation of the *GR* promoter 1_7 with profound and lasting effects on the stress response of the off-springs (Brown et al. 2007). The NGFI-A binding site in the *GR* promoter 1_7 (Figure 6), homologous to the human 1F, was highly methylated (>80%) in the offspring of low caring mothers whereas it was rarely methylated in the offspring of high caring rats (Weaver et al. 2004). As a result, binding of NGFI-A to the *GR* 1_7 promoter was inhibited in the hippocampus of offspring of low caring mothers and *GR* 1_7 expression was reduced (Brown et al. 2007). Interestingly, these effects were reversed by cross-fostering indicating a direct effect of maternal care on the epigenome of the offspring (Weaver et al. 2004). Infusion of L-methionine reversed these effects on methylation and NGFI-A binding to the exon 1_7 promoter in the rat brain (Szyf et al. 2005).

In Lewis and Fisher rats that naturally differ in their stress response and hippocampal GR levels, the 1_7 promoter was shown to be un- or poorly methylated throughout (mostly below 10% and never exceeding 30%), with no difference between the two strains. Feeding these rats a methyl-supplemented diet had no significant effect on the *GR* promoter 1_7 methylation levels (Herbeck et al. 2009).

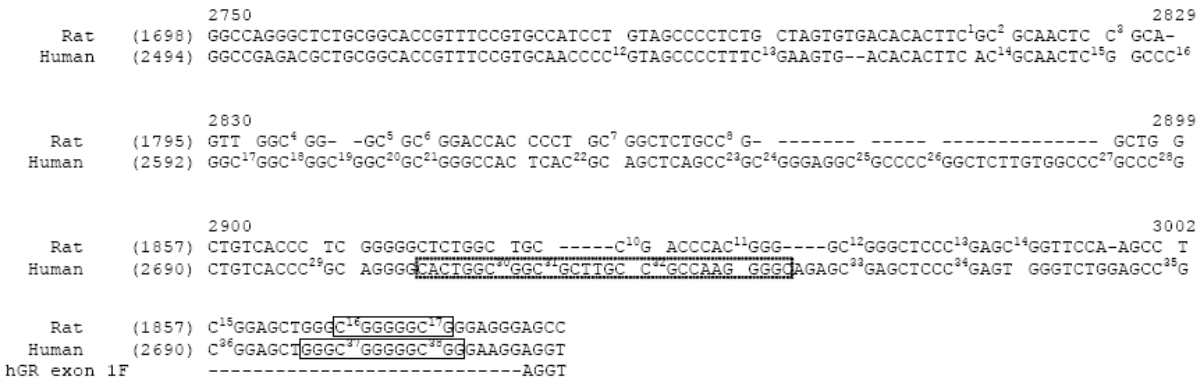

Figure 6. Alignment of the rat *GR* promoter 1_7 and the human *GR* promoter 1F.
Solid-lined boxes represent known canonical NGFI-A binding sites either in the rat (Daniels et al. 2009; Herbeck et al. 2009; Weaver et al. 2004) or the human (Alt et al. 2010b; Moser et al. 2007; Oberlander et al. 2008b; Turner et al. 2008) promoter. The broken-lined box in the human 1F promoter represents a hypothetical non-canonical NGFI-A binding site (McGowan et al. 2009).

Table 3. Methylation analyses of *GR* promoter regions.

GR promoter	Species	Model	Method	Tissue	Overall methylation levels	CpG specific comments †	Reference
1.7	r. nor.	LG-ABN	colony sequencing	hippocampus	<100%	80-100% at CpG 16-17 in Low LG-ABN 0-10% at CpG 16-17 in High LG-ABN	Weaver et al. 2004
1.7	r. nor.	met sup	colony sequencing	hippocampus	<15%	at CpG 16-17	Herberk et al. 2010
1.7	r. nor.	maternal sep	direct sequencing	hippocampus	not detected	at CpG 16-17	Daniels et al. 2009
1.10	r. nor.	PR diet	MS-PCR	liver	unknown	relative to control PR rats had 30% lower methylation	Lillycrop et al. 2007
1C	r. nor.	HV	methylation sensitive restriction enzyme PCR assay	umbilical blood	unknown	correlation between relative methylation and DNMT1 expression	Lillycrop et al. 2007
1D	h. sap.	HV	colony sequencing	PBMCs	<50%	stochastic and unique	Turner et al. 2008
1E	h. sap.	HV	colony sequencing	PBMCs	>25%	stochastic and unique	Turner et al. 2008
1F	h. sap.	HV	colony sequencing	PBMCs	<10%	at CpG 37-38	Turner et al. 2008
1H	h. sap.	HV	colony sequencing	PBMCs	<75%	stochastic and unique	Turner et al. 2008
1F	h. sap.	Dep	pyrosequencing	cord blood	<7%	<5% at CpG 37-38	Oberlander et al. 2008
1F	h. sap.	Dep, Al, Park, Dem	MS-PCR	hippocampus	<10%	only 1 subject had visible methylation all other donors unmethylated	Moser et al. 2007
1F	h. sap.	Suicide / abuse	colony sequencing	hippocampus	<40%	0% at CpG 37-38	McGowan et al. 2009
1B	h. sap.	MDD	pyrosequencing	hippocampus	0-12%		Alt et al. 2010
1E	h. sap.	MDD	pyrosequencing	hippocampus	4-9%		Alt et al. 2010
1F	h. sap.	MDD	pyrosequencing	hippocampus	0-4%	0-2% at CpG 37-38	Alt et al. 2010
1J	h. sap.	MDD	pyrosequencing	hippocampus	0-7%		Alt et al. 2010

† all CpG numbers as in Figure 6.
Abbreviations: r. nor, rattus norwegicus; h. sap, homo sapiens; LG-ABN, licking-grooming and arched-back nursing; met sup, methy-supplemented diet; maternal sep, maternal seperation; PR, protein-restricted; HV, healthy volunteer; Dep, depression; Al, Alzheimer's disease; Park, Parkinson's disease; Dem, dementia; MDD, major depressive disorder; MS-PCR, methylation-specific PCR

Using the maternal separation model to change the stress response in rat pups (Table 3), Daniels *et al*. observed elevated NGFI-A levels and significant behavioural changes. In this model, the 1_7 promoter, including both CpG sites within the NGFI-A binding site, was uniformly unmethylated even after applying the maternal separation stressor (Daniels et al. 2009).

Epigenetic programming of the *GR* is not limited to central tissues such as the hippocampus. It has also been proposed that dietary restriction could lead to changes in DNA methyl content, affecting epigenetic programming of the *GR* promoter both centrally and peripherally (Ernst et al. 2008). Feeding a protein-restricted diet to pregnant dams lead to a hypomethylation of the major *GR* promoter 1_{10} and to an increased expression of *GR* in the liver of these rat pups (Burdge et al. 2007).

These animal models showing changes in *GR* promoter methylation are not easily transferred to humans. Nevertheless maternal adversities like depression or protein restriction and their effects on the epigenome of offspring have been investigated (Table 3). Oberlander *et al*. showed that prenatal exposure to maternal depression leads to increased methylation levels of the *GR* promoter 1F at the NGFI-A binding site in cord blood of newborns (Oberlander et al. 2008). Like in most rat experiments, methylation levels were uniformly low (5-10 %) with small but significant differences between children of depressed and healthy mothers.

Several studies also investigated alterations in the human *GR* 1F promoter in specific disease populations based on the rat 1_7 data of Weaver *et al*. (Weaver et al. 2004). In neurological disorders such as Parkinson's disease, Alzheimer's or dementia no hypermethylation of the 1F promoter and the NGFI-A binding site could be found (Moser et al. 2007). We showed that in major depressive disorder there was no methylation of the NGFI-A binding site of the 1F promoter in several regions of human *post mortem* brains (Alt et al. 2010). However, in suicide victims with a history of child abuse, McGowan *et al*. found increased methylation patterns compared to suicide victims without abuse (McGowan et al. 2009). In this study, methylation of another putative NGFI-A binding site within promoter 1F resulted in decreased expression levels of 1F transcripts and overall GR levels. The known NGFI-A binding site was completely unmethylated in all of the suicide victims. Thus, it is possible that the other transcription factor binding sites are important for the transcriptional regulation of the *GR* 1F promoter and that these are more sensitive to epigenetic modifications. Interestingly, in all four of the above studies (Alt et al. 2010; McGowan et al. 2009; Moser et al. 2007; Oberlander et al.

2008), levels of methylation were always very low in comparison to those in the LG-ABN rats (Weaver et al. 2004). Investigating the complete *GR* CpG island, we were able to show highly variable methylation patterns among different *GR* promoters in peripheral blood mononuclear cells (PBMCs) of healthy donors, suggesting that epigenetic programming may not be restricted to the 1F promoter, but operates throughout the CpG island (LeClerc et al. 1991a). It remains unclear, however, what triggers changes in methylation, and when are the different tissues most susceptible to epigenetic programming. Despite some contradictions it seems that levels of methylation are consistently low in the brain, and somewhat higher and more variable at least in the blood mononuclear cells and liver.

Research Objectives

The Glucocorticoid receptor is involved in many physiological processes, such as the regulation of growth, metabolic homeostasis, immune functions and others. GR is ubiquitously distributed, but expression levels vary widely between tissues. GR levels are thought to be controlled by multiple alternative first exons. Seven of these exons are located in an upstream CpG island. The first objective of this thesis was to investigate the promoter activity of the intronic regions between these exons, and their susceptibility to CpG methylation.

SNPs are the most common form of genetic variability. The SNPs in the *GR* gene have been associated with the variation of GR function. SNPs occurring in upstream promoter regions of genes can potentially affect the process of transcription. The second objective was to indentify new SNPs in *GR* promoter regions by genotyping 221 donors to reveal potential new promoter-specific haplotype, and to examine their functional consequences.

The human *GR* promoter 1F is susceptible to methylation during stressful early life events resulting in lower 1F transcript levels. CpG methylation in promoter 1F is thought to interfere with transcription factor binding, subsequently inhibiting transcription. The third objective was to investigate the role of predicted transcription factors in regulating 1F transcription and the epigenetic sensitivity of promoter 1F was investigated by performing the single CpG dinucleotide methylation reporter gene studies.

GR plays an important role in HPA axis regulation in response to stress. The fourth objective was to determine the distribution pattern of the *GR* first exons and *GR* splice variants in different regions of the healthy human brain. Furthermore, methylation status of the *GR* promoters was investigated to examine the link between methylation and expression.

Outline

In **Chapter 1**, a broad notion of the context which this thesis was based on were described and explained and the overview about the transcriptional regulation of the glucocorticoid receptor has been described. In **Chapter 2**, the activity of the seven proximal *GR* promoter regions located in an upstream CpG island was measured. 221 donors were sequenced to identify promoter SNPs and these new SNPs were functionally characterised. The susceptibility of these promoters to DNA methylation was examined. In **Chapter 3**, the role of NGFI-A and several other transcription factors predicted to bind the 1F promoter was analysed in cells representing peripheral and central tissues. In **Chapter 4**, we focus on the role of E2F1 on regulation of *GR* exon 1F. The binding site of E2F1 on promoter 1F has been identified and single CpG dinucleotide methylation experiments were performed to investigate the epigenetic sensitivity of promoter 1F. In **Chapter 5**, the *GR* first exons, *GR* splice variants and methylation status in *GR* promoters in human health brain have been investigated. The GR/MR ratio in different brain tissues has been measured. In **Chapter 6**, the results of the major finding of this thesis was discussed and summarized. The future perspectives are given.

CHAPTER 2

Transcriptional control of the human glucocorticoid receptor: Identification and analysis of alternative promoter regions

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2.1 Abstract

Glucocorticoid receptor (GR) levels are thought to be controlled by multiple alternative first exons. Seven of these exons are located in an upstream CpG island. In this study, we investigated the promoter activity of the intronic regions between these exons, and their susceptibility to CpG methylation and sequence variability. The seven promoters were cloned into luciferase reporter genes, and their activity measured in ten cell lines. CpG islands of 221 donors were genotyped and the effects of these single nucleotide polymorphisms (SNPs) were investigated in a reporter gene assay. We showed that each of the first exons was independently controlled by a unique promoter located directly upstream. Promoter activities were cell type-specific, and varied considerably between cell types. Irrespective of the cell type, *in vitro* methylation effectively silenced all reporter constructs. Eleven SNPs were observed within the CpG island of 221 donors, and a new promoter-specific haplotype was revealed. Four of the minor alleles reduced the reporter gene activity, with cell type specific effects. This complexity within the CpG island helps to explain the variable, tissue-specific transcriptional control of the GR, and provides insight into the mechanisms underlying tissue specific deregulation of GR levels.

Keywords: Promoter activity, DNA methylation, Single nucleotide polymorphism (SNP), glucocorticoid receptor, alternative first exons

2.2 Introduction

Transcriptional mechanisms are critically important for cell- and tissue-specific gene expression patterns and protein diversity. Recently, the use of alternative promoters has been shown to play a pivotal role in generating the molecular complexity observed in higher species (Landry et al. 2003). Kimura (Kimura et al. 2006) showed that among more than 15000 human genes, 52% contained more than one first exon, and that on average genes have 3.1 alternative first exons. Tissue specific expression was observed for 17% of the alternative first exons. Variability in transcription start sites and alternative promoter usage, are frequent in CpG-rich regions (Illingworth and Bird 2009). CpG dinucleotides are the least frequent of all nucleotide pairs, and they are scattered in clusters, or CpG islands, throughout the genome. A CpG island is usually >200bp long and has a >50% G+C content, and an observed/expected CpG ratio of >0.6 (Gardiner-Garden and Frommer 1987). There are between 24000-27000 such CpG islands in the human genome, corresponding to about 1% of the total genome (Illingworth et al. 2008; Waterston et al. 2002). CpG islands have been found immediately upstream of 60-70% of all genes, in the regions that contain their promoters (Larsen et al. 1992; Saxonov et al. 2006; Weber et al. 2007). Overall 60-90% of CpGs are thought to be methylated (Ehrlich 2003; Gruenbaum et al. 1981; Razin et al. 1984), and their genomic location largely determines their methylation status. CpG dinuleotides outside of CpG islands are usually methylated, while those within CpG islands are thought to be protected from methylation (Cooper et al. 1983). DNA methylation can occur on both DNA strands at the 5'-cytosine of a CpG dinucleotide and is associated with stable gene silencing by preventing the binding of transcription factors. CpG methylation, especially in CpG islands, has been implicated in tissue-specific regulation of many genes (Song et al. 2005).

Another molecular feature that affects gene function is genetic variability, most commonly in the form of single nucleotide polymorphisms (SNPs). SNPs are scattered throughout the 23 pairs of chromosomes of the human genome, and roughly 11 million common polymorphisms (>1% frequency) are estimated to exist (Musunuru and Kathiresan 2008). A third generation human haplotype map catalogues from 1.3 to 4.1 million SNPs depending on ethnicity (Altshuler et al.

2010). Polymorphisms within gene promoter regions can have profound effects on transcriptional efficiency of genes (Blank et al. 2005; Myers et al. 2007; Wang et al. 2008) and may contribute to disease susceptibility of complex inherited traits. For example, a SNP in the interleukin-1 promoter region was reportedly associated with rheumatoid arthritis (Harrison et al. 2008) and an E-cadherine160 C/A promoter polymorphism was associated with increased susceptibility and altered disease progression of pancreatic carcinoma in Chinese patients (Fei et al. 2010). Similarly multiple sequence variants upstream of *IRGM* in some populations were associated with Crohn's disease (Prescott et al. 2010).

The glucocorticoid receptor gene (*GR*) (OMIM +138040; official symbol is *NR3C1* for nuclear receptor subfamily 3, group C, member 1) is a prime example of a gene with multiple alternative first exons susceptible to epigenetic DNA methylation. The GR and its glucocorticoid (GC) ligand are involved in many physiological processes, such as the regulation of growth, metabolic homeostasis, immune functions and others. GR is ubiquitously distributed, but expression levels vary widely between tissues (Kalinyak et al. 1987). The human *GR* contains 8 translated exons (exon 2-9) and 9 untranslated alternative first exons (Presul et al. 2007; Turner and Muller 2005). This 5'-transcript variability does not affect the protein sequences since the ATG translation start codon is located in exon 2, although it is thought to be responsible for regulating GR levels in a tissue- specific manner (Presul et al. 2007; Turner and Muller 2005). Exon 1A, together with its promoter, is located in a distal promoter region about 30kb upstream of the translation start site. Promoter 1A (Breslin et al. 2001) was involved in up-regulation of GR levels by glucocorticoids in T cells and down-regulation in other cell types (Pedersen and Vedeckis 2003). However, most alternative first exons (1B-1J) were found within a proximal methylatable CpG island. The more recently discovered exons (1D-1J) have a distinct tissue distribution with important consequence for tissue-specific regulation of GR expression (Turner and Muller 2005; Turner et al. 2006; Presul et al. 2007). This evolutionarily conserved CpG island upstream of exon 2 was also known to have promoter activity (Breslin and Vedeckis 1998; Geng et al. 2008; Nunez and Vedeckis 2002; Presul et al. 2007; Turner and Muller 2005) and a large number of transcription factors (Reviewed in Turner et al. 2010) have been identified binding to this region including the GR itself (Geng et al.

2008). The *GR* CpG island was initially shown to be susceptible to methylation in rodents (Weaver et al. 2004) and later in man (Moser et al. 2007) with significant consequences on the functioning of the hippocampus-pituitary-adrenal (HPA) axis, and the subsequent response to a physical or psychosocial stressor (reviewed in Turner et al. 2010).

In this study, we further investigated the intronic regions between the alternative first exons within the proximal CpG island of the *GR*. We showed that each of these regions has promoter activity and that each alternative first exon has its own promoter directly upstream. These promoters were susceptible to silencing by methylation and harbored SNPs that affected their activity.

2.3 Materials and Methods

2.3.1 DNA constructs containing wild type promoter regions

The *GR* promoters 1D, 1J, 1E, 1B, 1F, 1C and 1H were cloned (Figure 7) by PCR amplification from the BAC clone RP11-278J6 (AC091925) (Invitrogen, Merelbeke, Belgium) at positions -4825 to -4485, -4310 to -3994, -3950 to -3829, -3779 to -3615, -3536 to -3211, -3146 to -2751 and -2371 to -1899 relative to the ATG translation start codon in exon 2 using primers and conditions shown in Table 4. PCR products were digested with *Bgl*II and *EcoR*I (New England Biolabs, Frankfurt, Germany) and cloned into the pMetLuc-reporter vector containing the luciferase reporter gene (Clontech, Saint-Germain-en-Laye, France). All clones were sequence-verified using pMetLuc-sequencing forward primer: 5'-AACCGTATTACCGCCATGCA-3' and reverse primer: 5'- AACACCACCTTGATGTCCATGG-3'.

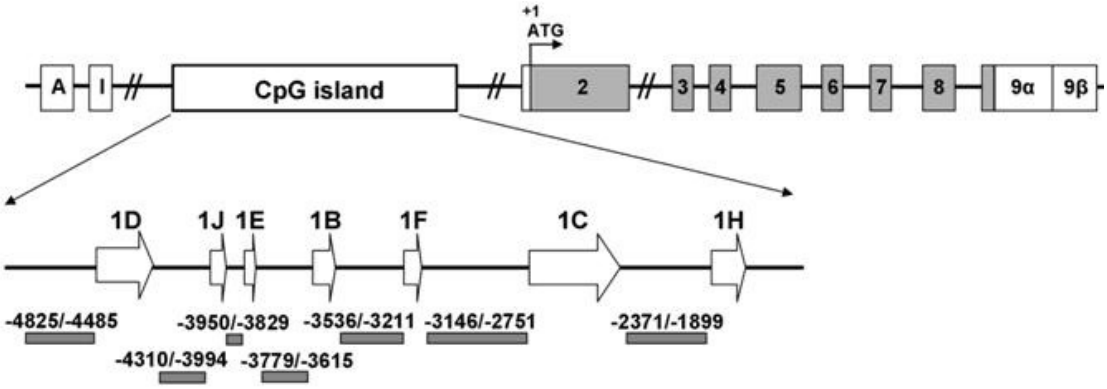

Figure 7. Structure of the proximal CpG island of the human glucocorticoid receptor.

Numbers represent the sequence fragments that were tested for promoter activity in the luciferase reporter gene assays. Numbering starts at the ATG of exon 2. The +1 position is the adenine. The start of the human exon 2 is at -13bp.

Table 4. PCR primers and their associated reaction conditions.

Promoter regions	Sequence[a]	Tm (°C)[b]	[Mg^{2+}](mM)	Primers (µM)	DNA polymerase used
Promoter 1D	Fwd: 5'-Taccggactc agatct GCGAGTATCTCTTCCTTTGCC-3' Rev: 5'-Gtctactgca gaattc ACAGCCACTCTCTCACCTCC-3'	62	4	0.5	Taq DNA polymerase(Invitrogen)
Promoter 1J	Fwd: 5'-Taccggactc agatct GGTAGGAGGCTCGGTCCCGGC-3' Rev: 5'- Gtctactgca gaattc GTTCCCGGTGCAGGCCCCAC-3'	63	2	1	
Promoter 1E	Fwd: 5'-Taccggactc agatct AGAAGAGGTCAGGAGTTTCGGAA-3' Rev: 5'- Gtctactgca gaattc GAGCTGGATTTCTTTGCACTTTT-3'	59	2	1	
Promoter 1B	Fwd: 5'-Taccggactc agatct GTGAGCACATTGGGCGGGAGGGGTG-3' Rev: 5'- Gtctactgca gaattc CGCCGCGCTCTCGCACTGGGA-3'	70	1	1	Diamond DNA polymerase(Bioline)
Promoter 1F	Fwd: 5'-Taccggactc agatct AAGTACGTATGCGCCGACCC-3' Rev: 5'- Gtctactgca gaattc CTCGCTACCTCCTTCCCGCC-3'	67	0.5	1	Phusion Hot Start DNA polymerase(Finnzymes)
Promoter 1C	Fwd: 5'-Taccggactc agatct CAGGTCGGCCCCCGCC-3' Rev: 5'- Gtctactgca gaattc CAAAATGGAGGAGGCGGCG-3'	66	0.7	1	
Promoter 1H	Fwd: 5'-Taccggactc agatct GCCAGAGGTAAGAAGCGAGGCGGGA-3' Rev: 5'- Gtctactgca gaattc GCCGGGGCCTCCCCGGAGCC-3'	70	0.7	0.1	

[a]Fwd, forward or sense primer ; Rev, reverse or antisense primer. Primer with restriction enzyme recognition sites (underlined) used for amplification of the promoter fragments.

[b]Tm, annealing temperature in PCR.

2.3.2 *In vitro* methylation of reporter plasmid

Luciferase reporter constructs containing promoter 1B, C, D, F and H were methylated *in vitro* using *Sss*I CpG methyltransferase (New England Biolabs). Briefly, 5µg plasmid, 1µl *Sss*I methylase (4U/µl), 1µl SAM (32mM) were incubated for 4 hours (37°C) in a 50µl reaction and subsequently inactivated (65°C, 20min). Plasmids were purified by phenol extraction followed by ethanol precipitation. Complete methylation was confirmed by methylation-specific restriction digestion using *Hpa*II and *Msp*I (Bryans et al. 1992). Only completely methylated plasmids were used for DNA transfection studies.

2.3.3 Glucocorticoid receptor genotyping

The CpG islands of 221 donors previously characterized for *GR* SNPs (Kumsta et al. 2009) were genotyped using standard PCR methods. Seven promoter fragments were amplified from genomic DNA using primers from Table 4 and PCR conditions from Table 5. PCR fragments were purified using the Jetquick PCR purification kit (Gemomed, DE). Purified products were sequenced using the BigDye 3.1 Terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, The Netherlands) on the ABI 3130 sequencer (Applied Biosystems) using the pMetLuc-sequencing primers. Sequences were analysed in Vector NTi (Invitrogen, Paisly, UK).

2.3.4 DNA constructs containing SNPs of promoter regions

Reporter constructs containing individual SNPs (ss184956515 (1D), rs3806855 (1B), rs10482606 (1C), ss184956511 (1H), ss184956512 (1H) and rs10482614 (1H)) were generated by *in vitro* site-directed mutagenesis using a standard protocol (Invitrogen). Mutagenesis primers are listed in Table 6. Constructs (rs3806854 (1B), ss184956513 (1F) and rs10482605 (1C)) that were not obtained by *in vitro* site-directed mutagenesis were generated by PCR amplification of donor genomic DNA using the primers in Table 4 and conditions provided in Table 5. PCR products were digested with *Bgl*II and *EcoR*I and ligated into pMetLuc-vector. All clones were sequence-verified.

Table 5. PCR conditions for cloning promoter regions.

Promoter Region	Tm (°C)	$[Mg^{2+}]$ (mM)	Primers (µM)	DNA polymerase used
Promoter 1D	68	1	0.5	Phusion Hot Start DNA polymerase (Finnzymes)
Promoter 1J	70	1	0.1	
Promoter 1E	66	1	0.1	
Promoter 1F	70	2	0.5	Platinum®Taq DNA Polymerase (Invitrogen)
Promoter 1B	70	1	1	Diamond DNA polymerase (Bioline)
Promoter 1C	54	2	0.5	
Promoter 1H	70	3	0.5	

Table 6. *In vitro* site-directed mutagenesis PCR primers.

SNPs	Fwd a	Rev
ss184956515(1D)	cgt-cag-tct-gag-cgc-ggc-ggA-agg-tga-gag-agt	ccg-ccg-cgc-tca-gac-tga-cgg-cgg-ctc-ccc
rs10482603(1J)	acg-ggc-acc-cct-cgc-ccc-acA-gcc-ctc-tcc-ttt	gtg-ggg-cga-ggg-gtg-ccc-gtg-cgg-gaa-gcc
ss184956514(1J)	ctt-ccc-ctt-gga-ctg-agg-ggA-aag-ctc-cta-aca	ccc-ctc-agt-cca-agg-gga-agg-gaa-ctc-gtg
rs3806855(1B)	ctt-ggc-agg-cgg-cgc-ctc-ctG-ctg-ccg-ccg-ccg	agg-agg-cgc-cgc-ctg-cca-agt-tca-acc-ccc
rs10482606(1C)	tgg-gag-cgc-gtg-tgt-gcg-agC-gtg-tgc-gcg-ccg	ctc-gca-cac-acg-cgc-tcc-cac-tcc-acc-ccc
ss184956511(1H)	tag-cct-cgg-gga-gtg-ggg-gtA-ggg-ggc-tgg-caa	acc-ccc-act-ccc-cga-ggc-taa-taa-aag-ttt
ss184956512(1H)	gaa-agg-gca-gcg-cgc-ggg-tgC-cag-cgc-tgg-cct	cac-ccg-cgc-gct-gcc-gtt-tcg-tca-ccg-tcg
rs10482614(1H)	gtc-gcc-cga-cac-ccg-ttt-tcA-tgg-tga-acg-cta	gaa-aac-ggg-tgt-cgg-gcg-acc-ccc-ttg-gag

[a]Fwd, forward or sense primer ; Rev, reverse or antisense primer. The capital letters indicate the SNPs in the promoter regions.

2.3.5 Cell culture and transient transfections

Ten cell lines were cultured as described in the Table 7. All culture media and reagents were obtained from Lonza (Verviers Belgium). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Twenty-four hours prior to transfection, cells were seeded in 24-well plates. Transfections were performed using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Detailed transfection conditions are shown in Table 7. Daudi cells were transfected using the Microporator according to the manufacturer's protocol (Digital Bio, Korea). Transfection efficiency was controlled by co-transfecting the pSEAP2-control vector (Clontech) expressing secreted alkaline phosphatase (SEAP). pMetLuc-control vector (Clontech) with a CMV promoter was used as a positive transfection control.

2.3.6 Reporter gene assay

Culture supernatants were recovered 48 hours post-transfection. Luciferase assays were performed using the Ready-To-Glow Secreted Luciferase Reporter system kit (Clontech). Luciferase gene expression was normalized to SEAP measured using the Great EscAPeTM SEAP Fluorescence Detection kit (Clontech) and subsequently expressed either relative to the empty pMetLuc-reporter vector, or as a percentage of the unmethylated or wildtype reporter vector.

2.3.7 Statistical analysis

Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* test. Linkage disequilibrium (LD) among the sixteen SNPs was estimated with D' and r^2 using LD Heatmap package and haplotype frequency analysis with the Haplostats package for the R statistical environment (Version 2.9.0). Statistical analysis and graphs were performed with SigmaPlot 9.0 (Systat Software GmbH, Erkrath, Germany). Statistical significance was considered for $p<0.05$. Results are shown as the mean±SEM.

Table 7. Ten cell lines culture conditions and transfection conditions.

Cell lines		DNA (ng)[1]	Density	Lipofectamine LTX (µl)	PLUS reagent (µl)		Medium
Cationinc lipid transfection							
Hela S3	Human cervix epithelial adenocarcinoma	500+500	4x10E4	0.75	-		DMEM+10%FBS+1%L-Glutamine
293FT	Human embryonic kidney	500+500	8x10E4	1.75	-		DMEM+10%FBS+1%L-Glutamine
HepG2	Human hepatocellular carcinoma	500+500	8x10E4	2.25	0.5		EMEM +10%FBS
Hep3B	Human hepatocellular carcinoma	500+500	8x10E4	2.5	1		EMEM +10%FBS
U373 MG	Human glioblastoma-astrocytoma	500+500	4x10E4	1.5	1.5		EMEM+20%FBS
HL60	Human promyelocytic leukemia cells	500+500	4x10E4	2.75	3		IMDM+20%FBS
SK-N-SH	Human neuroblastoma	500+500	1x10E5	1.75	1.5		EMEM+20%FBS
Jurkat	Human acute T cell leukaemia	500+500	1x10E5	1.75	1		RPMI 1640 without Phenol Red +10%FBS+1%L-Glutamine
THP1	Human acute monocytic leukemia cells	500+500	1x10E5	1.75	1		RPMI 1640 +10%FBS+ 1% Sodium Pyruvate.
Electroporation				PV[2]	PW[2]	PN[2]	
Daudi	Human Burkitt's lymphoma cell line	500+500	1x10E5	1475	20	1	RPMI 1640 +10%FBS+ 1% Sodium Pyruvate.

[1]. DNA amount is given as reporter gene (ng) and internal pSEAP control (ng).

[2]. PV, pulse volt: PW, pulse width: PN, pulse number.

2.4 Results

2.4.1 Activity of the GR promoters in human cell lines

The regions immediately upstream of each *GR* first exon shown in Figure 7 were cloned into a luciferase-reporter vector to demonstrate their promoter activity. Each of the promoters constructs except 1J and 1E was significantly active in at least one cell line (Figure 8a-j). In all cell lines, the pMetLuc-Control vector, controlled by a CMV promoter, was 3 (Hela S3) to 63 times (U373MG) more active than the activity of the empty pMetLuc-reporter. Although the constructs including the pMetLuc-Control vector showed a great variability in activity between cell types, the activity pattern of most promoters was similar across the different cell lines. Despite differential promoter usage, exon 1C had the strongest promoter in all cell lines except in U373MG and SK-N-SH cells where promoters 1H and 1D were most active. The other two most active promoters across the different cell lines were 1H and, to a lesser extent, 1B. The activity of promoters 1J, 1E and 1F was mostly low, while 1D was elevated in at least a few cell lines. Interestingly, although the promoter 1E and 1J were the weakest in all cells, they repressed the empty reporter gene activity in HepG2 and Hep3B cells. The analysis of promoter 1J is limited, since the CEM-C7H2 cells, the only cell line in which exon 1J has been reported, was not available for testing.

2.4.2 GR promoter activity after methylation

The *GR* CpG island is susceptible to highly variable levels of cytosine methylation (Turner et al. 2008; Weaver et al. 2004). In order to understand the effect of CpG methylation on the activity of the different promoters, reporter constructs were methylated *in vitro* by *Sss*I methyltransferase and transfected into four cell lines with different promoter activities. Complete methylation essentially repressed promoter activity in most cell lines to below 10% of the unmethylated vector (Figure 9b-f). When constitutive expression was low, as for promoter 1F and 1H in Jurkat cells, the relative loss of activity was low although still significant. This indicates that the activity of promoters upstream of GR alternative first exons is highly susceptible to epigenetic methylation. Complete methylation of all the CpG sites in the pMetLuc-Control vector, including the common

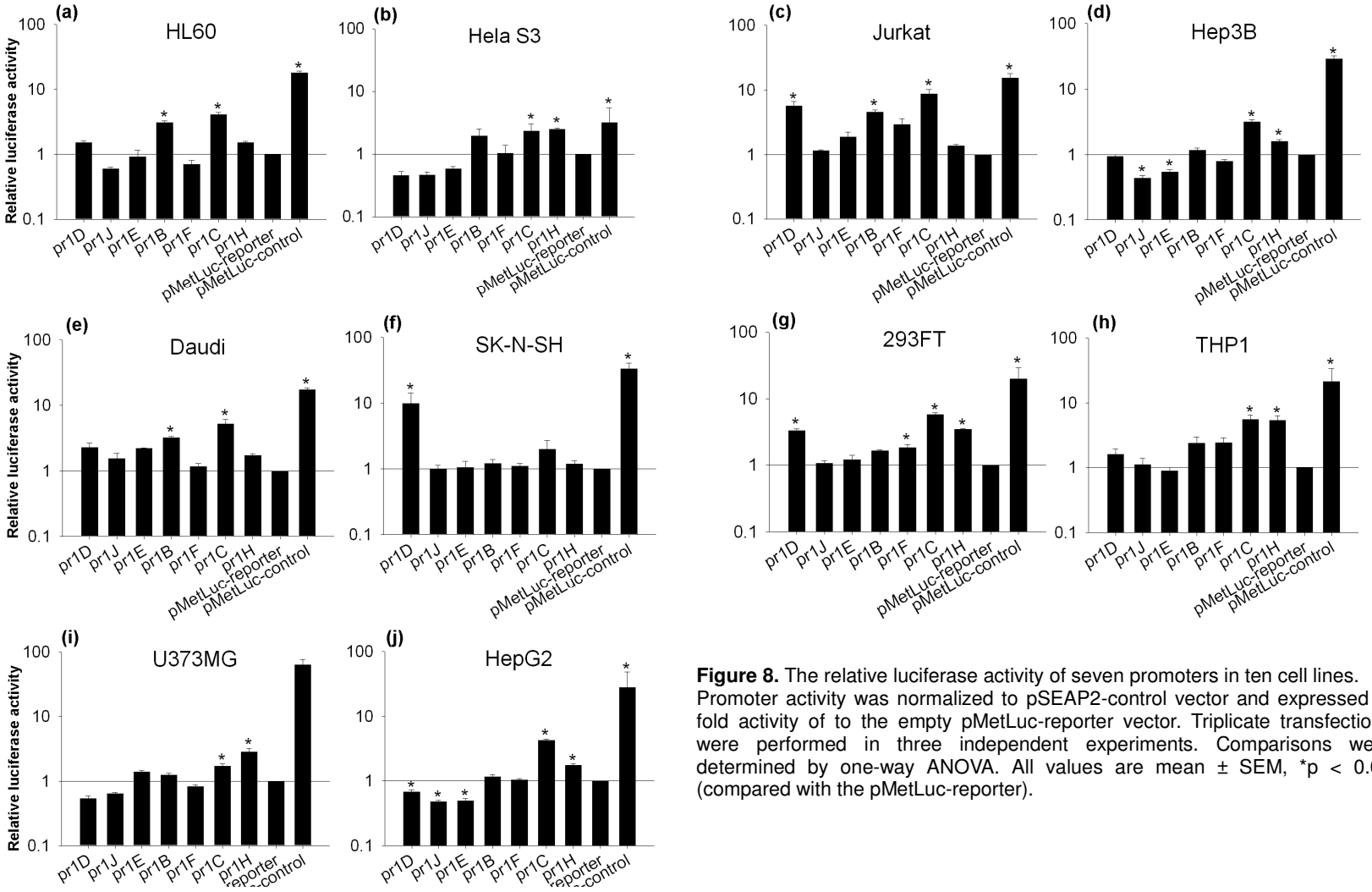

Figure 8. The relative luciferase activity of seven promoters in ten cell lines. Promoter activity was normalized to pSEAP2-control vector and expressed in fold activity of to the empty pMetLuc-reporter vector. Triplicate transfections were performed in three independent experiments. Comparisons were determined by one-way ANOVA. All values are mean ± SEM, *p < 0.05 (compared with the pMetLuc-reporter).

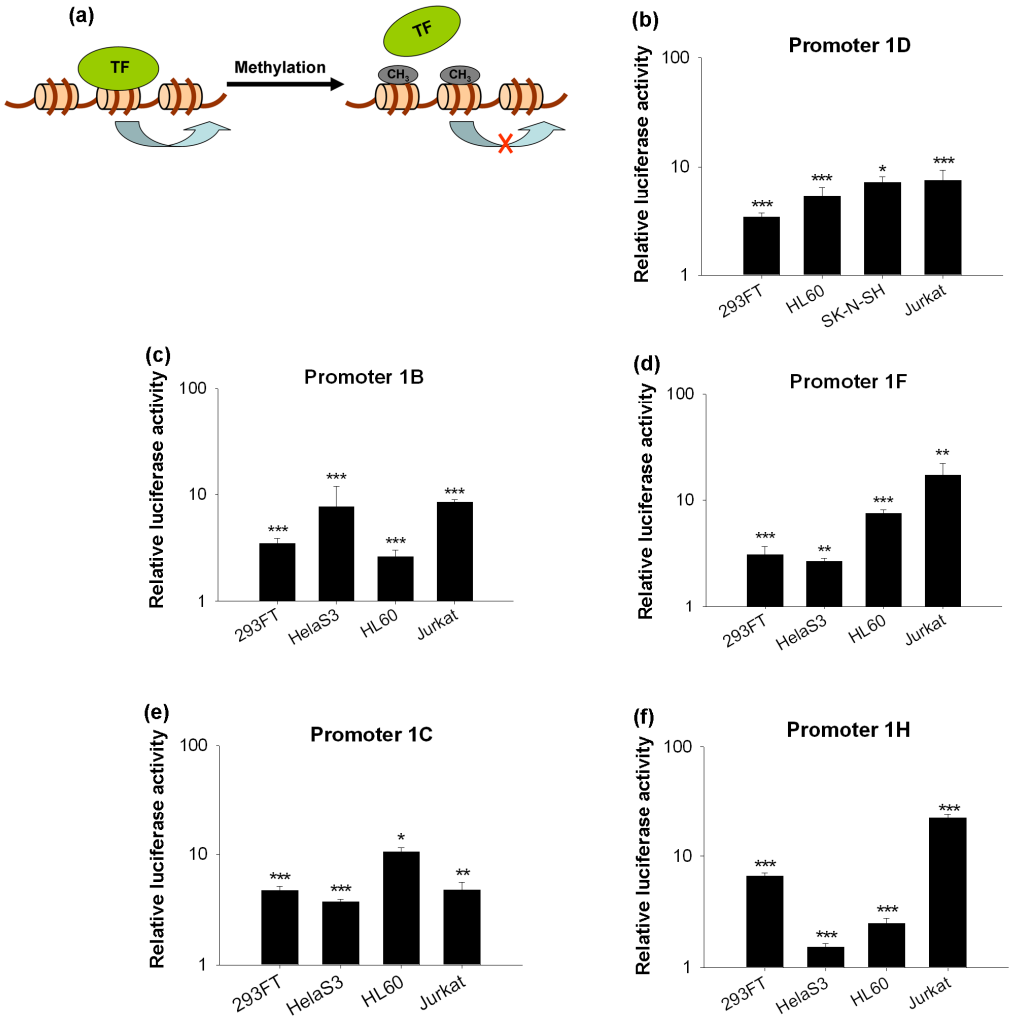

Figure 9. DNA methylation (CH_3) inhibits binding of transcription factors (TF) and gene activation (a). Relative luciferase activity of five promoters before and after complete methylation in four cell lines (b-f).

Promoter 1D contains 28 CpGs; promoter 1B, 17; promoter 1F, 43; promoter 1C, 63; promoter 1H, 54. Promoter activity was normalized to pSEAP2-control vector and expressed in percent of the unmethylated promoter activity. Triplicate transfections were performed in three independent experiments. Data are mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

essential promoter sequences, the luciferase coding sequence, the 3'-untranslated region and the poly-adenylation signal, had no effect on luciferase levels in either of the cell lines tested.

2.4.3 Genotyping of the different alternative GR promoters

The sequence variability within the above alternative *GR* promoter regions was investigated in 221 donors with 5 known *GR* SNPs (Kumsta et al. 2009). After genotyping, eleven SNPs were observed including 5 previously unreported SNPs (Table 8). SNPs were found in all promoter regions except 1E. Promoter 1B, 1C and 1J each harbored two SNPs and three SNPs were observed in promoter 1H. The frequency of the minor alleles within our population varied from 0.23% to 15.53%. These SNPs had not previously been investigated for linkage disequilibrium (LD) except for rs10482605 (NR3C1-1) in promoter 1C (Kumsta et al. 2009).

Using SNPs rs6189, rs6195, rs41423247 and rs6198 within the *GR* which were previously reported in these 221 donors (Kumsta et al. 2009), we were able to analyse the LD and haplotype structure of sixteen SNPs covering a genomic region of roughly 129kb including the CpG island and the complete coding sequence. LD was detected between the five new promoter SNPs (Figure 10a). The ss184956515 in promoter 1D was in LD with both rs10482603 in promoter 1J (D'=0.965, r^2=0.682) and ss184956512 in promoter 1H (D'=0.965, r^2=0.683). SNPs rs3806855 (D'=0.949, r^2=0.943) and rs3806854 (D'=0.937, r^2=0.925) both in promoter 1B are in strong LD, and constitute haplotypes together with rs10052957, rs10482614 and rs41423247. The five new promoter SNPs did not change the existing haplotype structure (Kumsta et al. 2009), but a new promoter-specific haplotype (number 4-2) was identified (Figure 10b).The LD between the five minor alleles in haplotype 4-2 was shown in Table 9. Haplotype 4-2 occurred in 13.3% of our population. This haplotype 4-2 (13.3%) and haplotype 4-1 (20.8%) represented the haplotype 4 (36.5%) described in the Kumsta study (Kumsta et al. 2009).

Table 8. Location and frequencies of SNPs in human *GR* exon 1 promoter regions.

Location	Alleles	dbSNP reference	Position [a]	Available samples/total	N° heterozygous	N° homozygous	Total minor alleles	Minor allele frequency
promoter 1D	G/A	ss184956515	-4497	219/221	2	0	2	0.46%
promoter 1J	G/A	rs10482603	-4209	219/221	1	0	1	0.23%
	G/A	ss184956514	-4154	219/221	2	0	2	0.46%
promoter 1E	-	-	-	219/221	-	-	-	-
promoter 1B	T/G	rs3806855	-3725	219/221	62	1	64	14.61%
	T/C	rs3806854	-3723	219/221	62	0	62	14.16%
promoter 1F	G/A	ss184956513	-3283	219/221	2	0	2	0.46%
promoter 1C	T/C	rs10482605	-3117	219/221	52	8	68	15.53%
	T/C	rs10482606	-2864	219/221	1	0	1	0.23%
promoter 1H	G/A	ss184956511	-2247	218/221	1	0	1	0.23%
	A/C	ss184956512	-2196	218/221	1	0	1	0.23%
	G/A	rs10482614	-1998	218/221	60	1	62	14.22%

[a] Locations with respect to ATG start codon. The start of the human exon 2 is at -13bp.

Table 9. Linkage disequilibrium between five investigated loci in haplotype 3.

Loci_1	Location	Loci_2	Location	D'	r^2
rs10052957 (*TthIII*I)	promoter1D	rs3806855	promoter 1B	0.999	0.605
		rs3806854	promoter 1B	0.999	0.616
		rs10482614	promoter 1H	0.971	0.595
		rs41423247 (*Bcl*I)	Intron 2	0.069	0.060
rs3806855	promoter1B	rs3806854	promoter 1B	0.999	0.981
		rs10482614	promoter 1H	0.949	0.943
		rs41423247 (*Bcl*I)	Intron 2	0.999	0.537
rs3806854	promoter1B	rs10482614	promoter 1H	0.937	0.925
		rs41423247 (*Bcl*I)	Intron 2	0.999	0.527
rs10482614	promoter 1H	rs41423247 (*Bcl*I)	Intron 2	0.968	0.517

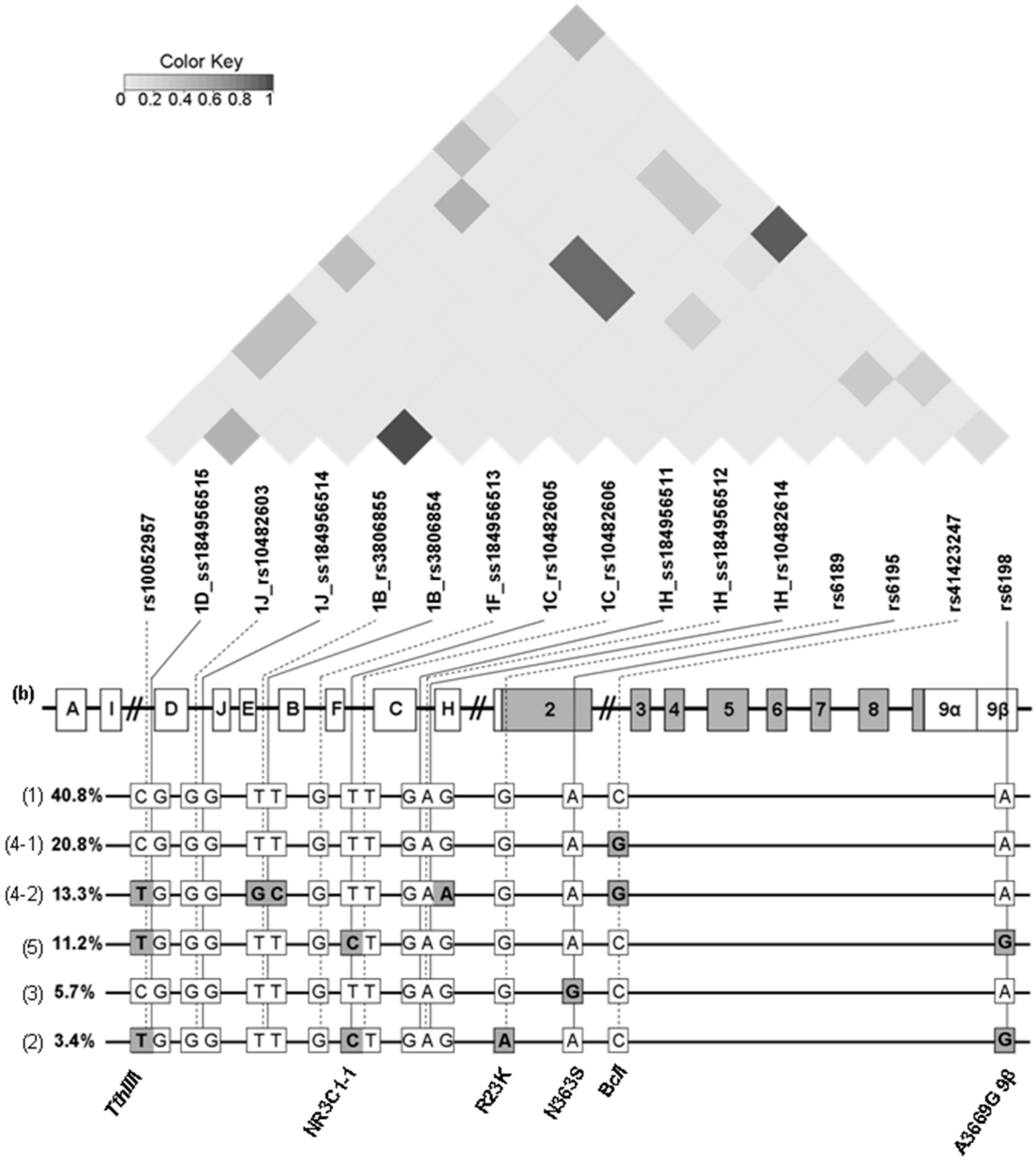

Figure 10. Linkage disequilibrium (LD) structure of the human *GR* (a). LDs between 2 sites are represented by the grey diamonds: darker grey, stronger LD; light grey, weaker LD; white diamonds, no LD. Genomic organization of the *GR* (b) is shown in the upper part of the panel. Open boxes indicate alternative first exons; the coding exons of the gene are drawn as grey boxes. The lower part of the panel shows (1-5) the observed haplotype structure and frequencies. Minor alleles are denoted by bold letters.

2.4.4 Effect of SNPs on promoter activity

To investigate the functional consequences of the promoter SNPs, they were introduced into the cloned promoters by site-directed mutagenesis.

In promoter 1B, the two minor alleles of rs3806854 and rs3806855 reduced promoter activity in some cell lines. After *post hoc* correction, the promoter activity associated with the minor G allele of rs3806855 was significantly reduced in Hela S3 ($p<0.001$), HL60 ($p=0.004$) and Jurkat ($p<0.001$) (Figure 11a) and the minor G+C alleles (combination of rs3806855+ rs3806854) significantly reduced promoter activity in Jurkat ($p=0.004$) and Hela S3 cells ($p=0.044$). Both minor alleles had no effect in 293FT, HL60 and U373MG cells. Whilst the minor G allele of rs3806855 alone had no effect on predicted transcription factor binding sites, the minor C allele of rs3806854 introduced a predicted ETF binding site.

For promoter 1H, the minor A allele of rs10482614 significantly impaired promoter activity in 293FT ($p<0.001$), Hela S3 ($p<0.001$), HL60 ($p=0.008$), Jurkat ($p=0.008$) and U373MG ($p=0.008$) cells (Figure 11b). This may, in part, be due to the predicted loss of the KID3 binding site in this reporter gene. No significant differences could be observed for the minor A allele of ss184956511 and the minor C allele of ss184956512 in these cell lines. The loss of three predicted transcription factor binding sites (CACD, WT1 and KID3) induced by the ss184956511 and the addition of CP2 had no obvious effect on promoter activity.

For promoter 1C, the minor C allele of rs10482606 was associated with significantly lower activity in 293FT ($p<0.001$), Hela S3 ($p<0.001$), and U373MG ($p=0.003$) (Figure 11c). The inclusion of the minor allele did not alter the predicted ZF5 binding site located within rs10482606. No significant differences could be observed for the minor C allele of rs10482605 in HL60 and Jurkat cell lines.

The minor alleles in promoter 1D and 1F had no effect on promoter activity in any of the 5 cell lines (Figure 11d and e).

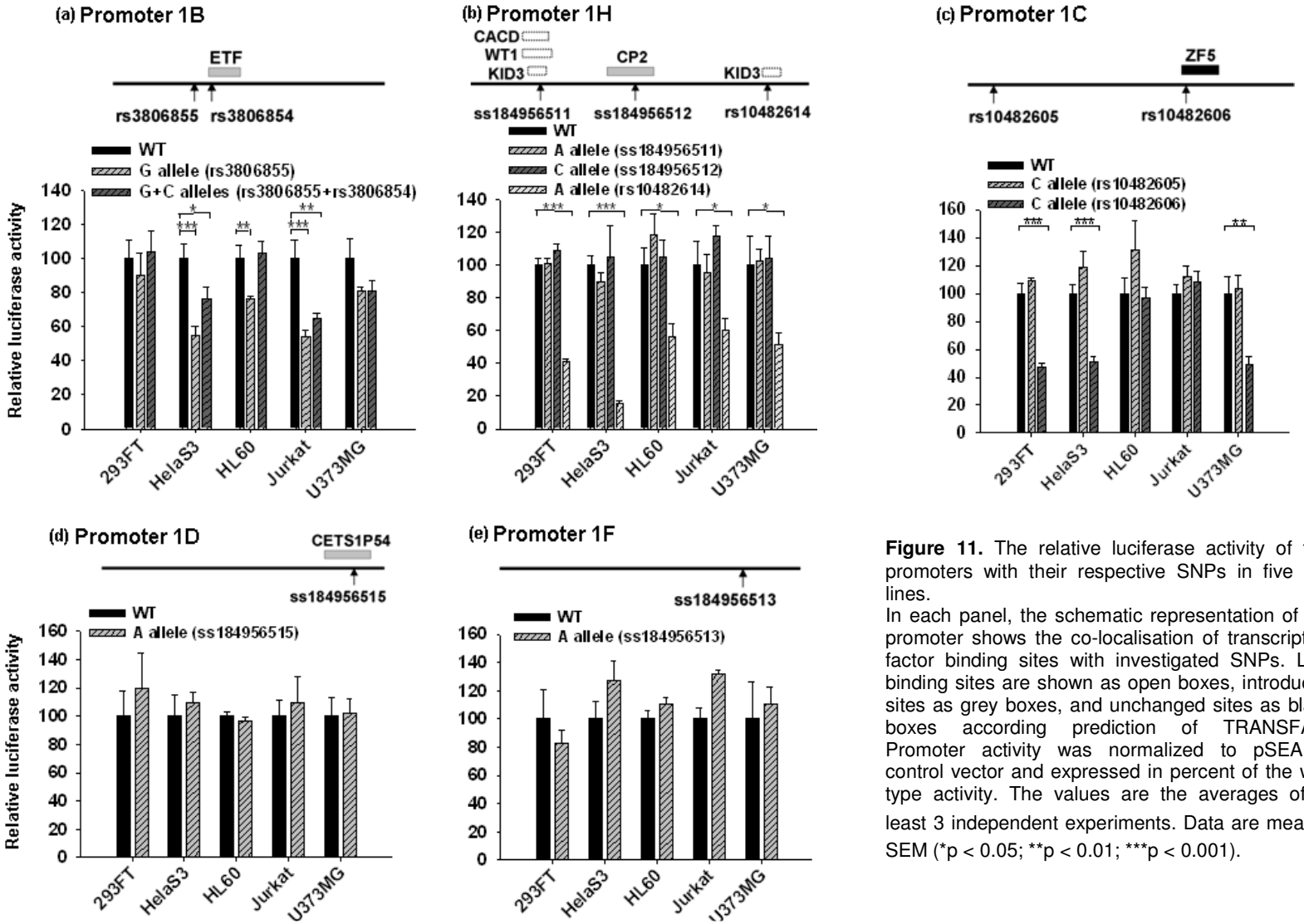

Figure 11. The relative luciferase activity of five promoters with their respective SNPs in five cell lines.
In each panel, the schematic representation of the promoter shows the co-localisation of transcription factor binding sites with investigated SNPs. Lost binding sites are shown as open boxes, introduced sites as grey boxes, and unchanged sites as black boxes according prediction of TRANSFAC. Promoter activity was normalized to pSEAP2-control vector and expressed in percent of the wild type activity. The values are the averages of at least 3 independent experiments. Data are mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.5 Discussion

We have previously suggested that alternative first exon usage is the major mechanism regulating expression of the *GR* (Turner and Muller 2005; Turner et al. 2006). We and others have suggested that each first exon has a unique promoter (Breslin and Vedeckis 1998; Geng et al. 2008; Nunez and Vedeckis 2002; Presul et al. 2007; Turner and Muller 2005; Turner et al. 2006), but this has not been systematically investigated. In this study, we have investigated the introns immediately upstream of each first exon for promoter activity. The cloned introns vary from 122 (1E) to 473 (1H) bp in length, and fit the classical convention for proximal promoters (Werner 1999; Bortoluzzi et al. 2005; Hay and Docherty 2006). We show that introns upstream of exons 1B, C, D, F, and H are active promoters. In line with our previous observation of transcripts containing exon 1C predominating (Turner and Muller 2005; Alt et al. 2010), the relative activity of this promoter was highest in almost all cell lines investigated. The activity of promoter 1H in six cell lines concords with the broad tissue distribution of this exon (Turner and Muller 2005). The promoter 1D was remarkably active in the SK-N-SH brain-derived cell line, which is in line with the observation of exon 1D in the hippocampus (Turner and Muller 2005) and the cerebellum (Presul et al. 2007). Similarly, the activity of promoter 1D in Jurkat cells agrees with the findings of Geng et al. (2008). The activity of promoters 1J and 1E was lowest and sometimes even reduced the activity of the pMetLuc-reporter, exon 1E and 1J correspondingly had the lowest abundance in all human tissues and cell lines investigated (Alt et al. 2010; Presul et al. 2007). Overall, the promoter activity mirrors the previously reported first exon transcript abundance data. The predominantly constitutive nature of promoter 1C is reflected in a promoter activity that is consistently stronger than that of the tissue specific exons 1B, D, E, F, J and H (Turner and Muller 2005; Alt et al, 2010; Presul et al, 2007). Therefore the activity of promoter 1C may be considered as constitutive whereas the other exons tend to be more tissue-specific. We suggest here that tissue-biased usage of first exons is regulated by tissue-biased activation of their promoters probably by tissue-specific transcription factors. Tissue-specific co-factors may be involved such as c-Myb and Ets observed by Geng et al. (2008). In order to investigate the distribution of potential response elements in the promoter regions identified here, we examined the evolutionary conserved transcription factor binding sites (TFBS) using *in silico* phylogenetic footprinting (ISPF) (Turner et al. 2008). Significantly different transcription factor binding sites were predicted in each promoter region, as shown in Turner et al. (2008), suggesting that the activities of each exon 1 depend on its own set of transcription

factors, as a mechanism of tissue-specific exon1 regulation, in line with our reporter gene data. However, we cannot exclude that other regions outside of those cloned may contribute to the promoter activity and that our results are biased by the reporter gene assay used.

Our data further showed that all alternative GR promoters in the CpG island are susceptible to methylation. Methylation effectively shuts down promoter activity. We demonstrated that irrespective of the cell lines or promoter construct used for transfection, *in vitro* methylation of the *GR* promoters reduced the reporter gene activity on average to about 7% of the unmethylated promoters (Figure 9b-f) suggesting that epigenetic regulation applies to all GR promoters. This reduction in activity was independent of the number of methylatable CpGs in a promoter ($p>0.05$). Epigenetic modifications are thought to play an important role in tissue-specific *GR* regulation. Initially, it was shown in rats that maternal care influences the methylation of two CpG dinucleotides, part of a confirmed NGFI-A binding site, in promoter 1_7 in the hippocampus (Weaver et al. 2004). More recently, it was found that hippocampal methylation of two different CpG dinucleotides in an hypothetical NGFI-A binding site in the human orthologue 1F correlated with childhood abuse and subsequent suicide (McGowan et al. 2009). In the limbic system, however, neither Moser (Moser et al. 2007) nor Alt (Alt et al. 2010) was able to detect methylation at these positions in numerous pathologies. *In vitro* 100% methylation of all CpGs in a reporter gene promoter may not represent the physiological situation, unfortunately a technique allowing methylation of individual CpG dinucleotides within a reporter gene is currently unavailable. In peripheral blood mononuclear cells of healthy donors, stochastic and unique partial methylation levels were detectable throughout the CpG island upstream of the *GR.* For the 130 CpGs investigated, >25% methylation was observed in at least one donor, and very few CpGs reached 75% methylation in any donor (Turner et al. 2008). In comparison, the relatively low methylation level in central nervous system tissues such as the hippocampus, suggests that epigenetic control may be more limited in central than in peripheral GC target tissues such as immune cells (Alt et al. 2010; McGowan et al. 2009; Moser et al. 2007). As *GR* promoter usage is largely tissue- or cell-type specific, methylation levels may have tissue-specific functional consequences. Thus, with each alternative exon having its own promoter, differential promoter methylation provides a mechanism for regulating the activity of each promoter individually.

By sequencing the *GR* CpG islands of 221 donors, five new SNPs were added to the seven known SNPs creating a total of twelve promoter variants. In a reporter gene system, four SNPs modulated

promoter activity (Figure 11a, b and c). Although it is not clear how these SNPs affect promoter activity, two SNPs (rs10482606 and rs3806854) introduced two new CpG dinucleotides sites in promoter 1C and 1B respectively, potentially susceptible to methylation. Others were associated with new or deleted transcription factor binding sites. Our initial report of the differential usage of first exons in different organs (Turner and Muller 2005) as well as our present study of the activity of alternative promoters in multiple cell lines provided evidence to suggest that effects of SNPs vary between tissues. This may apply particularly to the SNPs rs3806855 (1B), rs3806854 (1B) and rs10482614 (1H) with activities that widely vary between cell types. Thus, minor alleles may play an important role in determining GR levels in some tissues, but have little effect in others. For instance, the minor C allele of rs10482605 was observed to be significantly associated with reduced *GR* expression in the Mexican-American population (Niu et al. 2009). Kumsta et al. (2009) showed that this minor C allele reduced promoter activity in two brain-derived cell lines, however, Labuda et al. (2010) showed increased promoter activity in a human choriocarcinoma cell line. We did not find any effect of this minor C allele in any of the five cell lines derived from human kidney (293FT), cervix (Hela S3), peripheral blood (HL60) or brain (SK-N-SH and U373MG). This may be an example where a SNP affects promoter activity in a cell- or tissue-specific manner. Our linkage disequilibrium analysis showed that the above three functional SNPs, rs3086855 (1B), rs3086854 (1B) and rs10482614 (1H), were associated within haplotype 4-2 (Figure 10b), probably with tissues-biased effects. Based on the first exon usage in different human tissues (Turner and Muller 2005), it may be predicted that haplotype 4-2 will have an effect predominantly in the liver, lung and the immune system, since these are the main tissues using the promoters 1B and 1H (Turner and Muller 2005), down-regulated by this haplotype.

As the *GR* has multiple promoters, a SNP in one promoter will have only subtle overall effects on GR levels. Nevertheless, even small changes in tissue-specific, GR levels may have significant pathophysiological consequences. For instance, the minor C allele of rs10482605 (1C) has been associated with an increased risk of major depression (van West et al. 2006) and a higher complication rate in childhood acute lymphoblastic leukemia (Labuda et al. 2010). The rs10052957 in promoter 1D, located outside of the CpG island, was associated with higher basal cortisol secretion in men (Rosmond et al. 2000). Van Rossum et al. (2004) reported that the rs10052957 is in LD with the ER22/23EK polymorphism and is associated with a relative resistance to glucocorticoids and a healthy

metabolic profile. Thus, SNPs affect the functioning of important promoters, probably lowering specific transcript in a tissue-specific manner, leading to increased disease susceptibility. However, in these studies the associated levels of first exon transcripts were not quantified, as a measure of *in vivo* promoter activity of genotypes with and without the SNPs.

In summary, we have shown that each alternative first exon of the *GR* within the CpG island is independently controlled by its own unique promoter region and that methylation and sequence variability influences the promoter activity. This complexity within the regulatory region containing the multiple alternative first exons helps to explain the variable, tissue-specific transcriptional control of the GR. With an increasing diversity of the human transcriptome that is currently unfolding, we suggest that the mechanisms controlling GR transcription apply to many other genes with alternative untranslated first exons. Recent annotations of the human genome suggest that almost half of the protein-coding genes contain alternative promoters (Davuluri et al. 2008). This complexity appears to be shared with other higher species but not with lower organisms such as the fruit fly or worm (Lander et al. 2001). This complex regulation mechanism may, in part, explain the unexpectedly low number of gene in higher mammals.

CHAPTER 3

Transcriptional regulation of the glucocorticoid receptor transcript 1F: NGFI-A and E2F1?

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3.1 Abstract

The human *GR* promoter 1F is susceptible to methylation during stressful early life events resulting in lower 1F transcript levels. It was proposed that this is due to methylation of a hypothetical NGFI-A binding site in this promoter. In this study, the role of NGFI-A in regulating 1F transcription was analysed using transient transfections, reporter gene assays and chromatin immunoprecipitation in cells representing peripheral (293FT) and central (U373MG) tissue. Chromatin immunoprecipitation confirmed NGFI-A binding to the *GR* 1F promoter. NGFI-A induced 1F promoter activity in the reporter gene assay, but had no effect on endogenous transcript levels. Several other transcription factors predicted by *in silico* phylogenetic footprinting to bind the 1F promoter were analysed. E2F1 was identified to bind to and to strongly up-regulate the expression of the 1F reporter gene. The E2F1 binding site in promoter 1F also covers a CpG site highly methylated in association with childhood abuse. Together with our data this suggests an important role for E2F1 in *GR* 1F regulation. Here we provide for the first time evidence that E2F1 is a major element in the transcriptional complex capable of driving the expression of *GR* 1F transcripts.

Keywords: Alternative first exons, Behavioural epigenetics, Chromatin immunoprecipitation, Glucocorticoid receptor, Stress response, Transcription factor

3.2 Introduction

The human glucocorticoid receptor (GR) is an ubiquitously expressed steroid hormone receptor with a pivotal role in maintaining homeostasis under stress. The 5'-region of the *GR* gene (OMIM + 138040; *NR3C1*) consists of 9 alternative first exons designated exons 1A to 1I in humans (reviewed in (Turner et al. 2010)). This 5'-heterogeneity remains untranslated as the ATG start codon lies within exon 2, but it orchestrates translational regulation (Pickering and Willis (Pickering and Willis 2005).

Glucocorticoids are the end product of the hypothalamic-pituitary-adrenal (HPA) stress axis. In the rat, the HPA axis feedback sensitivity is thought to be dependent upon expression levels of *Gr* first exons, which in turn have been shown to be susceptible to environmental influences such as early life stress in both rodents and man (Weaver et al. 2004); (McGowan et al. 2009). Rodents experiencing poor maternal care, measured by licking/grooming and arched-back nursing (LG-ABN), had higher methylation levels throughout promoter 1_7 with the first CpG dinucleotide in the Ngfi-a binding site (also known as Egr-1; Zif-268; Krox-24) being especially susceptible to methylation or demethylation (Weaver et al. 2004). In humans, negative early life experience induced methylation at another, hypothetical, NGFI-A binding site (CpG 32) in the 1F promoter (McGowan et al. 2009).

However, alterations in *GR* levels are not exclusively due to promoter methylation. We previously showed that, although methylation levels were highly variable in peripheral blood mononuclear cells (PBMCs) between individuals, the 2 key CpG sites (37-38) of the promoter 1F NGFI-A binding site were methylated at 10% or less (Turner et al. 2008). In the normal brain or the brain of patients with major depressive disorder (MDD), we and others detected at maximum 4% methylation in the 1F promoter, and only 0-2% in the NGFI-A binding site (Alt et al. 2010; Moser et al. 2007). However, we showed decreased levels of 1F and *NGFI-A* expression in the hippocampus of MDD patients, suggesting a link between *NGFI-A* and *GR* 1F expression, although without measurable promoter methylation.

In this present study, we further investigated the role of NGFI-A in the transcriptional regulation of the *GR in vitro*. We confirmed that NGFI-A binds to the 1F promoter, poorly activates the 1F reporter gene but does not induce endogenous 1F transcripts. As NGFI-A seems to be unable to directly affect 1F transcription we tested several other transcription factors predicted to bind the 1F promoter. We found

the transcription factors ELK1 and E2F1 to weakly and strongly up-regulate 1F promoter activity respectively. Therefore, we suggest that E2F1 is required for driving the expression of the *GR* 1F transcripts.

3.3 Materials and Methods

3.3.1 Cell cultures

Human embryonic kidney (293FT) cells were cultured at 37°C and 5% CO_2 in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum, 1% non essential aminoacids, 1% Ultraglutamine and 1% sodium pyruvate (Gibco-BRL, Breda, The Netherlands). Human glioblastoma-astrocytoma (U373MG) cells were cultured in EMEM medium (Lonza) containing 20% fetal bovine serum. Although 293FT cells do not express functional GR, they were selected because of the high promoter 1F activity observed. U373MG cells were used because of their low 1F promoter activity (Cao-Lei et al. 2011).

3.3.2 GFP expression vector construction

The full length *NGFI-A* coding sequence was amplified from pCMV6-XL5-EGR1 (Origene, Rockville, MD, USA), all other transcription factor coding sequences were subcloned from specific pCMV-SPORT6 clones (ImaGenes, Berlin, Germany). Amplification primers included adaptor sequences with different restriction sites (NGFI-A: *Nhe*I, *Sal*I; E2F1, RFX1 and HES1: *Nhe*I, *EcoR*I; ELK1: *Nhe*I, *Xho*I; EBF1: *Nhe*I, *SacI*I). All restriction enzymes were derived from NEB (Hitchin, UK). The PCR product was inserted into pIRES2-AcGFP1 (Clontech, Mountain View, CA, USA). Plasmids were sequenced using appropriate primers and the BigDye 3.1 terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, The Netherlands). Sequencing was performed on an ABI 3130 sequencer (Applied Biosystems). PCR primer sequences, restriction enzymes, annealing temperatures and $MgCl_2$ concentrations are shown in Table 10. The human *GR* promoter 1F (-3536/-3211) luciferase construct was generated as described previously (Cao-Lei et al. 2011). PCR primer sequences, restriction enzymes, annealing temperatures and $MgCl_2$ concentrations are shown in Table 10.

Table 10. Primer sequences and PCR conditions.

	Primer sequences	Tm (°C)	MgCl2 (mM)	Primers (uM)	Product (bp)	DNA Polymerase
expression vector constructs						
pNGFI-A-Ac-GFP	fwd: 5'-GAACCGTCAGATCCGCTAGCATGGCCGCGGCCAAGGCCGAGAT-3' rev: 5'-GCCCGCGGTACC GTCGAC TTAGCAAATTTCAATTGTCCTG-3'	64	2	0.5	1587	PI-TP
pE2F1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCCGTGAGCGTCATGGCCTTGG-3' rev: 5'-GTCGACTGCAGAATTCATCTCTGGAAACCCTGGTCCCTC-3'	66	0.5	0.3;0.5;0.7	1388	Ph-TP
pRFX1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCTCATTTTCCCCACCGTTGGCATGG-3' rev: 5'-GTCGACTGCAGAATTCACCCTGGCGTGGAGGGGTGGC-3'	64	1.5	0.3;0.5;0.7	3043	PI-TP
pELK1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCAACAGTACCCCTGGGATGGC-3' rev: 5'-GAAGCTTGAGCTCGAGCCAGAAGGGGTGGTGGTGGT-3'	66	0.7	0.3;0.5;0.7	1388	Ph-TP
pEBF1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCGAGGAGATTTTCCACAAGAA-3' rev: 5'-GATCCCGGGCCCGCGGCCAACCTCTTCATTAATACA-3'	64	0.5	0.5;0.7	1879	Ph-TP
pHES1-Ac-GFP	fwd: 5'-CGTCAGATCCGCTAGCCGTGAAGAACTCCAAAAATA-3' rev: 5'-GTCGACTGCAGAATTCGTTGGGGAGTTTAGGAGGAG-3'	60	0.5	0.7	968	Ph-TP
pMetLuc-1-F	fwd: 5'-TACCGGACTCAGATCTAAGTACGTATGCGCCGACCC-3' rev: 5'- GTCTACTGCAGAATTC CTCGCTACCTCCTTCCCGCC-3'	67	0.5	1	326	Ph-TP
plasmid sequencing primers						
pIRES-AcGFP	fwd: 5'-ATAGTCCTGTCGGGTTTCGCC-3' rev: 5'- CTCTTGAAGAAGTCGTGCTGCT-3'					
pMetLuc-1-F	fwd: 5'- AACCGTATTACCGCCATGCA-3' rev: 5'- AACACCACCTTGATGTCCATGG-3'					
pE2F1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-GCTTCGGCCAGTAACGTTAG-3' fwd-2: 5'-ACTGAATCTGACCACCAAGC-3' rev-2: 5'-CGGTACATGCACACACACAT-3'					
pRFX1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-GCTTCGGCCAGTAACGTTAG-3' fwd-2: 5'-TGGTACCCAGCAGGTGCACT-3' rev-2: 5'-CACGGGAGGGGGCCTGCCTG-3' fwd-3: 5'-ACCCGCCGTCTGGGCACCAG-3' rev-3: 5'-CAGGCCGCCCACTGCTCCAG-3'					
pELK1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-AAGCTCTTCCGATTTCAGGT-3' fwd-2: 5'-CTGTCTGGAGGCTGAAGAGG-3' rev-2: 5'-GCTTCGGCCAGTAACGTTAG-3'					
pEBF1-Ac-GFP	fwd-1: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev-1: 5'-TTCTTGTGGAAAATCTCCTCC-3' fwd-2: 5'-GATCCAGTGATAATTGACAG-3' rev-2: 5'-ACACTGTACAGGGCCTCGGC-3' fwd-3: 5'-AAGCACTGTATGGGATGCCA-3' rev-3: 5'-GCTTCGGCCAGTAACGTTAG-3'					
pHES1-Ac-GFP	fwd: 5'-GCAGAGCTGGTTTAGTGAAC-3' rev: 5'-GCTTCGGCCAGTAACGTTAG-3'					
expression analysis						
b-actin	fwd: 5'-GGCCACGGCTGCTTC-3' rev: 5'-GTTGGCGTACAGGTCTTTGC-3'	60	2	1	208	PI-TP
GAPDH	fwd: 5'-GAAGGTGAAGGTCGGAGTC-3' rev: 5'-GAAGATGGTGATGGGATTTC-3'	60	2	1	228	PI-TP
PPIA	fwd: 5'-TCCTGGCATCTTGTCCATG-3' rev: 5'-CCATCCAACCACTCAGTCTTG-3'	60	1	1	90	PI-TP
NGFI-A	fwd: 5'-ACCGGCCCAGCAAGACGCC-3' rev: 5'-GGGCACAGGGGATGGGTATG-3'	60	2	0.5	437	PI-TP
E2F1	fwd: 5'-TGAATATCTGTACTACGCAG-3' rev: 5'-AGATGATGGTGGTGGTGACA-3'	56	4	0.4	341	PI-TP
GR 1F	fwd: 5'-GTAGCGAGAAAAGAAACTGG-3' rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	60	1.75	0.5	511	PI-TP
exon 2	fwd: 5'-GAGGGGAGATGTGATGGACTTCT-3' rev: 5'-GCTGCTGCGCATTGCTTA-3'	55	2.5	0.8	170	PI-TP
ChIP PCR						
1-F	fwd: 5'-TACCGGACTCAGATCTAAGTACGTATGCGCCGACCC-3' rev: 5'- GTCTACTGCAGAATTC CTCGCTACCTCCTTCCCGCC-3'	64	3	0.1	370	D-TP
GR promoter 1A	fwd: GATTGTAAATTGGGCTCTGAGCT rev: GGCAGAAGTTGCAAAGGA	58	2	1	493	PI-TP

Diamond Taq polymerase: D-TP
Platinum Taq polymerase: PI-TP
Phusion Taq polymerase: Ph-TP

3.3.3 Transfection and luciferase reporter gene assay

Cells were seeded onto a 24-well plate at $4x10^4$ cells per well and transfected with 0.5ug of pMetluc-1F and pIRES-AcGFP encoding the different transcription factors and independent GFP, or the empty pIRES vector expressing GFP only, using Lipofectamine LTX (Invitrogen, Merelbeke, Belgium). Cells were harvested 48h post transfection and luciferase activity was measured using the Ready-To-Glow Secreted Luciferase Reporter System (Clontech). Transfection efficiency was confirmed to be above 80% by fluorescence microscopy of GFP. Secreted alkaline phosphatase (SEAP) was co-transfected for normalisation of luciferase levels SEAP levels were measured using the Great EscAPe TM SEAP Fluorescence Detection Kit (Clontech) following the manufacturers instructions. All transfections were performed in triplicate and three independent experiments were performed from different cell passages.

3.3.4 Preparation of cDNA in 293FT and U373MG cells and real-time qPCR

Messenger RNA was purified 48h post-transfection using a μMACS™ mRNA Isolation Kit (Miltenyi Biotech, Utrecht, The Netherlands). RNA quantity was measured on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). First-strand synthesis of total cDNA and real time PCR were carried out as previously described (Alt et al. 2010). To exclude genomic or plasmid DNA contamination effects water controls were included in the reverse transcription which revealed no presence of contaminating genomic DNA. Additionally, the β-actin PCR was designed to give a 208 bp fragment from the mRNA, or 300bp in the presence of genomic DNA. Amplification of the backbone region of the pIRES vector showed no presence of contaminating plasmid DNA. Primers and PCR conditions are shown in Table 10.

3.3.5 Protein extraction and Western blotting

Whole cell protein was extracted 48h post transfection with a cell lysis solution (pH 8.5) containing 7M urea, 2M thiourea, 4% CHAPS, 30mM Tris, 12% isopropanol, a protease inhibitor cocktail and a nuclease mix. All extractions were performed in triplicate from different cell passages. Protein concentrations were quantified with the 2D-quant kit (Amersham Biosciences, Diegem, Belgium). Proteins were resolved on a 4-16% Bis-Tris Novex gel (Invitrogen) and transferred to a nitrocellulose membrane (GE healthcare, Diegem, Belgium). Membranes were subsequently incubated with rabbit

polyclonal human NGFI-A (sc-110), human E2F1 (sc-193) antibodies and a mouse monoclonal human β-Actin (sc-47778) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and visualised with anti-rabbit Cy5 and anti-mouse Cy3 labelled secondary antibodies (Pierce, Rockford, IL) using a standard protocol (Towbin et al. 1979).

3.3.6 Sequential chromatin immunoprecipitation (sChIP)

Sequential ChIP to investigate the co-occupancy of NGFI-A and E2F1 on promoter 1F was performed as described before (Medeiros et al. 2009). Briefly, 293FT cells were cross-linked (1% formaldehyde) and sheared (Bioruptor 200, Diagenode). Sequential immunoprecipitations were performed using rabbit polyclonal antibodies. Initial immunoprecipitations for E2F1 (sc-193) or NGFI-A (sc-110) were purified with protein-A - Sepharose beads (Sigma-Aldrich). After washing, sepharose beads were resuspended, and the second immunoprecipitation was performed and purified using protein-A Magnetic beads (Invitrogen). Cross-linking was reversed and immunoprecipitated DNA purified with phenol/chloroform. Normal rabbit non-immune IgG (sc-2027) was used as a negative control. Immunoprecipitation specificity was verified by pre-incubation of the first antibody with its cognate peptide prior to precipitation. The *GR* exon 1F promoter region of the uncrosslinked DNA was subsequently quantified by PCR.

3.3.7 Statistical analysis

The gene specific threshold cycle (Ct) was used for semi-quantitative assessment of RT-PCR data using a stable internal reference gene (*β-actin*). The amount of mRNA in the transfected cells relative to the one in the untransfected cells was calculated as $2^{-(\Delta\Delta Ct)}$ according to Livak and Schmittgen (Livak and Schmittgen 2001). PCR efficiencies showing high linearity ($R^2 > 0.993$) after 7-log dilution series varied between E = 1.85 – 2.15.

All results were analysed using a one way analysis of variance (ANOVA) followed by Tukey post-hoc correction. Statistical analyses were performed using Sigma Stat for Windows (Erkrath, Germany). Differences were considered significant when $p<0.05$ after *post-hoc* correction.

3.4 Results

3.4.1 Overexpression of NGFI-A and 1F transcript levels

After transient transfection, real-time PCR revealed that a 1500 fold (p=0.012) (U373MG) or even 1900 fold (p=0.009) (293FT) overexpression of NGFI-A mRNA (Data not shown), and quantification by Western blot (Fig. 12E-F) of both NGFI-A and E2F1 showed a 20 fold increase in protein levels. This overexpression had no effect on the level of endogenous exon 1F or total GR expression in 293FT or U373MG cells (Fig. 12A-B). Protein functionality was confirmed by induction of b-myb (DeGregori et al. 1995) by the overexpressed transcription factors. Maximum transcription factor and b-myb expression was observed 24 hours post transfection. Co-transfection of the NGFI-A expression vector with the 1F reporter gene showed a poor induction of only 2.5 fold in 1F promoter activity in 293FT cells and none in U373MG cells (Fig. 13A-B). Thus, NGFI-A is either a week inducer of the native 1F transcript or additional transcription factors are necessary for its activation.

3.4.2 NGFI-A binding to the human GR promoter 1F

To confirm that NGFI-A binds to the *GR* promoter 1F, we performed chromatin immunoprecipitation in 293FT and U373MG cells. As the chromatin structure can block the promoters accessibility, the well known active chromatin markers acetyl-lysine-9 (K9) residues of histones H3 and H4 (Kadonaga 1998) were used as positive controls. Successful amplification of promoter 1F confirmed the euchromatic character of promoter 1F and its accessibility for transcription factor binding (Fig. 13D). After NGFI-A specific immunoprecipitation, the promoter 1F was amplified from genomic DNA demonstrating that NGFI-A was bound to promoter 1F. No product was amplified in the negative control PCR for promoter 1A. Immunoprecipitation of the NGFI-A promoter 1F complex was inhibited by pre-incubation of the antibody with its blocking peptide, confirming the specificity of the IP.

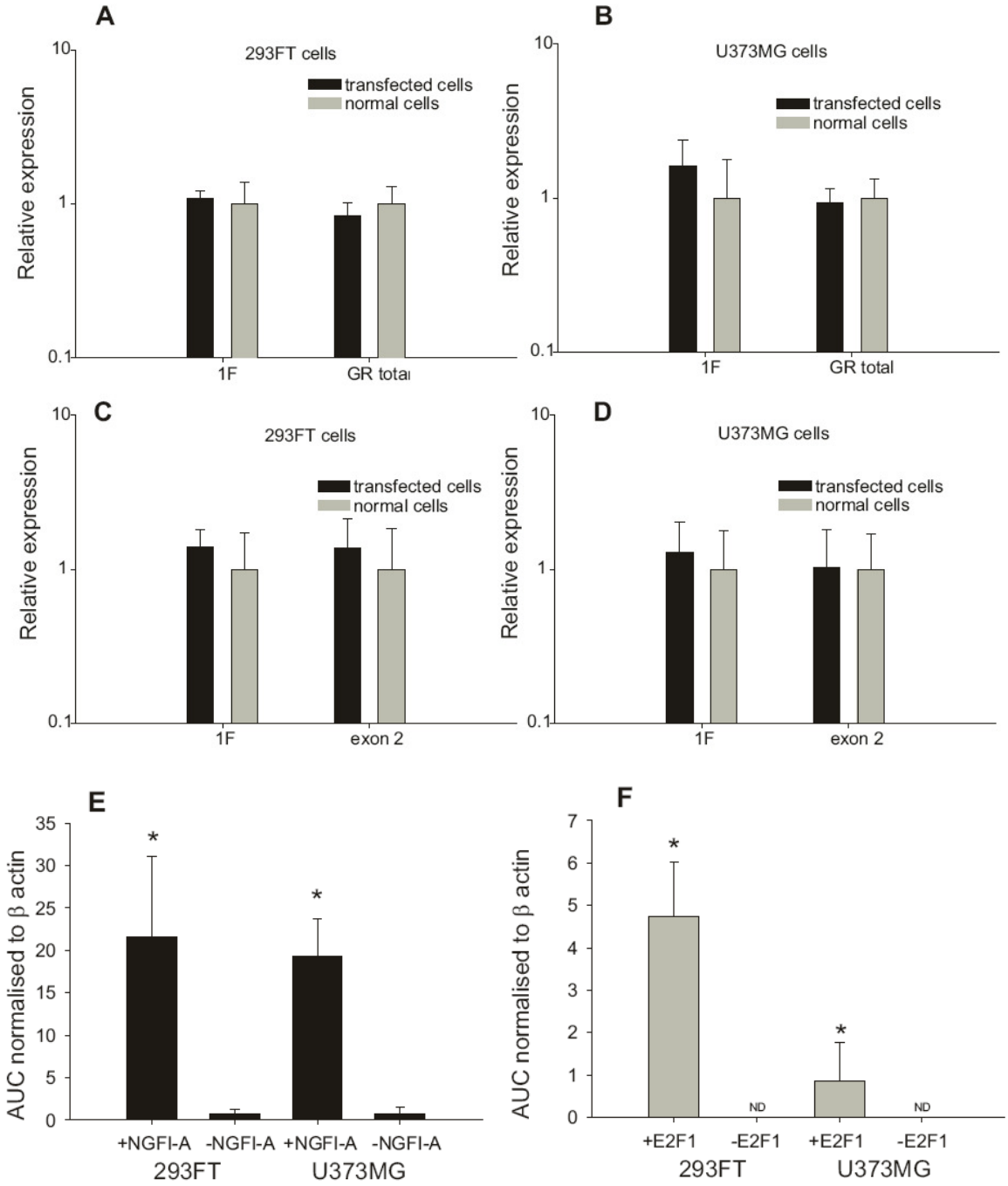

Figure 12. The effect of NGFI-A and E2F1 overexpression on human 1F transcript expression.
Relative mRNA expression of exon 1F and total *GR* in 293FT (A) and U373MG (B) cells after transfection with a NGFI-A expression vector was measured by real-time PCR.
Relative mRNA expression of exon 1F and total *GR* in 293FT (C) and U373MG (D) cells after transfection with an E2F1 expression vector was measured by real-time PCR.
Protein levels after transfection with an NGFI-A (E) or E2F1 (F) overexpression vector as measured by Western blot. Relative expression values (A-D) are given as $2^{-(\Delta\Delta Ct)}$ with the geometric means normalised to *β-actin*. * $P<0.05$. All results are from three PCR replicates on three independent experiments. Western blots (E-F) were quantified in triplicate and the area under the curve (AUC) normalised to β-actin.

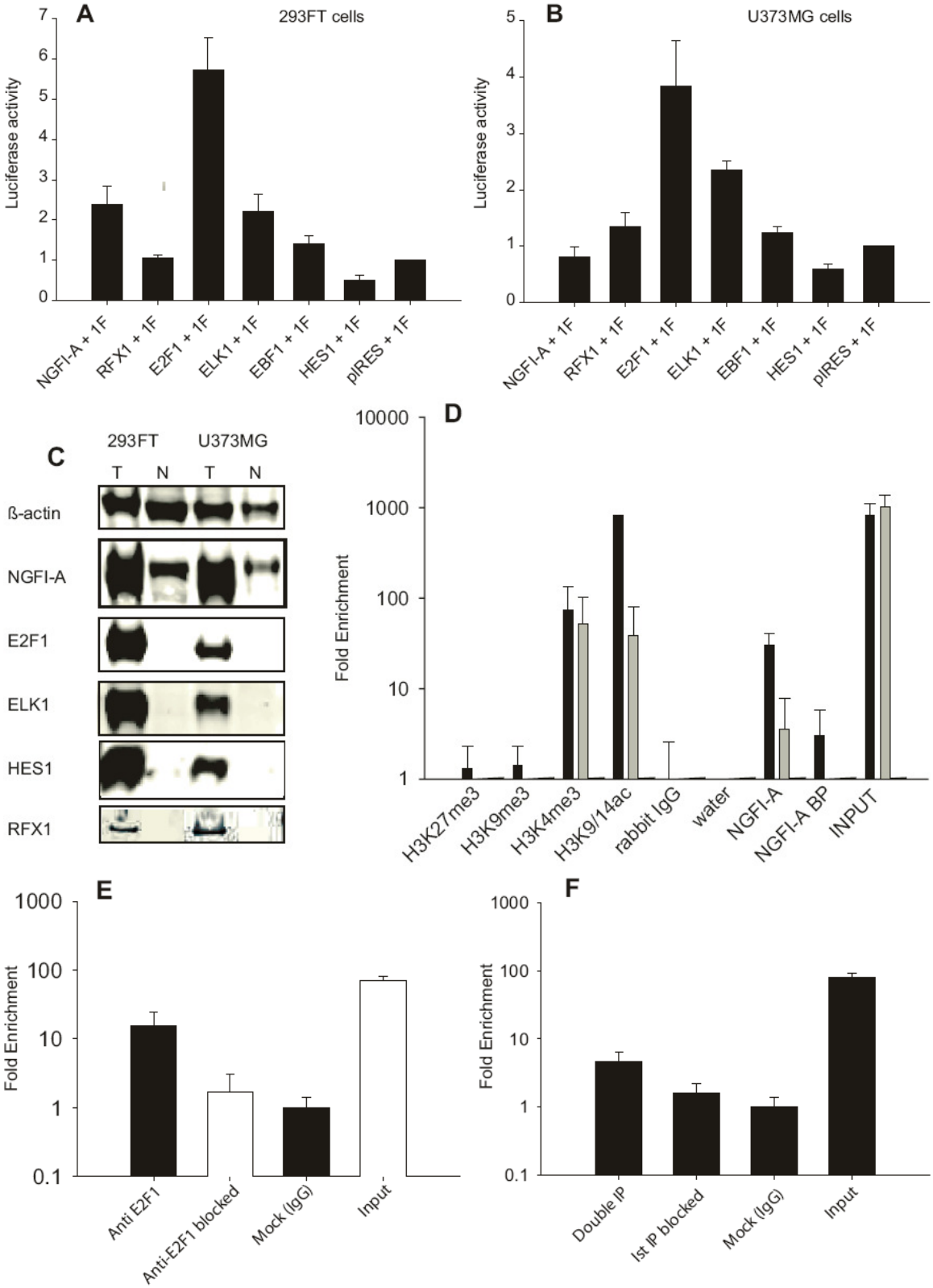

Figure 13. Induction of 1F promoter activity by other predicted transcription factors and Chromatin immunoprecipitation.
Luciferase activity (mean ± SD) of the 1F reporter gene co-transfected with different transcription factor expression vectors and normalised to the co-transfection of the promoter 1F reporter gene with the empty expression vector pIRES+1F in 293FT (A) and U373MG cells (B).
Western blot analysis of different transcription factors in transfected (T) and untransfected (N) cells to confirm overexpression (C).

Chromatin immunoprecipitation (D) of genomic DNA from 293FT cells (black bar) as well as U373MG cells (grey bar) using different histone antibodies as markers for inactive (H3K27me3, H3K9me3) or active (H3K4me3, H3K9/14ac) chromatin. Precipitation with a NGFI-A specific antibody (sc-110) was followed by amplification of the 1F promoter region (370bp). Antibody specificity was confirmed by blocking with the cognate peptide (NGFI-A BP). Chromatin immunoprecipitation (E) showed specific binding of E2F1 to promoter 1F in 293FT cells.
E2F1 and NGFI-A co-localize on the 1F promoter (F) as shown by double ChIP in 293FT cells. The double ChIP is the mean of both E2F1/NGFI-A and NGFI-A/E2F1 immunoprecipitations. All experiments were performed in triplicate from differing cell passages. Reporter gene transfections were performed in triplicate *per* experiment.

3.4.3 Effects of different transcription factors on the promoter 1F reporter gene

Computational methods (Turner et al. 2008) previously predicted several transcription factor binding sites evolutionary conserved in promoter 1F of eukaryotes. The strongest of these predictions (RFX1, E2F1, ELK1, EBF1 and HES1) were cloned into the pIRES-AcGFP vector and individually co-transfected with the promoter 1F reporter gene. Successful overexpression of the different transcription factors was confirmed by Western Blot analysis (Fig. 13C). In 293FT cells NGFI-A, E2F1 and ELK1 lead to an increase in luciferase activity (Fig. 13A). E2F1 increased 1F transcription almost 6 fold while the overexpression of ELK1 and NGFI-A resulted in a weak 2.5 fold increase of 1F promoter activity. In U373MG cells the overexpression of NGFI-A did not up-regulate 1F activity (Fig. 13B). ELK1 weakly increased 1F promoter activity by 2.5 fold, as in 293FT cells. E2F1 overexpression increased the 1F promoter activity more pronouncedly by ~ 4 fold. Reporter gene activity induced by co-overexpression of E2F1 and NGFI-A was similar to that induced by E2F1 alone (Data not shown). All other transcription factors analysed had no significant effect on reporter gene activity. Similarly, there was no reporter gene activity when co-transfecting the expression vectors with the pMet-Luc construct without the promoter, suggesting that our observations are due to interactions with the promoter 1F sequence, rather than the backbone of the reporter gene plasmid.

3.4.4 E2F1 binds to the endogenous 1F promoter

Since E2F1 induced the 1F promoter in the reporter gene assays, we also evaluated its effect on endogenous 1F levels in 293FT and U373MG cells. Although E2F1 was strongly overexpressed, as confirmed by Western Blot analysis ($p<0.001$ in 293FT cells; $p=0.019$ in U373MG cells; Fig. 12F and 13C), this did not increase 1F transcripts in 293FT cells (Fig. 12C) nor in U373MG cells (Fig. 12D). E2F1 overexpression was lower in U373MG than 293FT cells, possibly due to cell specific factors. Although E2F1 was not able to increase endogenous 1F expression levels, chromatin immunoprecipitation with an E2F1 antibody showed that in both cell lines E2F1 binds to the

endogenous 1F promoter and did not bind to promoter 1A. Immunoprecipitation specificity of E2F1 was confirmed by pre-incubating the antibody with its cognate peptide (Fig. 13E).

3.4.5 E2F1 and NGFI-A Sequential ChIP

Since ChIP demonstrated that both E2F1 and NGFI-A bind promoter 1F, sequential immunoprecipitation was performed to evaluate promoter co-occupancy. Irrespective of the sequence of immunoprecipitation, promoter 1F DNA was enriched. The mean enrichment for both immunoprecipitation sequences was lower than for E2F1 alone (4.5 vs 15.3 fold respectively) (Fig 13E-F), and significantly above the mock IgG, or that of blocking the first antibody with its cognate peptide, confirming at least partial co-occupancy of the promoter 1F (Fig. 13F).

3.5 Discussion

It has been shown previously that NGFI-A is involved in the transcriptional regulation of the promoter of the human alternative exon 1F and its rat homologue 1_7 and that both are subject to epigenetic regulation (McGowan et al. 2009; Weaver et al. 2004). However, we and others have observed a poor correlation between promoter methylation and altered 1F expression levels (Alt et al. 2010; Moser et al. 2007) suggesting that other mechanisms play a role, especially since promoters are rarely dependent on a single transcription factor (reviewed in (Turner et al. 2010)).

Using chromatin immunoprecipitation, we confirmed that the DNA is accessible for transcriptional regulation and that NGFI-A binds to the human promoter 1F, at least in the two cell types tested. The two cell lines, 293FT and U373MG have been selected since they represent the highest and lowest promoter 1F activity amongst a series of cell lines tested (Cao-Lei et al. 2011). Although 293FT cells, as the original HEK293 cells from which they were derived, seem to have no functional GR, they are known to express full length immunoreactive GR, and have been used before to study mechanisms of endogenous GR translation (Yudt and Cidlowski 2001; Zhou and Cidlowski 2005). Previous studies using HEK 293 cells reported a 4 fold increase in the 1F promoter activity (McGowan et al. 2009) and in the rat 1_7 (Weaver et al. 2007) when reporter genes were co-transfected with the human and the rat NGFI-A, respectively. However, in neither of these studies, the natural 1_7 or 1F transcripts of the cells were measured. We showed that NGFI-A overexpression does not induce the endogenous 1F transcript. Thus, our data suggests that NGFI-A may be part of the transcriptional complex that activates the reporter gene, but that this is not sufficient to activate the natural 1F promoter *in vitro*. This interpretation is also supported by the co-immunoprecipitation of both transcription factors on promoter 1F. Because of endogenous NGFI-A expression in both cell lines, we can not exclude a role of NGFI-A in the E2F1 overexpression experiments, but this was not supported by co-overexpression experiments.

Analysis of the human *GR* 1F promoter suggested that NGFI-A is only one of a series of transcription factors that regulates the expression of the 1F transcript. In our reporter gene system ELK1 had a similar or even higher activation capacity compared to NGFI-A. The transcription factor E2F1, one of our most robust predictions, showed a significantly higher transcriptional activity than NGFI-A in both cell lines. E2F1 overexpression significantly increased promoter 1F both in 293FT cells (6 fold) and

U373MG cells (4 fold) in our reporter gene system. Moreover, we demonstrated binding of E2F1 to promoter 1F by immunoprecipitation, and that it occupies the promoter concomitantly with NGFI-A. In both cell lines there is no evidence for regulation of the 1F transcript by NGFI-A. The evidence is stronger that the two additional transcription factors, E2F1 and ELK1, play a role in regulating the 1F transcript. The interactions of these transcription factors with the promoter 1F need to be further characterised to elucidate the mechanisms underlying promoter 1F activity.

The E2F binding site predicted by *in silico* phylogenetic footprinting (Turner et al. 2008) covers CpG position 32 that, in human suicide victims with a history of childhood abuse, was highly methylated (40%) in comparison to suicide victims without childhood abuse (15%) (McGowan et al. 2009). The *post-mortem* samples from the abused victims had lower 1F transcript levels in agreement with their higher methylation levels. The authors suggested that CpG 32 was part of a second, hypothetical NGFI-A binding site. We suggest that E2F1 binds in this region and that reduced E2F1 binding as a result of DNA methylation could be responsible for the lower 1F expression observed previously (McGowan et al. 2009).

In conclusion, the region immediately up-stream of exon 1F is the target of multiple transcription factors. We did not observe a dominant effect of NGFI-A in our reporter gene system and both ELK1 and E2F1 were more effective, with the latter significantly outperforming all the other transcription factors tested. Using chromatin immunoprecipitation, we demonstrated that the promoter 1F chromatin structure is compatible with transcription and that both NGFI-A and E2F1 bind to the endogenous *GR* 1F promoter. Therefore, we suggest E2F1 as a major element in the complex necessary to control *GR* 1F transcription *in vitro* and *in vivo*.

CHAPTER 4

Transcriptional regulation and epigenetic sensitivity of the human glucocorticoid receptor transcript 1F

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4.1 Abstract

The complicated promoter region of the glucocorticoid receptor (GR) plays a pivotal role in the regulation of GR levels. Among the multiple promoters, in particular, promoter 1F is susceptible to methylation by adverse early life events. CpG methylation in promoter 1F is thought to interfere with transcription factor binding, subsequently inhibiting transcription. By overexpression of selected transcription factors predicted by *in silico* phylogenetic footprinting (ISPF), we identified E2F1 and showed that it binds to promoter 1F by chromatin immunoprecipitation (ChIP). E2F1 knockdown abrogated 1F promoter activity, whilst its overexpression induced a remarkable upregulation. By sequential promoter deletion and mutagenesis we identified two *bona fide* E2F1 binding sites in this promoter region. Using a single nucleotide methylation reporter gene, we showed that such limited CpG methylation did not have a significant effect on promoter 1F activity, and that single CpG dinucleotide methylation can not mediate the inhibition of transcription.

Keywords: Glucocorticoid receptor promoter 1F, Promoter activity, Single nucleotide methylation, E2F1

4.2 Introduction

Glucocorticoid (GC) hormones affect all tissues and cell types via the ubiquitously expressed glucocorticoid receptor (GR, OMIM +138040; official symbol NR3C1 for nuclear receptor subfamily 3, group C, member 1). GR mediated GC actions regulate multiple cell functions including growth, metabolic homeostasis and immune functions. Cellular GC responses are highly dependant on GR levels in the target tissues. GCs are the final product of the HPA axis which is tightly controlled by GR levels, especially in the hippocampal and hypothalamic feedback loop.

We and others have shown that the complicated 5' structure of the GR plays a pivotal role in the regulation of GR levels (Barrett et al. 1996; Breslin et al. 2001; Breslin and Vedeckis 1998; Geng and Vedeckis 2004; Nunez and Vedeckis 2002; Turner et al. 2010; Wei and Vedeckis 1997). The human GR contains 8 translated exons (exon 2-9) and 9 untranslated alternative first exons (Presul et al. 2007; Turner and Muller 2005). All of the alternative first exons identified are located in either the proximal or the distal promoter region. Exons 1B to 1H have their own individual promoter regions (Cao-Lei et al. 2011) and are located in a 3kbp long upstream CpG island containing 11 orthologous alternative first exons with a high sequence homology between humans, rats and mice (Bockmuhl et al. 2011; McCormick et al. 2000; Turner and Muller 2005). The GR is susceptible to epigenetic modulation. For instance, in rats intrauterine growth retardation affected the epigenetic of hippocampal GR expression (Ke et al. 2010). In the human placenta it was shown that the birth weight was associated with GR exon1F promoter methylation (Filiberto et al. 2011). The central feedback mechanisms of the HPA axis have been shown to be sensitive to environmental influences, with exon 1_7 (orthologue of the human 1F) playing an important role. Early life events produced life-long epigenetic changes in promoter 1_7, with increased DNA methylation of two CpG dinucleotides (CpG 16-17), that belong to a confirmed NGFI-A binding site (Weaver et al. 2004). In pups receiving low maternal care 80 to 100% methylation at CpG 16 was observed in contrast to 0–10% in high-care pups. These epigenetic changes appeared to be limited to particular adverse early life events since early separation from

the mother did not induce epigenetic methylation of the 1_7 promoter (Daniels et al. 2009). Also Norway rat derived strains with naturally differing stress responses and hippocampal GR transcript levels, had very low methylation levels in promoter 1_7 (Herbeck et al. 2009). In humans, Oberlander et al reported that prenatal exposure to maternal mood led to increased methylation levels of the NGFI-A binding site in promoter 1F from cord blood of newborns (Oberlander et al. 2008). Similarly, hippocampal methylation of two different CpG dinucleotides, in an hypothetical NGFI-A binding site, further upstream, correlated with prior childhood abuse and subsequent suicide (McGowan et al. 2009). In contrast, the NGFI-A binding site in post mortem human hippocampal tissue from a wide range of neurological disorders was uniformly unmethylated (Moser et al. 2007), and no methylation of the NGFI-A binding site was found in either brains of patients with major depressive disorder or in matched controls (Alt et al. 2010). Investigating the transcriptional control of the human GR promoter 1F we showed that NGFI-A does not induce expression of GR 1F transcripts in vitro despite its binding to the promoter 1F (Alt et al., 2011 submitted), suggesting that other transcription factors may be involved in the regulation of the 1F promoter.

In the present study, we further investigated the activity of six transcription factors predicted by using *in silico* phylogenetic footprinting (ISPF). We showed that E2F1 is involved in the regulation of GR promoter 1F and investigated the interactions of this transcription factor with the 1F promoter.

4.3 Materials and Methods

4.3.1 Plasmid construction

4.3.1.1 Promoter 1F truncated sequences

The *GR* promoter 1F (-3536 to -3211 relative to the ATG translation start codon) was cloned into pGL4.10(luc2) vector (Promega Benelux, Leiden, The Netherlands) by PCR amplification from the BAC clone RP11-278J6 (AC091925) (Invitrogen, Merelbeke, Belgium). Truncated promoter 1F sequences covering the regions -3536 to -3440 (1F_97), -3439 to -3307 (1F_133), -3306 to -3211 (1F_96), -3536 to -3307 (1F_97+133) and -3439 to -3211 (1F_133+96) (Figure 14) were subsequently generated by PCR using primer containing suitable restriction digestion sites. All amplifications were performed in 25µl reactions with 20mM Tris-HCl (pH 8.4), 50mM KCl, 200mM deoxynucleoside triphosphates, 1x SyBR Green and 2.5U Platinum Taq DNA polymerase (Invitrogen) on an Opticon 2 thermal cycler (Bio-Rad, Nazareth Eke, Belgium). Primers and corresponding annealing temperatures are shown in Table 11. Products were digested with *Nhe*I (5') and *Hind*III (3') (New England Biolabs, Frankfurt, Germany) and ligated into the pGL4.10 vector. All clones were sequence-verified using the BigDye 3.1 Terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, NL) on an ABI 3130 sequencer (Applied Biosystems) with the pGL4.10-sequencing forward primer 5'- tgttggatgctcatactcgt -3' and reverse primer 5'- aatggcgctgggcccttctt -3'.

Figure 14. Schematic representation of promoter 1F, the location of the CpG dinucleotides and NGFI-A, E2F, E2F1 and HES1 binding sites. Restriction sites and the truncated regions of promoter 1F used in this study are shown. CpG numbering as *per* McGowan *et al* (McGowan et al. 2009).

Table 11. PCR primers and their associated reaction conditions.

Promoter regions	Sequence[a]	Tm (℃)[b]	[Mg^{2+}](mM)	Primers (μM)	DNA polymerase
Promoter 1F	Fwd: 5'-catttctctggcctaactggcgtacgtatgcgccgac-3' Rev: 5'-accggattgccaagcttccttcccgcccccgcccag-3'	64	1	0.5	Phusion Hot Start DNA polymerase(Finnzymes)
1F_97	Fwd: 5'-catttctctggcctaactggcgtacgtatgcgccgac-3' Rev: 5'-accggattgccaagcttgcagcgtctcggccgc -3'	61	0.7	0.1	
1F_133	Fwd: 5'-catttctctggcctaactggcggcaccgtttccgtgc -3' Rev: 5'-accggattgccaagcttgcgggcgggccacaag -3'	64	1	0.5	
1F_96	Fwd: 5'-catttctctggcctaactggctgtcacccgcaggggcac -3' Rev: 5'-accggattgccaagcttccttcccgcccccgcccag-3'	64	0.7	0.1	
1F_97+133	Fwd: 5'-catttctctggcctaactggcgtacgtatgcgccgac-3' Rev: 5'-accggattgccaagcttgcgggcgggccacaag -3'	64	0.7	0.1	
1F_133+96	Fwd: 5'-catttctctggcctaactggcggcaccgtttccgtgc -3' Rev: 5'-accggattgccaagcttccttcccgcccccgcccag -3'	58	0.7	0.1	

[a]Fwd, forward or sense primer; Rev, reverse or antisense primer. Primer with restriction enzyme recognition sites (underlined) used for amplification of the promoter fragments. [b]Tm, annealing temperature in PCR.

Table 12. Mutations in human *GR* exon 1 promoter 1F.

Mutations[a]	Sequence
Mut1F_E2F_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcggcgct**AAAAAAAAAAA**ggcagagcgagctccgagtgggt
Mut1F_E2F_Rev	acccactcgggagctcgctctgcc**TTTTTTTTTTT**agcgccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Mut1F_E2F1_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctgggcggg**AAAAAAAAAA**aggtagcgaggaattctgcagtcgac
Mut1F_E2F1_Rev	Gtcgactgcagaattcctcgctacct**TTTTTTTTTT**cccgcccagctccgcggctccagacccactcgggagctcgctctgcccc
Mut1F_NGFI-A_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagct**AAAAAAAAAAAAA**aaggaggtagcgaggaattctgcagtcgac
Mut1F_NGFI-A_Rev	Gtcgactgcagaattcctcgctacctcctt**TTTTTTTTTTTTT**agctccgcggctccagacccactcgggagctcgctctgcccc
Mut1F_NGFI-A+E2F1_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagct**AAAAAAAAAAAAAAAAA**aggtagcgaggaattctgcagtcgac
Mut1F_NGFI-A+E2F1_Rev	Gtcgactgcagaattcctcgctacct**TTTTTTTTTTTTTTTTT**agctccgcggctccagacccactcgggagctcgctctgcccc
Controls	**Sequences**
WT(37-38)_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctgggcgggggcgggaaggaggtagcgaggaattctgcagtcgac
WT(37-38)_Rev	Gtcgactgcagaattcctcgctacctccttcccgcccccgcccagctccgcggctccagacccactcgggagctcgctctgcccc
WT(30-31-32)_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcggcgcttgccgccaaggggcagagcgagctccgagtgggt
WT(30-31-32)_Rev	acccactcgggagctcgctctgcccttggcggcaagcgccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg

[a]Fwd, forward oligodeoxynucleotides; Rev, reverse oligodeoxynucleotides. The enzyme recognition sites are boxed. The mutated sequences are in blod.

4.3.1.2 Mutagenesis in promoter 1F

Mutations in the transcription factor binding sites were inserted into the pMetLuc-1F vector by restriction-ligation of synthetic double stranded DNA. Briefly, sense and antisense synthetic oligodeoxynucleotides (20nmol) (Eurogentec, Seraing, Belgium) (Table 12) were heated to 95°C (5 min) and cooled to room temperature at a rate of 0.1°C/s in the appropriate restriction digest buffer and the resultant double strand DNA was digested with either *Blp*I and *Sac*I (New England Biolabs) or *Sac*I and *EcoR*I (Figure 14) for 1h at 37°C. Digested oligodeoxynucleotides were ligated into pMetLuc-1F (Cao-Lei et al. 2011) by T4 DNA ligase (New England Biolabs). All plasmids were transformed into Escherichia coli and purified using a Plasmid Maxi Kit (Qiagen, Venlo, NL), and sequence-verified using the pMetLuc-sequencing forward primer 5'-aaccgtattaccgccatgca-3' and reverse primer 5'-aacaccaccttgatgtccatgg-3'.

4.3.1.3 Single nucleotide methylation in promoter 1F

pMetLuc-1F was methylated in defined CpG positions including positions 30, 31, 32, 30+31, 30+32, 31+32, 30+31+32, 37, 38, and 37+38 (Figure 14). For this propose, sense and antisense oligodeoxynucleotides (20nmol) with 5'-methyl-cytosine were synthesized (Eurogentec) and were heated in the appropriate restriction digest buffer to 95°C for 5 min and cooled to room temperature to generate double stranded methylated DNA. After restriction digestion with either *Blp*I and *Sac*I or *Sac*I and *EcoR*I restriction enzymes (Table 13), products were ligated into similarly digested pMetLuc-1F. To avoid changes in methylation due to subcloning in Escherichia coli, ligation products were transfected directly into 293FT cells after purification using the Jetquick PCR purification kit (Genomed, Loehne, Germany). Ligation efficiency was verified by Real-time PCR using flanking primers (Forward: 5'-aaccgtattaccgccatgca-3', Reverse: 5'-aacaccaccttgatgtccatgg-3'). All plasmids containing methylated dinucleotides were verified by pyrosequencing after bisulfite treatment.

Table 13. Methylations in human *GR* exon 1 promoter 1F.

Methylations[a]	**Sequences**
Met1F_37_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctggg**cg**ggggcgggaaggaggtagcgaggaattctgcagtcgac
Met1F_37_Rev	Gtcgactgcagaattcctcgctacctccttcccgcccc**cg**cccagctccgcggctccagacccactcgggagctcgctctgcccc
Met1F_38_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctgggcgggggg**cg**ggaaggaggtagcgaggaattctgcagtcgac
Met1F_38_Rev	Gtcgactgcagaattcctcgctacctccttcc**cg**cccccgcccagctccgcggctccagacccactcgggagctcgctctgcccc
Met1F_37+38_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctggg**cg**ggggg**cg**ggaaggaggtagcgaggaattctgcagtcgac
Met1F_37+38_Rev	Gtcgactgcagaattcctcgctacctccttcc**cg**cccc**cg**cccagctccgcggctccagacccactcgggagctcgctctgcccc
Met1F_30_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactgg**cg**gcgcttgccgccaaggggcagagcgagctccgagtgggt
Met1F_30_Rev	Acccactcgggagctcgctctgcccttggcggcaagcgc**cg**ccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_31_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcgg**cg**cttgccgccaaggggcagagcgagctccgagtgggt
Met1F_31_Rev	Acccactcgggagctcgctctgcccttggcggcaag**cg**ccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_32_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcggcgcttgc**cg**ccaaggggcagagcgagctccgagtgggt
Met1F_32_Rev	Acccactcgggagctcgctctgcccttgg**cg**gcaagcgccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_30+31_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactgg**cg**g**cg**cttgccgccaaggggcagagcgagctccgagtgggt
Met1F_30+31_Rev	Acccactcgggagctcgctctgcccttggcggcaag**cg**c**cg**ccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_30+32_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactgg**cg**gcgcttgc**cg**ccaaggggcagagcgagctccgagtgggt
Met1F_30+32_Rev	Acccactcgggagctcgctctgcccttgg**cg**gcaagcgc**cg**ccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_31+32_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcgg**cg**cttgc**cg**ccaaggggcagagcgagctccgagtgggt
Met1F_31+32_Rev	Acccactcgggagctcgctctgcccttgg**cg**gcaag**cg**ccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg
Met1F_30+31+32_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactgg**cg**g**cg**cttgc**cg**ccaaggggcagagcgagctccgagtgggt
Met1F_30+31+32_Rev	Acccactcgggagctcgctctgcccttgg**cg**gcaag**cg**c**cg**ccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg

Controls	**Sequences**
WT(37-38)_Fwd	Ggggcagagcgagctccgagtgggtctggagccgcggagctgggcgggggcgggaaggaggtagcgaggaattctgcagtcgac
WT(37-38)_Rev	Gtcgactgcagaattcctcgctacctccttcccgccccccgcccagctccgcggctccagacccactcgggagctcgctctgcccc
WT(30-31-32)_Fwd	Cactcacgcagctcagccgcgggaggcgccccggctcttgtggcccgcccgctgtcacccgcaggggcactggcggcgcttgccgccaaggggcagagcgagctccgagtgggt
WT(30-31-32)_Rev	acccactcgggagctcgctctgcccttggcggcaagcgccgccagtgcccctgcgggtgacagcgggcgggccacaagagccggggcgcctcccgcggctgagctgcgtgagtg

[a]Fwd, forward oligodeoxynucleotides; Rev, reverse oligodeoxynucleotides. The enzyme recognition sites are boxed. The methylated sequences are in blod.

4.3.1.4 Overexpression vectors

The previously described pIRES-AcGFP expression vectors for NGFI-A, E2F1, ELK1, HES1, RFX1 and EBF1 were used (Alt *et al*., 2011 submitted). All vectors produced two independent proteins from one mRNA, the cloned transcription factor and a GFP, using an internal ribosome entry site. Transfection efficiency was monitored by fluorescence microscopy examination of GFP from the pIRES-AcGFP vector. Transfections with an efficiency > 80% were used.

4.3.2 Cell culture and transient transfections

The 293FT (Human embryonic kidney) cell line was cultured in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum, 1% non-essential aminoacids, 1% ultraglutamine and 1% sodium pyruvate (Gibco-BRL, Breda, NL) and was maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 24- or 96- well plates 24h prior to transfection with Lipofectamine LTX (Invitrogen) using the conditions in Table 14. All transfections were performed in triplicate and three independent experiments were performed. Culture supernatants were recovered 48 hours post-transfection.

Table 14. Transfection conditions.

Transfection	Plate	Cell Density	DNA (ng)			Lipofectamine LTX (µl)
			Vector containing promoter 1F	Internal control	Vector containing transcription factor	
Co-transfection						
pGL-deletion 1F	96-well plate	$1x10^4$	100	10	-	0.4
Triple transfection						
pGL-deletion 1F	96-well plate	$1x10^4$	100	10	100	0.6
pMetLuc-mutated 1F	24-well plate	$8x10^4$	500	500	500	5.25
pMetLuc-methylated 1F	24-well plate	$8x10^4$	500	500	500	5.25

4.3.3 Luciferase reporter gene assay

For transfections with pGL vectors, luciferase assays were performed using the Dual-Glo Luciferase Assay system (Promega Benelux) following the manufacturers protocol. Firefly luciferase levels were normalized to Renilla luciferase levels.

Transfections with pMetLuc vectors were monitored using the Ready-To-Glow Secreted Luciferase Reporter system kit (Clontech, Saint-Germain-en-Laye, France) following the manufacture's protocol. Luciferase gene expression was normalized to secreted alkaline phosphatase (SEAP) measured

using the Great EscAPeTM SEAP Fluorescence Detection kit (Clontech) and subsequently expressed either relative to the empty pMetLuc-reporter vector or to the pMetLuc-1F wild-type. Luminescence was measured in all cases with a TECAN infinite ® 200 Luminometer (TECAN, Giessen, NL).

4.3.4 Chromatin Immunoprecipitation (ChIP) Assays with transiently transfected 293FT cells

Fourty-eight hours post-transfection with pMetLuc-1F, cells were cross-linked with formaldehyde, sheared (Bioruptor 200, Diagenode, Liège, Belgium), and chromatin immunoprecipitations were performed as previously reported (Alt *et al*., 2011 submitted). Rabbit polyclonal anti-human E2F1 (sc-193) and goat polyclonal anti-human HES1 (sc-13842) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used for ChIP. Normal rabbit (sc-2027) and goat (sc-2028) non-immune IgG were used as negative controls. Immunoprecipitation specificity was verified by pre-absorbing the antibodies with their cognate peptides prior to precipitation. PCR primers and amplification conditions for the immunoprecipitated plasmid and genomic DNA are listed in Table 15. PCR product sizes were verified by 2% agarose gel electrophoresis.

Table 15. PCR primers and their associated reaction conditions of ChIP PCR.

ChIP PCR	Sequence[a]	Tm (°C)[b]	[Mg^{2+}] (mM)	Primers (µM)	DNA polymerase
Plasmid promoter 1F	Fwd: 5'-aaccgtattaccgccatgca-3' Rev: 5'-aacaccaccttgatgtccatgg-3'	64	0.5	0.5	Phusion Hot Start DNA polymerase
Endogenous promoter 1F	Fwd: 5'-taccggactcagatctaagtacgtatgcgccgaccc-3' Rev: 5'-gtctactgcagaattcctcgctacctccttcccgcc-3'	64	3	0.1	Diamond DNA polymerase

[a]Fwd, forward or sense primer; Rev, reverse or antisense primer.
[b]Tm, annealing temperature in PCR.

4.3.5 Knock-down of endogenous E2F1 by RNA interference

GeneSolution siRNA Hs_E2F1_1 (target sequence: caggaccttcgtagcattgca) and negative control siRNA (target sequence: aattctccgaacgtgtcacgt) were purchased from Qiagen. Twenty-four hours prior to transfection, $2x10^4$ 293FT cells were seeded per well in a 24-well plate and were transfected with HiPerFect (Qiagen) according to the manufacturer's instructions using 37.5ng of siRNA and 3µL of HiperFect per well. Twenty-four hours post-transfection, cells were collected and analyzed by Real-time PCR and Western blot for the disappearance of *E2F1* mRNA and protein. Real-time PCRs were

performed in 25µl reactions containing 250mM Tris-HCl (pH 8.3), 50mM KCl and 200mM deoxynucleoside triphosphates (dNTPs) 1x SyBR Green and 2.5U Platinum TaqDNA polymerase (Invitrogen) on an Opticon 2 thermal cycler (Bio-Rad). Cycling conditions were as follows: 95°C for 2 min, 40 cycles, at 95°C for 20s, annealing temperature (Table 16) for 20s and 72°C for 25s. *E2F1* and *Exon 1F* expression was normalized to *β-Actin* using the method of Livak and Schmittgen (Livak and Schmittgen 2001). Western blots were performed using a standard protocol (Towbin et al. 1979). Membranes were incubated with rabbit polyclonal anti-human E2F1 (sc-193) antibodies and a mouse monoclonal anti-human β-Actin (sc-47778) antibody (Santa Cruz) and visualised with anti-rabbit Cy5 and anti-mouse Cy3 labelled secondary antibodies (Pierce, Rockford, IL).

Table 16. PCR primers and their associated reaction conditions of knockdown PCR.

Knockdown PCR	Sequence[a]	Tm (°C)[b]	[Mg^{2+}] (mM)	Primers (µM)	DNA polymerase
β-Actin	Fwd: 5'-ggccacggctgcttc-3' Rev: 5'-gttggcgtacaggtctttgc-3'	60	2	1	Platinum®Taq DNA Polymerase
E2F1	Fwd: 5'-gaagtccaagaaccacatcc-3' Rev: 5'-gatctgaaagttctccgaag-3'	60	2	1	
Exon 1F	Fwd: 5'-gtagcgagaaaagaaactgg-3' Rev: 5'-cagtggatgctgaactcttgg-3'	60	1.75	0.5	

[a]Fwd, forward or sense primer; Rev, reverse or antisense primer.
[b]Tm, annealing temperature in PCR.

4.3.6 Statistical analysis

Differences between two groups were analysed using the Student's-T test. Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* test. Statistical analysis and graphs were performed with SigmaPlot 9.0 (Systat Software GmbH, Erkrath, Germany). Statistical significance was considered for $p < 0.05$. Results are shown as the mean ± SEM.

4.4 Results

4.4.1 Effect of selected transcription factors on promoter 1F

To investigate the distribution of potential response elements within the *GR* 1F promoter, the evolutionary conserved transcription factor binding sites (TFBSs) predicted by ISPF (Turner et al. 2008) were examined. Transfection with cloned transcription factors activated the pGL4.10 reporter system in a manner similar to our previous report using pMetLuc (Alt *et al.*, 2011 submitted) although with minor differences, showing that in 293FT cells only E2F1 resulted in a 5 fold increase of 1F promoter activity ($P < 0.05$) (Figure 15). HES1 had very little effect on promoter activity. However, NGFI-A and ELK1 which have previously been shown to have a marginal activating effect on the 1F promoter (2.3 and 2.1 fold respectively) in the pMetLuc reporter system, had no effect on the 1F promoter in the pGL4.10 system.

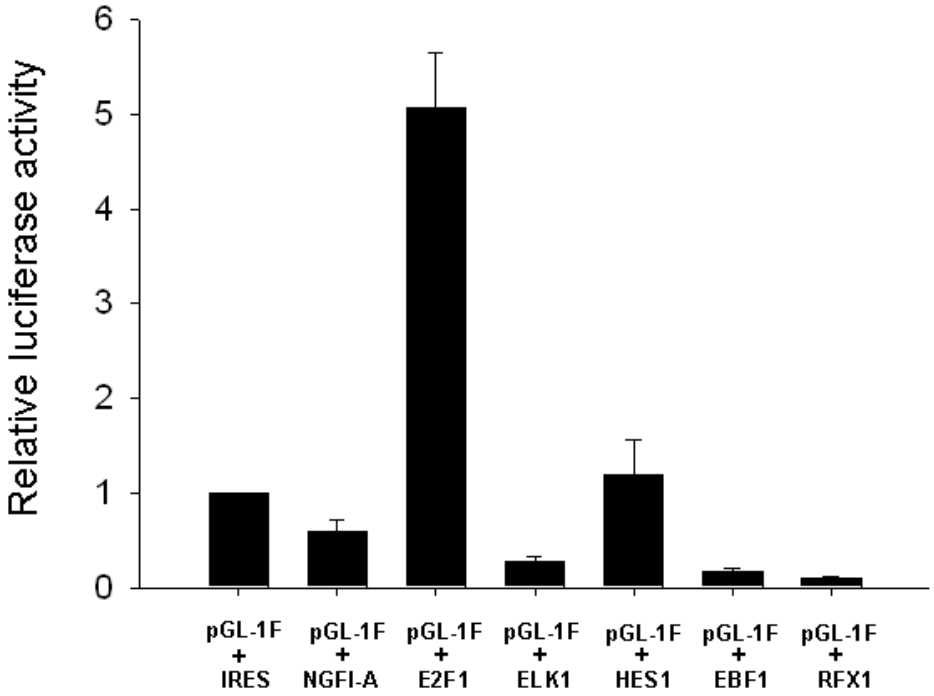

Figure 15. Relative induction of pGL-1F reporter gene by ISPF predicted transcription factors. Luciferase activity was normalized to pGL4.73 (hRluc/SV40) internal control vector and expressed in fold activity of the pGL-1F reporter. Mean ± SEM of triplicate transfections of three independent experiments are shown. Significance was determined by one-way ANOVA.

4.4.2 Knocked down of E2F1 by siRNA

To confirm the transcriptional regulation of exon1F by E2F1, its expression was knocked down by transfection of 293FT cells with specific siRNA. As shown in Figure 16A, siRNA effectively knocked down E2F1 mRNA 24h post-transfection to 25% of their initial level. However, the protein level was only reduced by 23% at 24h (Figure 16B), and returned after 48h to normal levels. Nevertheless this reduction in E2F1 was sufficient to down-regulate exon 1F transcription. Messenger RNA transcripts of exon 1F, representing only about 1% of total *GR* transcript (Alt et al. 2010), were reduced to a level that was no longer detectable (Figure 16C), suggesting a direct and highly sensitive regulation of 1F by E2F1.

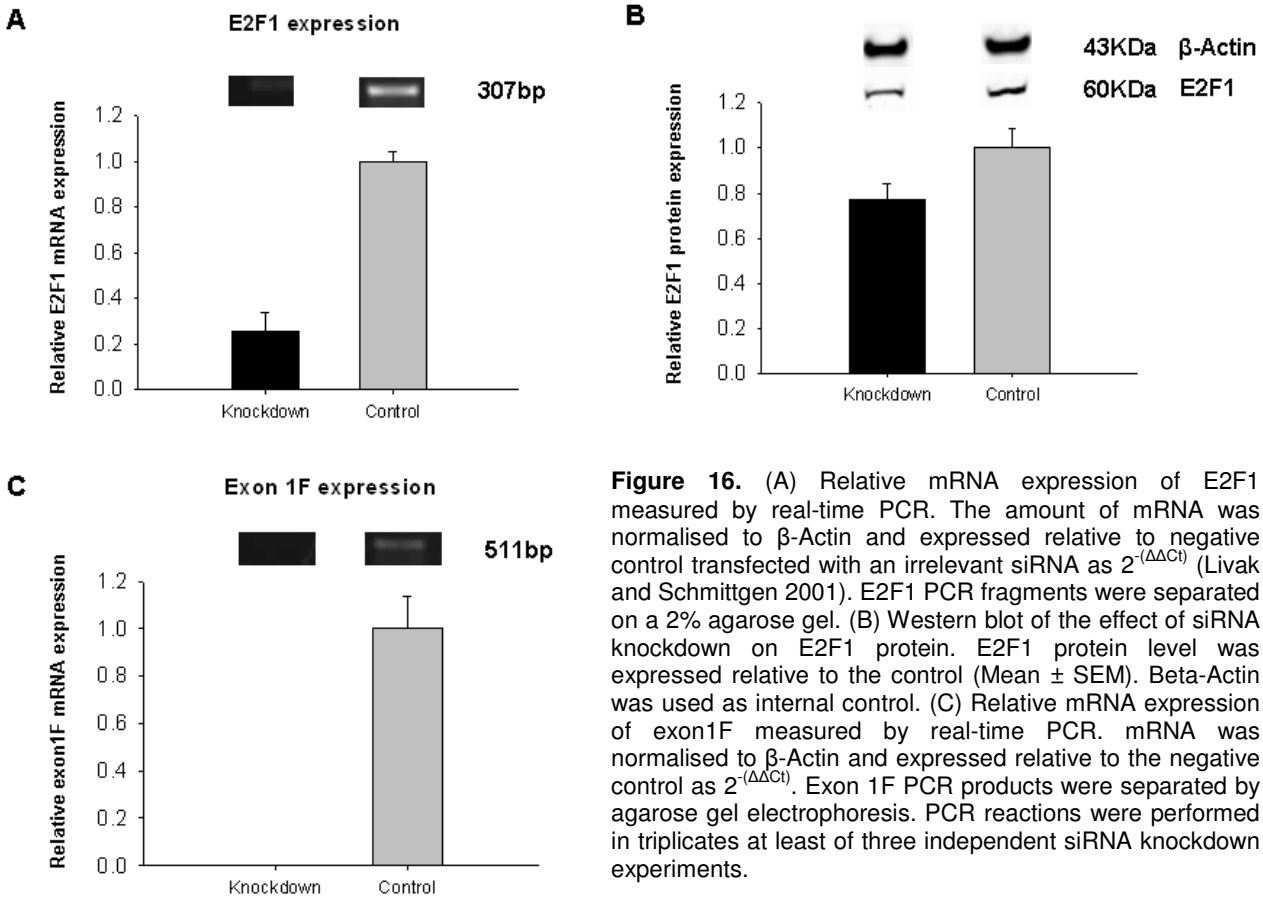

Figure 16. (A) Relative mRNA expression of E2F1 measured by real-time PCR. The amount of mRNA was normalised to β-Actin and expressed relative to negative control transfected with an irrelevant siRNA as $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen 2001). E2F1 PCR fragments were separated on a 2% agarose gel. (B) Western blot of the effect of siRNA knockdown on E2F1 protein. E2F1 protein level was expressed relative to the control (Mean ± SEM). Beta-Actin was used as internal control. (C) Relative mRNA expression of exon1F measured by real-time PCR. mRNA was normalised to β-Actin and expressed relative to the negative control as $2^{-(\Delta\Delta Ct)}$. Exon 1F PCR products were separated by agarose gel electrophoresis. PCR reactions were performed in triplicates at least of three independent siRNA knockdown experiments.

4.4.3 Effect of transcription factors on truncated sequences of promoter 1F

To identify active regions within the promoter 1F, a series of truncated promoter constructs were prepared. All constructs (1F_96, 1F_133+96, 1F_133, 1F_97+133 and 1F_97) showed significant baseline activity ($p < 0.05$) (Figure 17A), however the region -3536 to -3307 (1F_97+133) showed the highest baseline promoter activity, statistically similar to the complete promoter 1F.

Overexpression of NGFI-A had no significant effect on any of the truncated promoter constructs, suggesting that in this pGL4.10 based system, NGFI-A plays no role in inducing promoter 1F activity (Figure 17B).

E2F1 overexpression increased both the activity of the complete promoter 1F (6.8 fold, $p < 0.05$) (Figure 17C), and of the 1F_133+96 construct (10 fold, $p < 0.05$) in comparison to the natural activity. While some of the other truncated constructs (1F_133 and 1F_97+133) showed a trend to higher activity, this was not statistically significant. Thus, the 3' two-thirds of the promoter seem to mediate E2F1 activity.

Overexpression of HES1 had very little effect on the complete promoter 1F, but it efficiently increased the 1F_97, 1F_97+133, 1F_133 and 1F_96 promoter activity ($p < 0.05$) (Figure 17D). Interestingly activity of 1F_133+96 was repressed in comparison to 1F_133 and 1F_96 alone, suggesting more complex interactions of transcription factors within this promoter sequences.

Since both E2F1 and HES1 upregulated the complete or partial promoter 1F, they were also tested by co-transfection. HES1 reduced E2F1 activity in the case of 1F and 1F_133+96, E2F1 inhibited the effect of HES1 activity on 1F_97, 1F_133 and 1F_97+133, suggesting that E2F1 has an additional binding site on the 5' end of the promoter (Figure 17E).

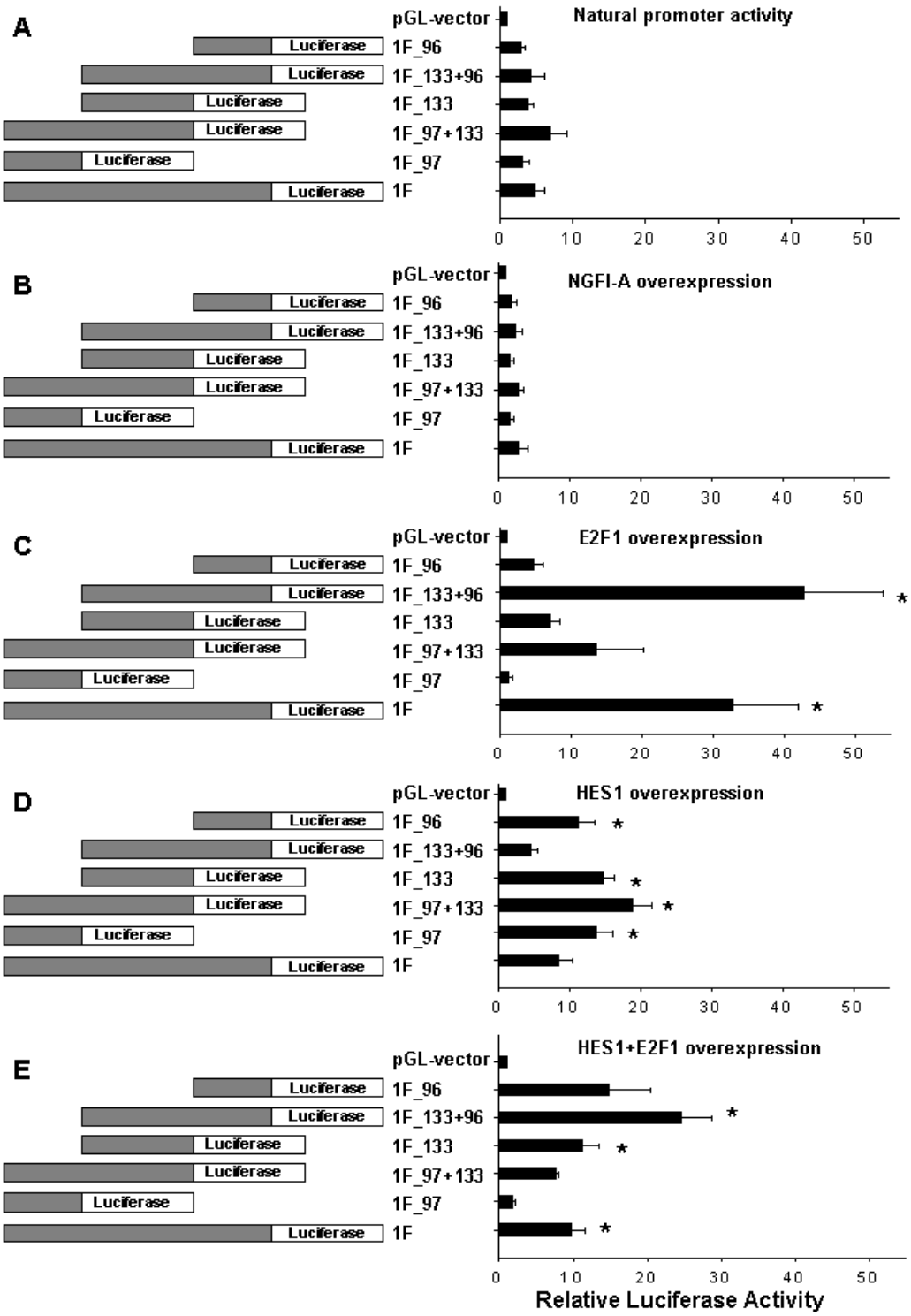

Figure 17. (A) Natural luciferase activity of truncated sequences of promoter 1F. (B-E) Luciferase activity of truncated promoter 1F constructs with overexpression of NGFI-A, E2F1, HES1 and E2F1+HES1. Truncated sequences are named according to the size of the sequence. Promoter activity was normalized to pGL4.73 (hRluc/SV40) internal control vector and expressed in fold activity of the empty pGL4.10 vector. Triplicates of three independent experiments were performed. Comparisons were determined by one-way ANOVA. All values are mean ± SEM. *$p < 0.05$.

4.4.4 Direct binding of E2F1 and HES1 to promoter 1F

To verify that our reporter gene system was representative of the genomic situation, pMetluc-1F was transfected into 293FT cells and the binding of endogenous E2F1 protein to both the reporter gene and the genomic DNA was tested by ChIP. A strong PCR signal was seen after specific amplification of both plasmid (Figure 18A) and genomic DNA (Figure 18B) when anti-E2F1 and anti-HES1 antibodies were used. Only a low signal was seen after blocking either antibody by its cognate peptide, demonstrating the specificity of the reaction. Thus E2F1 and HES1 bind both to the endogenous and exogenous promoter, however, the recruitment of E2F1 seems to be stronger in the endogenous promoter than in plasmid DNA. In contrast, there is no a significant difference in recruitment of HES1 in both endogenous and exogenous promoter 1F.

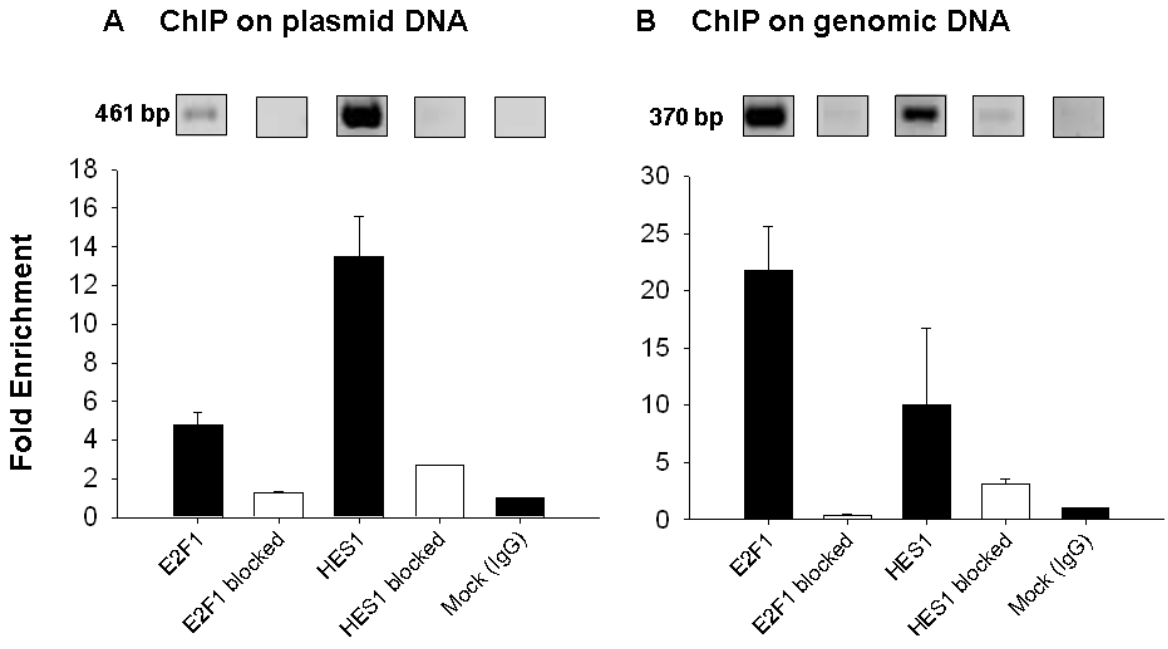

Figure 18. ChIP analysis of E2F1 and HES1 transcription factor binding to plasmid DNA (A) and to genomic DNA (B). Real-time PCR was performed to determine the DNA fold enrichment relative to the background signal of the negative control. PCR products were visualized on agarose gel. Specificity of the reaction was confirmed by peptides blocked antibody. Normal rabbit IgG were used as negative control. Repetitive experiments of three independent ChIPs are shown.

4.4.5 Effect of mutation on promoter 1F

Since E2F1 had a significant effect on the 1F_133+96 in the 5' truncated sequence, we mutated the partially overlapping E2F1/NGFI-A binding sites, and also the predicted E2F binding sites in the pMetLuc-1F constructs. Our original pMetLuc-1F construct was used because of the presence of unwanted restriction sites in the backbone of pGL4. The wild-type pMetLuc-1F promoter activity induced by E2F1 overexpression was significantly higher than that of any of the mutated pMetLuc-1F constructs (Figure 19A). Mutating only E2F1 binding site had the weakest effect on promoter activity (44% reduction), whilst mutations in the E2F and NGFI-A binding site resulted both in similar reductions (> 60% reduction) on promoter 1F expression. Simultaneous mutations in the E2F1/NGFI-A binding sites reduced promoter activity by 65%, E2F/NGFI-A by over 70%, and E2F/E2F1 by 63%, suggesting that these three binding sites explain most, but not all, of the promoter 1F activity. Nevertheless, mutating three all of these binding sites simultaneously resulted in only a 63% reduction of the promoter activity.

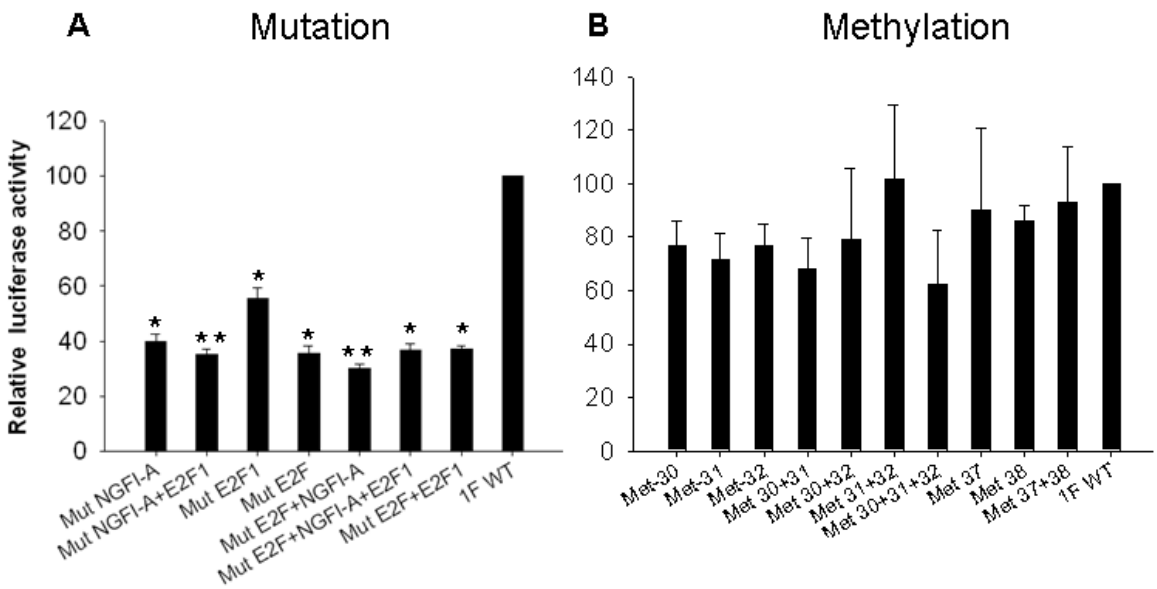

Figure 19. Effect of over-expression of E2F1 on luciferase activity of mutated promoter 1F constructs (A) and methylated promoter 1F constructs (B). Luciferase activity was normalized to that of each without overexpression and expressed in percent of the pMetLuc-1F wild-type. Triplicates of three independent experiments were performed. Comparisons were determined by one-way ANOVA. All values are mean ± SEM. *p < 0.05.

4.4.6 Effect of single nucleotide methylation on promoter 1F

As single CpG methylation of position 37, 38 (position 16, 17 in the rat) (Weaver et al. 2004) and 32 (McGowan et al. 2009) have been suggested to play an important role in control of 1F transcription, selective methylation of these CpG dinucleotides alone or in combination were performed. However, methylation of the individual CpGs 30, 31 and 32 had no statistically significant effect compared to the unmethylated pMetLuc-1F control (Figure 19B). Simultaneous methylation of all three positions, however, reduced promoter activity to 62% of the unmethylated control, although without reaching statistical significance. These results suggest that single CpG dinucleotide methylation in promoter 1F does not prevent the binding of E2F1 or reduce transcription.

4.5 Discussion

The most important finding of this study is that the epigenetically sensitive promoter 1F requires E2F1 for activation, contains at least 2 *bona fide* E2F1 binding sites, and that single nucleotide methylation of this promoter plays a less important role than previously thought.

By overexpressing E2F1, we obtained the highest promoter 1F induction with a 5 fold up-regulation in comparison to the natural activity of 1F promoter in 293FT cells. This E2F1 induction has also previously been observed using another reporter gene system in both 293FT cells (> 5 fold) and U373MG cells (~ 4 fold) (Alt *et al.*, 2011 submitted). Truncated reporter gene constructs showed that elements in the central region (133bp) and in the 3' end region (96bp) of promoter 1F were necessary for complete transcriptional activity in a manner similar to that observed after NGFI-A overexpression by McGowan *et al* (McGowan et al. 2009). Both E2F1 and E2F binding sites in the 1F_96 region had been predicted by ISPF (Turner et al. 2008). Although the 1F_133 region revealed no consensus binding sites for E2F1 or other E2F family members, this does not exclude the binding of E2F, indeed members of this family have been shown by ChIP to bind to several other genes without a consensus E2F binding site in their promoter (Weinmann et al. 2001). Mutation of the predicted E2F1 and E2F binding sites in this region showed a 40 and 60% reduction in promoter activity respectively, suggesting that they are both *bona fide* binding sites, although they are not responsible for the complete promoter activity. Similarly, ChIP suggests that E2F1 binds in a specific manner to the promoter, with similar results on the promoter 1F sequence in both the endogenous and reporter gene system. Knockdown experiments confirmed the prominent role of E2F1 in 1F transcription with a complete loss of activity after a 75% reduction at the mRNA level and a mere 23% reduction at the protein level. Taken together, these data suggest that E2F1 is essential for promoter 1F regulation.

However, the role of E2F1 is still not fully elucidated, since over expression of E2F1 did not upregulate endogenous 1F expression (Alt *et al.*, 2011 submitted). One possible explanation is that E2F1 is necessary but not sufficient to stimulate endogenous exon 1F transcription, and that other transcription factors must be upregulated. This is not surprising since many promoters require multiple members of family of transcription factors for activity (Henke et al. 2009; Takahashi et al. 2008).

Similar to E2F1, HES1 binds to promoter 1F as demonstrated by ChIP in both the endogenous promoter and reporter gene systems. It was predicted to bind to the end of the 1F_133 region. HES1

activated the truncated but not the complete promoter 1F constructs with the exception of 1F_133+96, suggesting the implication of the other modulating transcription factors and/or some repressor activity of HES1 which has been already observed in other genes (Davis and Turner 2001; Fischer and Gessler 2007; Hartman et al. 2004; Kageyama et al. 2007). Concurrent overexpression of E2F1 with HES1 showed considerably lower activity than E2F1 alone, suggesting that HES1 repressed E2F1 induced activation, compatible with the binding of both transcription factors to promoter 1F. In contrast, NGFI-A was inactive in the full length and the truncated promoter sequences, including in the 1F_96 region, where it was shown to bind in the rat (Weaver et al. 2004); and the 1F_133 region, where it was suspected to bind to in the human (McGowan et al. 2009). In a previous study with another reporter gene system (Alt *et al*., 2011 submitted), NGFI-A had also no effect in U373MG and had only a marginal effect in 293FT cells. Also ELK1 showed some minor activity in the previous study which was not observed in 293FT cells for the full length 1F construct in the present study. These minor differences in activity for the poorly active transcription factors can be only explained by the different reporter gene systems used. Nevertheless, the robust and reproducible activity of E2F1 suggests that this transcription factor plays a key role in regulating exon 1F transcription.

The E2F1 predicted binding site overlaps by six nucleotides with the known NGFI-A binding site identified in the 3' end region (Weaver et al. 2004). Mutating the suspected E2F, NGFI-A and E2F1 binding sites showed that they all play a role in 1F transcription. The mutations in the E2F/NGFI-A and NGFI-A/E2F1 binding sites reduced promoter activity by a 65%-70%, suggesting that the region in which E2F1 overlaps with the NGFI-A binding site (tgggc^{37}gggggc^{38}ggg, -3228 to -3215) and that the region covering the E2F binding site (cggcgcttgcc^{32}gccaagg, -3285 to -3268) are necessary for promoter 1F activity. Also, after deleting this region, the truncated promote 1F_97+133bp showed a similar reduction as compared to the whole promoter 1F. The two CpGs (position 37 and 38 here) identified in the rat NGFI-A site by Weaver *et al* (Weaver et al. 2004) fall into the former region and those of McGowan *et al* (McGowan et al. 2009) (CpG 30, 31 and 32) fall into the latter. Both of these earlier studies suggested that methylation of single CpG dinucleotide within these sites may be sufficient to abrogate 1_7 and 1F activity. We have previously shown that complete promoter methylation abrogates the activity of all the *GR* CpG island promoters (Cao-Lei et al. 2011). Similarly, patch methylation of a 225bp and 125bp promoter 1F construct, in which a completely methylated promoter was ligated into an unmethylated reporter gene backbone, reduced their activity to ~ 25% of

the corresponding unmethylated sequence (McGowan et al. 2009). To determine if the single CpG dinucleotide was as transcriptionally important as suspected, we employed a single nucleotide methylation approach, which showed that independent methylation of CpGs 30, 31, 32, 37 and 38 was unable to reduce the reporter gene activity. Also simultaneous methylation of CpG 30, 31 and 32 had little effect on reducing the promoter activity compared to the unmethylated control. Therefore, it seems unlikely that single CpG dinucleotide methylation can mediate the inhibition of transcription. This is in line with observations that single CpG dinucleotide methylation in the transcription factor Sp1 binding site, often associated with promoters of housekeeping genes, has no effect on binding of Sp1 and activation of transcription (Harrington et al. 1988; Holler et al. 1988). Detailed analysis of the complete rat chromosome 18 (containing the *Nr3c1*) in the original maternal care paradigm used by Weaver *et al* (2004) revealed that differential histone acetylation, DNA methylation and gene expression occurs in clusters across broad genomic regions (McGowan et al. 2011), suggesting that the phenotypic effect observed may be due to factors other than single nucleotide methylation.

Since the E2F family of transcription factors plays a pivotal role in regulating the expression of genes such as *cyclins A* and *D1* involved in the G1/S transition of the cell cycle (Dyson 1998; Fan and Bertino 1997; Inoshita et al. 1999), 1F transcripts may be preferentially activated during this phase. The observation of low 1F levels (roughly 1% of the total *GR*), may reflect the low numbers of cells transitioning between the phases in unsynchronized samples and hence the low or undetectable effect of E2F1 overexpression. E2F1 has been shown to be critically involved in neurogenesis of E2F1 knock out mice (Cooper-Kuhn et al. 2002). Since E2F1 is an essential transcription factor, it would be detrimental if its target genes were to be silenced by methylation of a few CpG dinucleotides in its promoter. Thus, our observation of the limited impact of methylation of individual CpG would appear to align with this perception.

In conclusion, we showed that over-expression of NGFI-A did not affect promoter 1F activity in the reporter gene assay despite binding to this promoter. HES1 bound to promoter 1F but did not activate the complete promoter, rather acting as a repressor of E2F1. In contrast transcription factor E2F1 strongly up-regulated promoter 1F, binds to the promoter 1F, and 1F_133+96 is necessary for promoter 1F regulation by E2F1. Mutations in E2F, NGFI-A, and E2F1 binding sites confirm that they are *bona fide* binding sites. Inhibition of transcription can not be mediated by methylation of single CpG dinucleotide in these binding sites, however, simultaneously methylation of 3 CpG dinucleotides

(30+31+32) has a larger, albeit still not a significant effect. Thus, we demonstrated that E2F1 is an element of the transcriptional complex critical for the expression of *GR* 1F transcripts and identified the binding site and its lack of susceptibility to single nucleotide epigenetic methylation.

CHAPTER 5

Glucocorticoid receptor gene expression and promoter methylation status throughout the human brains.

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Manuscript in preparation.

5.1 Abstract

Negative feedback regulation of the hypothalamic-pituitary-adrenal axis occurs through a dual-receptor system of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) and their distribution in the brain differs.

It is reported that *GR* alternative promoter usage is a potential mechanism controlling GR levels, *GR* 3' splice variants distribution, overall response to GC in the brain. The methylation status in these *GR* promoters in human brain is not clear. In this study, the *GR* first exons, *GR* splice variants and methylation status of the *GR* promoters in healthy human brains have been investigated. The GR/MR ratio in different brain tissues was measured. We showed that the *GR* first exons are expressed throughout the human brain with no region specific usage patterns. *GR* 3' splice variants (GRα and GR-P) were equally distributed in all the brain regions. These data mirrored the consistently low levels of methylation in the brain, and the observed homogeneity throughout the studied regions.

Keywords: glucocorticoid receptors, mineralocorticoid receptors, *GR* first exon transcripts, *GR* 3' splice variants, methylation status, human brain

5.2 Introduction

The hypothalamus-pituitary-adrenocortical (HPA) axis, one of the major stress response systems, maintains homeostasis and adaptation during challenges (de Kloet et al. 2005). Glucocorticoids (GCs) such as cortisol or corticosterone, downregulate the HPA axis activity in a negative feedback loop at the level of the paraventricular nucleus, the pituitary gland and the hippocampus via its cognate receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The level of, and balance between the two receptors will determine how a tissue responds to GCs. MR has a 10-fold higher affinity for GC than the GR (Reul and de Kloet 1985). The lower affinity of the GR for cortisol means that it is selectively activated during circadian and stress induced cortisol zeniths, whilst the MR will be activated (in the absence of protective 11β-hydroxysteroid dehydrogenase) during the nadirs (Conway-Campbell et al. 2007). The imbalance between the two receptors may be involved in the vulnerability to diseases, especially these associated with an altered HPA axis such as major depressive disorder (MDD) (de Kloet et al. 1998). In the rodent brain, MRs had a high expression in the limbic brain regions, such as hippocampus (Kretz et al. 2001; Reul and de Kloet 1985), whilst GRs are ubiquitously expressed, with the highest density in hypothalamic CRH neurons and pituitary corticotrophs (Morimoto et al. 1996; Reul and de Kloet 1985).

The human *GR* (OMIM +138040; *NR3C1*) is located within chromosome 5 and contains eight constant exons (exon2-9) and nine untranslated alternative first exons 1A-1I (Breslin et al. 2001; Presul et al. 2007; Turner and Muller 2005). This 5'-non-coding region does not affect the encoded protein sequences due to the ATG translation start codon located in exon 2. Exon 1A and 1I are under the control of the distal promoter region. The proximal exons 1B to 1H are controlled by individual promoters (Cao-Lei et al. 2011) located in a 3kp long upstream CpG island that has a high sequence homology between mice, rats and humans (Bockmuhl et al. 2011; McCormick et al. 2000; Turner and Muller 2005). Numerous transcription factors controlling GR expression have been identified (reviewed in Turner et al. 2010). Tissue specific *GR* first exon expression has been demonstrated in several studies (Alt et al. 2010; Presul et al. 2007; Russcher et al. 2007; Turner and Muller 2005), and corresponding tissue specific promoter activity shown in Cao-Lei et al. (Cao-Lei et al. 2011).

Splicing at the 3' end of the GR generates three mRNA transcripts encoding the GR isoforms, GRα, GRβ and GR-P. GRα and GRβ are generated by two alternatively spliced exon 9, 9α and 9β, encoding the C-terminal ligand binding domain (LBD). GRα comprising 777 amino acids, is the most active form

of the receptor mediating the majority of genomic GC action. The shorter GRβ (742 amino acids), with a LBD that is unable to bind GC, is thought to be a dominant negative regulator of the GRα (Bamberger et al. 1996; Oakley et al. 1996). GRα levels are universally higher than GRβ, and a decreased GRα: GRβ ratio has been invoked in GC resistance (Lu and Cidlowski 2005). GR-P mRNA lacks both exons 8 and 9, with exon 7 continuing into the following intron after splicing, truncating the LBD resulting in the inability of the 676 amino acid isoform to bind GC (Krett et al. 1995). The function of GR-P is still not fully understood although it is reported to enhance GRα activity (de Lange et al. 2001). Whilst the exact influence of the 5' region on the splicing of pre-mRNA is unknown, the alternative first exons have been reported to influence splicing and the resultant isoform (Alt et al. 2010; Russcher et al. 2007).

The activity of all the CpG island promoters has been shown to be mediated by DNA methylation (Cao-Lei et al. 2011), although promoter 1F has received the most attention (Alt et al. 2010; McGowan et al. 2009; Moser et al. 2007; Oberlander et al. 2008). DNA methylation, the most common epigenetic modification, is the covalent addition of a methyl group to the 5th position of the cytosine in a CpG dinucleotide via DNA methyltransferases (DNMTs). CpG dinucleotides are the least frequent of all nucleotide pairs, and are found in clusters throughout the genome termed CpG islands (Gardiner-Garden and Frommer 1987). The CpG islands frequently contain transcription start sites and 5' regions of housekeeping genes as well as tissue-specific genes. CpG dinucleotides within CpG island are usually unmethylated or hypomethylated (Cooper et al. 1983), however, in many disease such as cancer, CpG pair within a CpG island can be abnormally hypermethylated. A number of studies have shown that hypermethylation of promoter CpG islands are a common epigenetic event in human cancers. The activity of all the *GR* CpG island promoters can be abrogated by the complete promoter methylation *in vitro* (Cao-Lei et al. 2011).

Methylation of *GR* promoters especially 1F, and its rat orthologue promoter 1_7, is thought to be a key link between the environment and GR expression. Early life adverse events, such as the lack of maternal care, produced epigenetic changes in promoter 1_7 in rodents, and the NGFI-A binding site in this promoter is particularly susceptible to methylation with level varying from 0 to 100% (Weaver et al. 2004). However, no methylation in promoter 1_7 was reported in maternal separation rats and controls (Daniels et al. 2009), and levels never exceeded 20% in rats with a methylation supplementation diet (Herbeck et al. 2009). In humans, hippocampal methylation of the region orthologous to the rat NGFI-

A binding site was similarly unmethylated (Alt et al. 2010; Moser et al. 2007) or at < 5% (Oberlander et al. 2008). However, methylation of a separate NGFI-A binding site from that orthologous to the rat (Weaver et al. 2004) was observed in *post mortem* hippocampi of suicide victims to correlate with prior childhood abuse (McGowan et al. 2009).

There are very limited data about methylation distribution of the other *GR* alternative promoter regions, *GR* first exons and 3' splice variants distribution in the human brain. Here, we have examined the methylation level of human *post mortem* tissues representing 27 brain fields as well as the pituitary gland and investigated brain-region-specific expression of *GR* first exons and 3' splice variants. We found that the *GR* first exon expression patterns were similar throughout the brain tissues with only the expression of exon 1A3 being elevated in the pituitary gland. The individual promoters were poorly methylated throughout the brain and did not correlate with the expression of their associated exons. GRα and GR-P expression showed no differences amongst the tissues investigated.

5.3 Materials and Methods

5.3.1 Subjects

Human *post mortem* tissues from five donors were obtained from the Chiang Mai University (Thailand) following permission from the patient or a close relative for brain autopsy and for the use of anonymised brain material and clinical information for research purposes. Donor clinicopathological information and the tissues obtained are shown in Table 17 and Table 18 respectively. The Hippocampus (CA1-CA3) was not available for one donor (01). The 28 tissues were dissected as soon as possible *post mortem* to stabilise mRNA prior to analysis. One centimetre autopsy sections were cut and a 5 mm diameter punch biopsy obtained from each brain region. All biopsies were completely submerged in > 5 volumes RNA*later* (Qiagen) and stored at -20°C until analysis. The study was approved by the ethical committee of the Faculty of Medicine, Chiang Mai University.

5.3.2 DNA/RNA extraction

A ~ 1.5 mm^3 cube was excised from each sample and both genomic DNA and total RNA were extracted using AllPrep DNA/RNA mini kit (Qiagen, Venlo, Netherlands) based on the manufacturer's protocol. Genomic DNA and total RNA were measured on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Genomic DNA was stored at -20°C and used for bisulfite modification and pyrosequencing. RNA was stored at -80°C and used for real-time quantitative RT-PCR.

5.3.3 cDNA synthesis

Synthesis of cDNA was carried out at 50°C for 60 min using 200 U Superscript III RT (Invitrogen, Merelbeke, Belgium) and 2.5 mM dT20 primer in a 50 µl reaction containing 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol and 500 mM deoxynucleoside triphosphates (dNTPs). A negative control without reverse transcriptase and a second with sterile PCR water as template was performed to control for genomic DNA contamination. After cDNA synthesis, samples were stored at -20°C until further analysis.

Table 17. Five donors clinicopathological Information.

number	gender	Age (year)	Clock time of death	Post-mortem delay(h:min)	Cause of Death
01	male	54	00:20	10:10	
02	male	43	03:00	7:00	Blunt chest injury
03	female	59	12:00	3:30	Car accident
04	male	24	03:10	9:50	Car accident
05	male	20	05:30	7:00	Gun shot
Mean±SD	-	40	-	7:30	-

Table 18. List of the abbreviations of 28 human tissues.

N° tissues	28 tissues	Abbreviations
01	Pineal gland	PG
02	Pituitary gland	Pit
03	Superior frontal gyrus	SFG
04	Middle frontal gyrus	MFG
05	Inferior frontal gyrus	IFG
06	Superior parietal gyrus	SPG
07	Superior occipital gyrus	SOG
08	Middle temporal gyrus	MTG
09	Inferior temporal gyrus	ITG
10	Cingulate gyrus	CG
11	Calcarine sulcus (upper bank)	CAS (upper bank)
12	Calcarine sulcus (lower bank)	CAS (lower bank)
13	Caudate nucleus	CN
14	Nucleus accumbens	NA
15	Putamen	Put
16	Globus pallidus	GP
17	Amygdala	Amg
18	White matter	WM
19	Cerebellum	Cb
20	Substantia nigra	SN
21	Locus coeruleus	LC
22	Paraventricular nucleus	PVN
23	Ventromedial hypothalamus (ventrolateral)	VMHvl
24	Ventral subiculum pyramidal layer	SUBv-sp
25	Hippocampus (CA1-CA3)	Hi (CA1-CA3)
26	Hippocampus (CA4)	Hi (CA4)
27	Dentate gyrus	DG
28	Parahippocampal gyrus	PHG

5.3.4 Real-time PCR

All primers were designed using VectorNTi (Invitrogen, Paisley, UK) and synthesised by Eurogentec (Seraing, Belgium). Primers and PCR conditions are shown in Table 19.

Table 19. PCR primers and their associated reaction conditions.

Expression analysis	Sequence[a]	Length (bp)	Tm (°C)[b]	$[Mg^{2+}]$ (mM)	Primers (µM)
β-Actin	Fwd: 5'-GGCCACGGCTGCTTC-3' Rev: 5'-GTTGGCGTACAGGTCTTTGC-3'	208	60	2	1
Exon 1B	Fwd: 5'-GCCGGCACGCGACTCC-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	478	62	2	0.5
Exon 1C	Fwd: 5'-GCTCCTCTGCCAGAGTTGAT-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	464	62	2	0.1
Exon 1E	Fwd: 5'-CGTGCAACTTCCTTCGAGT-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	468	60	3	0.1
Exon 1F	Fwd: 5'-GTAGCGAGAAAAGAAACTGG-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	511	60	2	1
Exon 1J	Fwd: 5'-CCGGGGTGGAAGAAGAG-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	466	60	2	0.1
Exon 1H	Fwd: 5'-CTGACAGCCCGCAACTTGGA-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	531	65	3	1
Exon 1A3	Fwd: 5'-GCTTCATTAAAGTGTCTGAGAAGG-3' Rev: 5'-CAGTGGATGCTGAACTCTTGG-3'	464	57	2.25	0.5
Exon 2	Fwd: 5'-GAGGGGAGATGTGATGGACTTCT-3' Rev: 5'-GCTGCTGCGCATTGCTTA-3'	161	55	2.5	0.8
Exon 3/4	Fwd: 5'-CTCAACAGCAACAACAGGACCAC-3' Rev: 5'-GATGCAATCATTCCTTCCAGCA-3'	166	58	2	1
GRα	Fwd: 5'-TGTTTTGCTCCTGATCTGA-3' Rev: 5'-TCGGGGAATTCAATACTCA-3'	386	54	2	0.5
GRβ	Fwd: 5'-TGTTTTGCTCCTGATCTGA-3' Rev: 5'-TGAGCGCCAAGATTGT-3'	393	60	3	0.3
GR-P	Fwd: 5'-TGTTTTGCTCCTGATCTGA-3' Rev: 5'-CCTTTGTTTCTAGGCCTTC-3'	220	60	3	0.5
Total MR	Fwd: 5'-CTGAGTTCCTTTCCTCCTGTC-3' Rev: 5'-GCCACAGGTGACTACCCCAT-3'	225	61	2	0.6

[a]Fwd, forward or sense primer ; Rev, reverse or antisense primer. [b]Tm, annealing temperature in PCR.

Quantitative PCR was performed on an Opticon 2 thermal cycler (Bio-Rad) using 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates (dNTP), 1x concentrated SYBR Green (Cambrex, Verviers, Belgium) and 2.5 U Platinum Taq DNA polymerase (Invitrogen). Cycling conditions were as follows: 40 cycles, 95°C for 2 min, 95°C for 20s, 72°C for 20s with a final elongation for 10min at 72°C. For each run the specificity of amplification was verified by melting curve analysis and electrophoresis of PCR products on a 2% agarose gel and visualised with SYBR Safe (Invitrogen) under UV light.

5.3.5 Analysis of RT-PCR data

Relative PCR quantification was performed using the comparative threshold cycle method (Livak and Schmittgen, 2001). The housekeeping gene *β-actin* was chosen as the internal reference gene for normalisation as we have previously reported it to be stable throughout the limbic system (Alt et al.

2010). *GR* first exon expression, normalized to *β-actin*, was referenced to an internal standard, total GR, providing the percentage of total GR per sample.

5.3.6 Bisulphite treatment and promoter amplification

The bisulfite modification of the DNA was performed using the EpiTect-Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. Bisulfite-converted DNA was quantified using a NanoDrop 1000 spectrophotometer and subjected to PCR amplification of the specific promoter regions with the primers and under the conditions previously described (Turner et al. 2008). The PCR products were separated by gel electrophoresis and visualized under UV light to confirm product size.

5.3.7 Methylation quantification by pyrosequencing

Specific PCR products were subjected to quantitative pyrosequencing analysis using a PyroMark ID system (Qiagen) following the manufacturer's protocol. All sequencing primers were designed using PyroMark Assay Design software version 2.0.1.15 (Qiagen). Biotin labelled DNA was first immobilized on streptavidin-sepharose beads (GE Healthcare, Diegem, Belgium). After washing and denaturation, the biotinylated single stranded DNA was released into annealing buffer containing the sequencing primer. Positive and negative controls were performed in each run. The frequency of DNA methylation of each CpG dinucleotide was subsequently analyzed using the Pyro Q-CpG software (version 1.0.9, Biotage). Sequencing primers were shown in Table 20.

Table 20. Sequencing primers for *GR* promoters.

Promoter	Sequencing primers	Promoter	Sequencing primers
1B	S1:TTTTTYGAGTGTGAGTATATTG	1H	S1: TTTTTGTTAGAGGTAAGAAG
	S2: TTTYGTAGATTYGGGGAAGA		S2: GGGAGTTTATAAATTTTTATTAGT
1D	S1: TTGAGAATTAAGGAAGGA		S3: TGGGGGGTTGGTAAG
	S2: CCCCTACTCTAACATCTTAA		S4: GTYGAGGGGGAGGAA
	S3: CTCRACCACAACCAC		S5: TYGTTTTYGTGGTGA
1E	S1: GGGGTAGGGGTTTTATG		S6: RCCCTTCTCAAACCA
	S2: GAGGGTAGTAAATGTTAA		
	S3: GGAAGAAGAGGTTAGGAG		
1F	S1: TCACTTCRAAAAAAAC		
	S2: AACRAACRAACCACA		
	S3: GTTGTTATTYGTAGGGGTAT		

5.3.8 Statistical analysis

Differences between groups were evaluated using one-way repeated measures analysis of variance (ANOVA) followed by a Tukey *post hoc* test. Associations were calculated using Pearson's correlation coefficient which was corrected according to Bonferroni. Statistical analysis and graphs were performed with SPSS version 16.0 (SPSS Inc., Chicago IL, USA) and with SigmaPlot 9.0 (Systat Software GmbH, Erkrath, Germany). Statistical significance was considered for $p < 0.05$. R statistical environment (Version 2.9.0) was employed to draw association and methylation status heatmap plots.

5.4 RESULTS

5.4.1 Expression of *GR* first exons

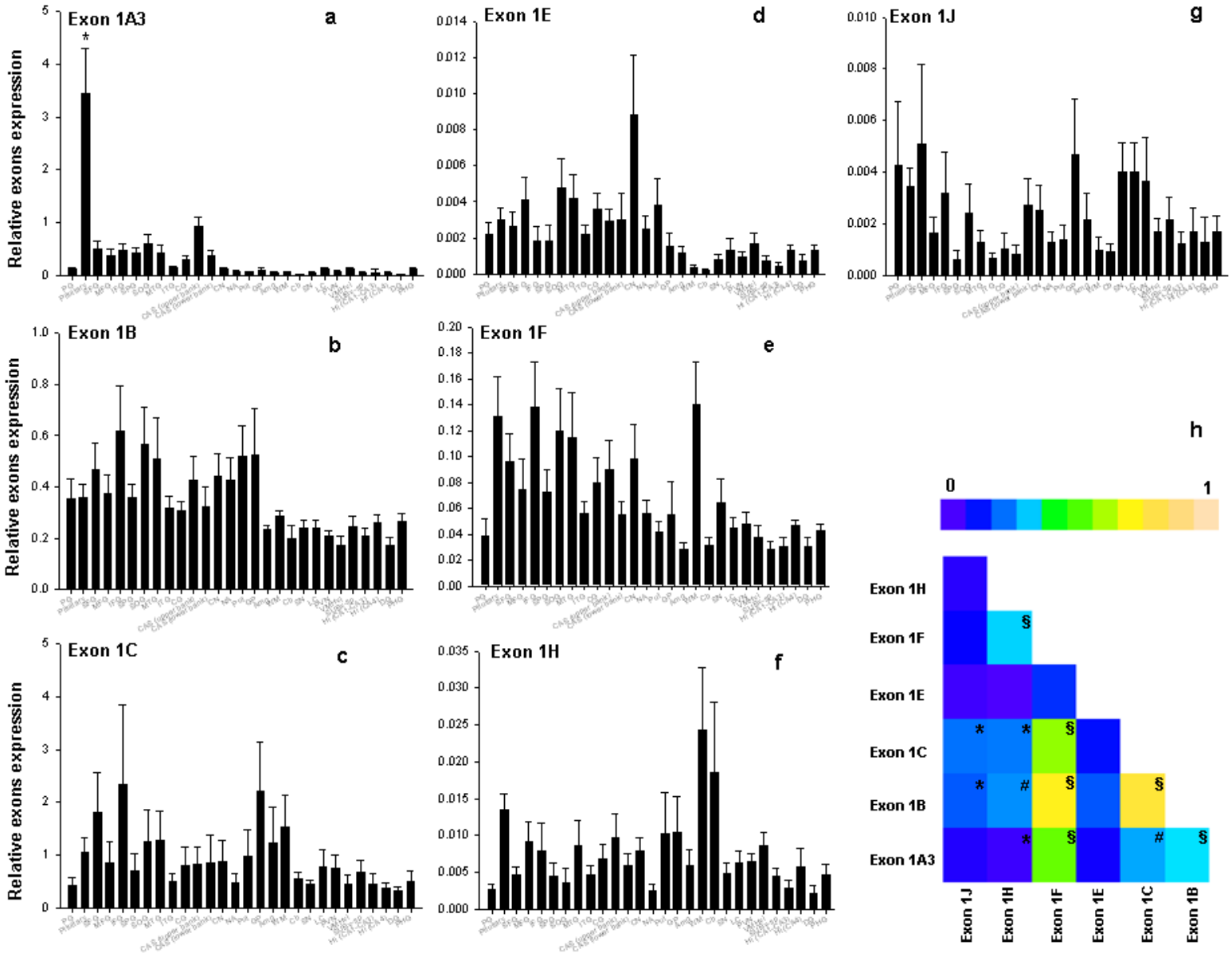
GR first exon transcripts are differentially expressed in the human brain tissues and pituitary gland. Amongst seven first exons transcripts investigated, exon 1C was the most abundant, followed by exon 1B and exon 1A3 (Figure 20c, b and a). Transcripts of 1E and 1J were the least abundant (Figure 20d, g). Statistical analysis did not show significant differences amongst 28 tissues in all seven first exon transcripts investigated, except exon 1A3 in which a markedly high expression was observed in pituitary gland (Figure 20a). Pearson correlation was performed to explore the associations amongst the seven first exon transcripts expression (Figure 20h). Exon 1B positively correlated with exon 1F ($r = 0.718$, $P < 0.001$) and exon 1C ($r = 0.773$, $P < 0.001$); exon 1F positively correlated with exon 1C ($r = 0.574$, $P < 0.001$) and exon 1A3 ($r = 0.525$, $P < 0.001$). The other correlations between different first exons including exon 1F/1H, exon 1B/1A3, exon 1B/1H, exon 1C/1A3, exon 1C/1J, exon 1C/1H, exon 1B/1J, and exon 1A3/1H showed significant but low correlation levels ($r < 0.50$).

5.4.2 Expression of *GR* 3' splice variants

GRα and GR-P were detected in all brain regions investigated. As expected, the functional GRα was predominantly expressed in all regions (Figure 21). GR-P is expressed with a ~ 1000 fold lower than GRα all the brain regions. The expression of neither GRα nor GR-P was significantly different amongst all 28 tissues and the ratio between GRα and GR-P was constant. Expression of GRβ was the lowest, approaching the lower detection limit of the PCR assay. There was a significant positive correlation between GRα and GR-P expression ($r = 0.429$, $P < 0.001$) (data not shown).

Figure 20. *(page 95) GR* first exon distribution in brains and the expression correlations in *GR* first exons. (a-f) Relative *GR* first exons expression in all 28 tissues. The amount of *GR* first exon mRNA normalised to β-actin and relative to a specific reference sample (total GR) was expressed as $2^{-(\Delta\Delta Ct)}$. (h) The expression correlation between each two first exons was represented by the shade depends on the Pearson correlation coefficient (r). Correlation levels are expressed by colour (0% (blue) to 100% (yellow)), levels from the scale at the panel top. Results are shown as the mean ± SEM. * $P < 0.05$, # $P < 0.01$, § $P < 0.001$.

Figure 20.


Exon 1A3
a
Relative exons expression
Exon 1E
d
Exon 1J
g
Exon 1B
b
Exon 1F
e
h
0
1
Exon 1C
c
Exon 1H
f
Exon 1H
Exon 1F
Exon 1E
Exon 1C
Exon 1B
Exon 1A3
Exon 1J
Exon 1H
Exon 1F
Exon 1E
Exon 1C
Exon 1B

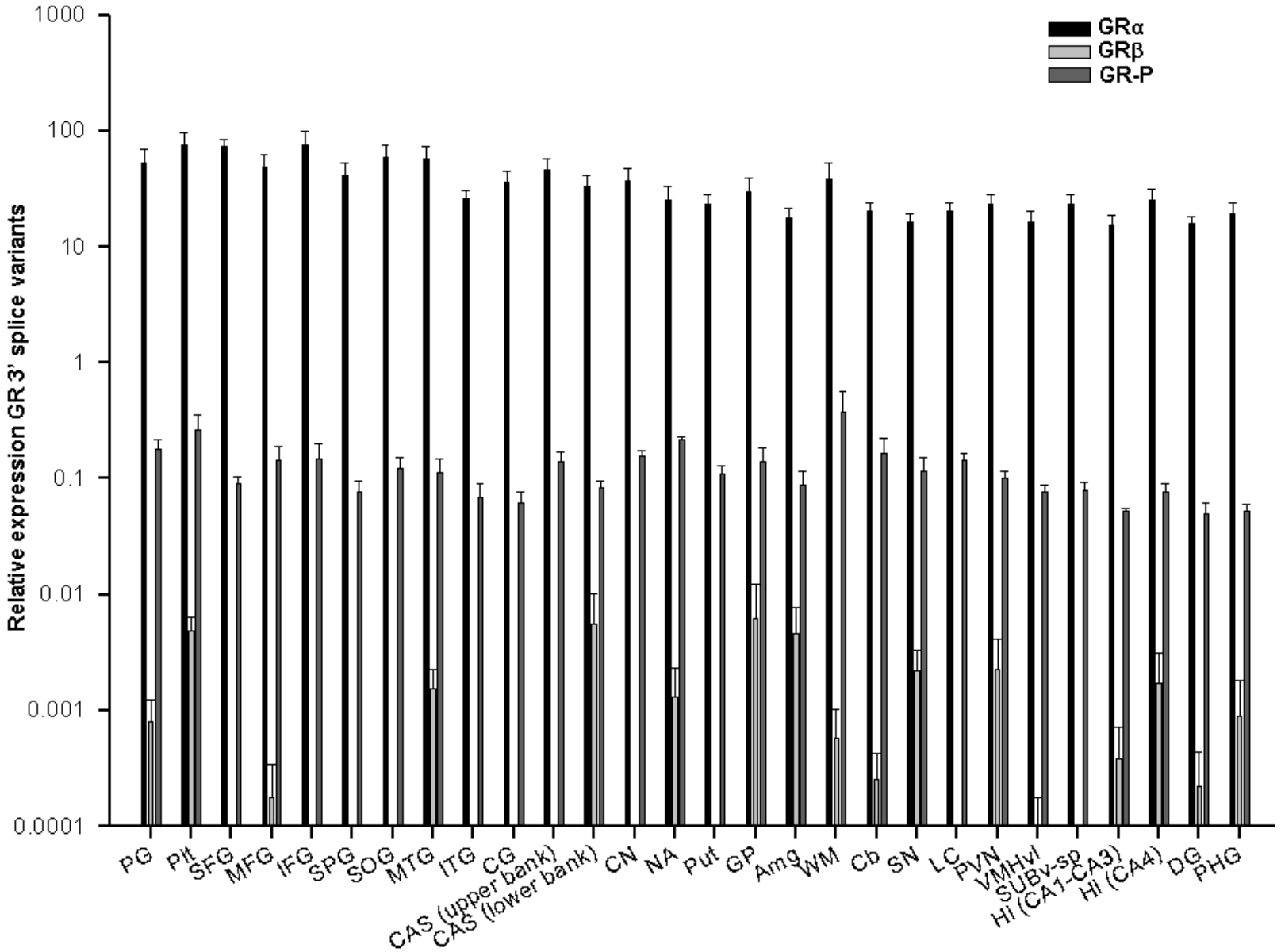


Figure 21. The *GR* 3' splice variants expression in 28 human tissues. Relative mRNA expression of all GRα, GRβ and GR-P normalised to β-actin as $2^{-(\Delta Ct)}$. Results are shown as the mean±SEM.

5.4.3 Relative expression of total GR and MR mRNA

Total GR and MR were detected in all 28 human tissues and GR expression was always higher than MR (Figure 22a). The highest GR/MR ratio was found in pituitary gland and cerebellum with at least 4 times more GR mRNA than MR. In the pineal gland, ventral subiculum pyramidal layer and hippocampus (CA1-CA3 and dentate gyrus), the GR/MR ratio was below 2. Elsewhere, the GR/MR ratio was between 2 and 4. The pituitary gland had a statistically significant difference with pineal gland, superior occipital gyrus, middle temporal gyrus, inferior temporal gyrus, putamen, globus pallidus, amygdala, white matter, substantia nigra, ventral subiculum pyramidal layer, hippocampus (CA1-CA3), hippocampus (CA4), dentate gyrus and parahippocampal gyrus. A statistically significant difference was also observed in cerebellum as compared to pineal gland, ventral subiculum pyramidal layer, hippocampus (CA1-CA3) and dentate gyrus. MR and GRα expression were positively correlated ($r = 0.714$, $P < 0.001$) as shown in Figure 22b.

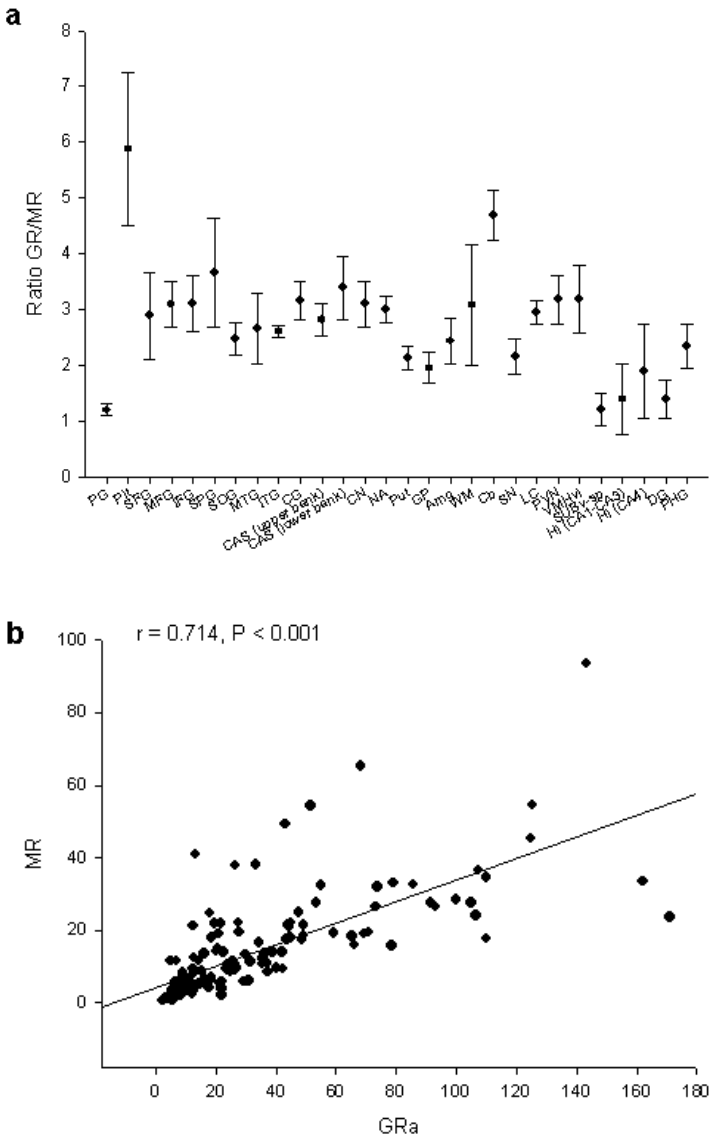


Figure 22. (a) The GR/MR ratio in 28 human tissues. Results are shown as the mean ± SEM. (b) The correlation between MR and GRα. Pearson's correlation coefficient was shown after Bonferroni correlation.

5.4.4 Association between promoter usage and *GR* 3' splice variants

To investigate the association between promoter usage and alternative splicing of the *GR* gene, correlations were examined between the first exons and the 3' splice variants. GRα positively associated with both exon 1B (r = 0.661, P < 0.001) and exon 1A3 (r = 0.610, P < 0.001) (Figure 23a, b). A high positive correlation was observed between GRα and exon 1F (r = 0.873, P < 0.001) (Figure 23c), and a low positive correlation was found between GRα and exon 1C (r = 0.474, P < 0.001) (Figure 23b). GR-P had a low positive correlation with both exon 1F (r = 0.411, P < 0.001) and exon 1C (r = 0.424, P < 0.001) expression respectively (Figure 23e, f). No positive correlation was found between GRβ and any of the first exons.

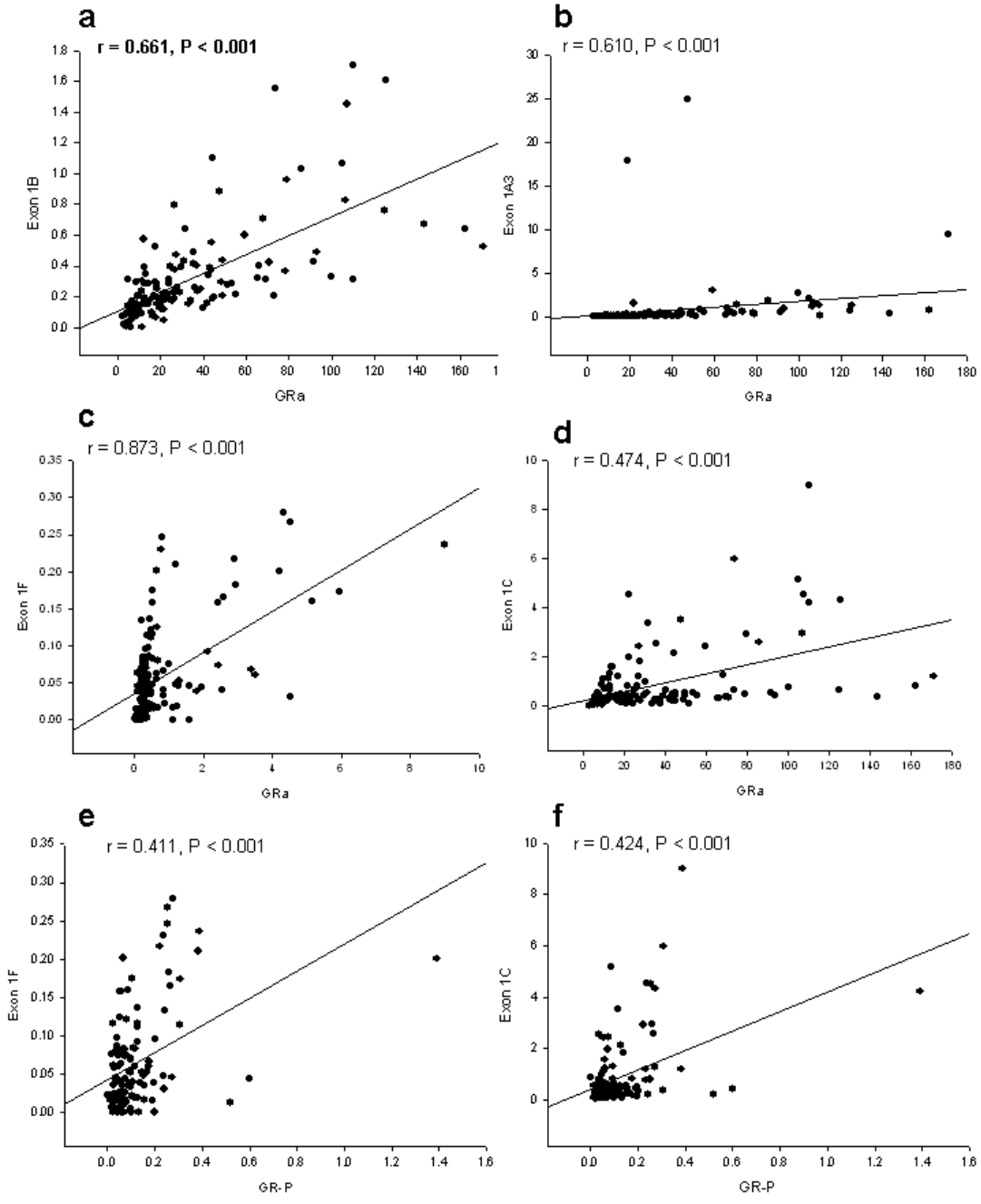


Figure 23. Pearson correlations between mRNA expression levels and *GR* 3' splice variants, *GR* first exon transcripts. Pearson's correlation coefficient was shown after Bonferroni correlation.

5.4.5 CpG Methylation analysis

Due to the difficulties in amplifying long stretches of identical nucleotides after bisulphite modification, it was not possible to get a complete sequence for some promoters. By pyrosequencing, we analysed the methylation degree of 21 CpG sites in promoter 1D, 12 in the region comprises part of promoter 1J, exon 1J and promoter 1E, 22 in promoter 1F and 42 in promoter 1H. Within five donors, the methylation level was variable in different human tissues.

For all five donors, methylation of individual CpG dinucleotides was low, never exceeding 25% in any of the 28 human tissues. Figure 24a shows the methylation level of individual CpG sites in three hippocampal regions and the paraventricular nucleus of the hypothalamus. The methylation of the CpG sites in these promoters was very low (< 25%) or undetectable in the five donors in these tissues. The other regions showed similar methylation levels (Figure 24b-g).

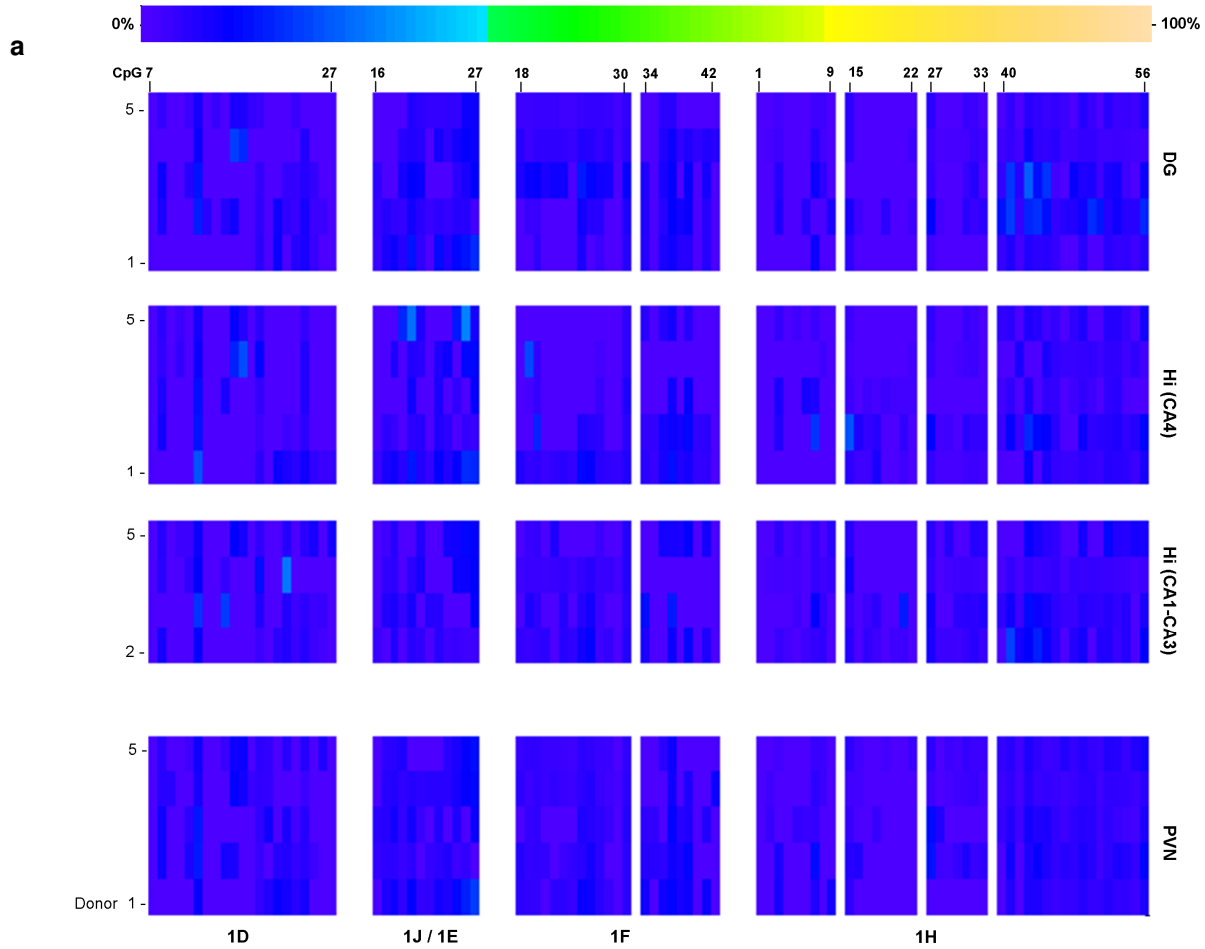
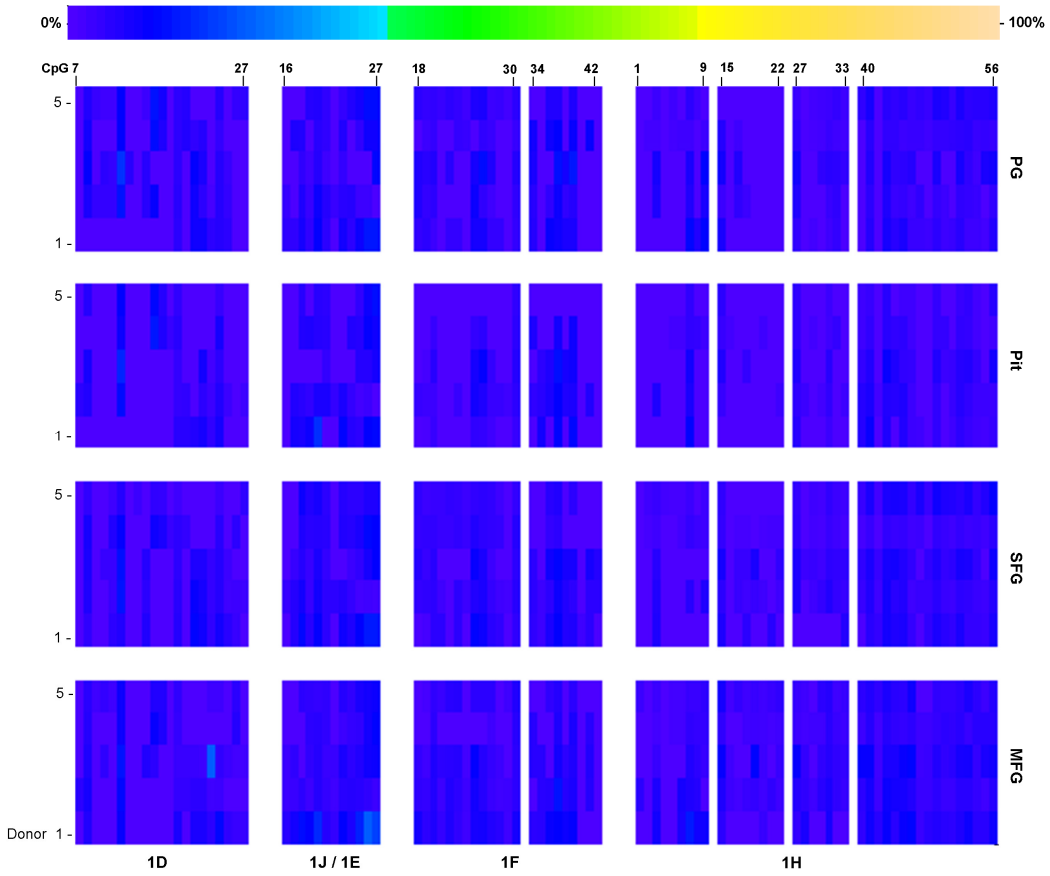
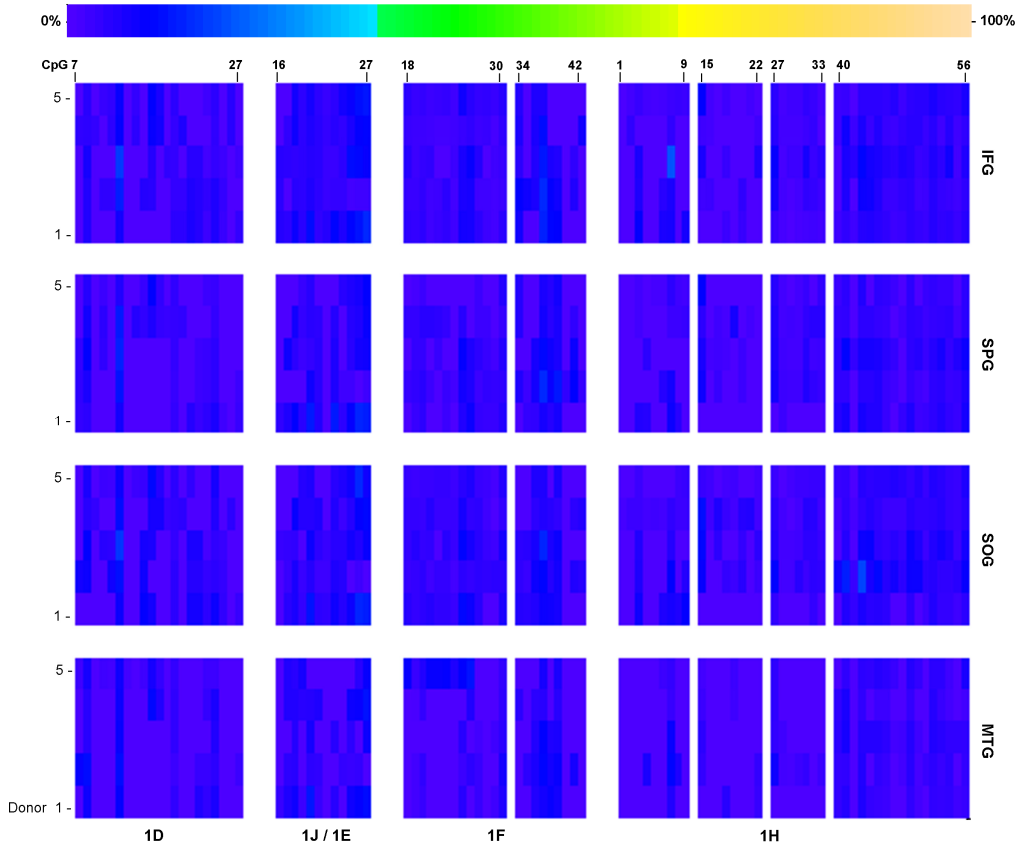


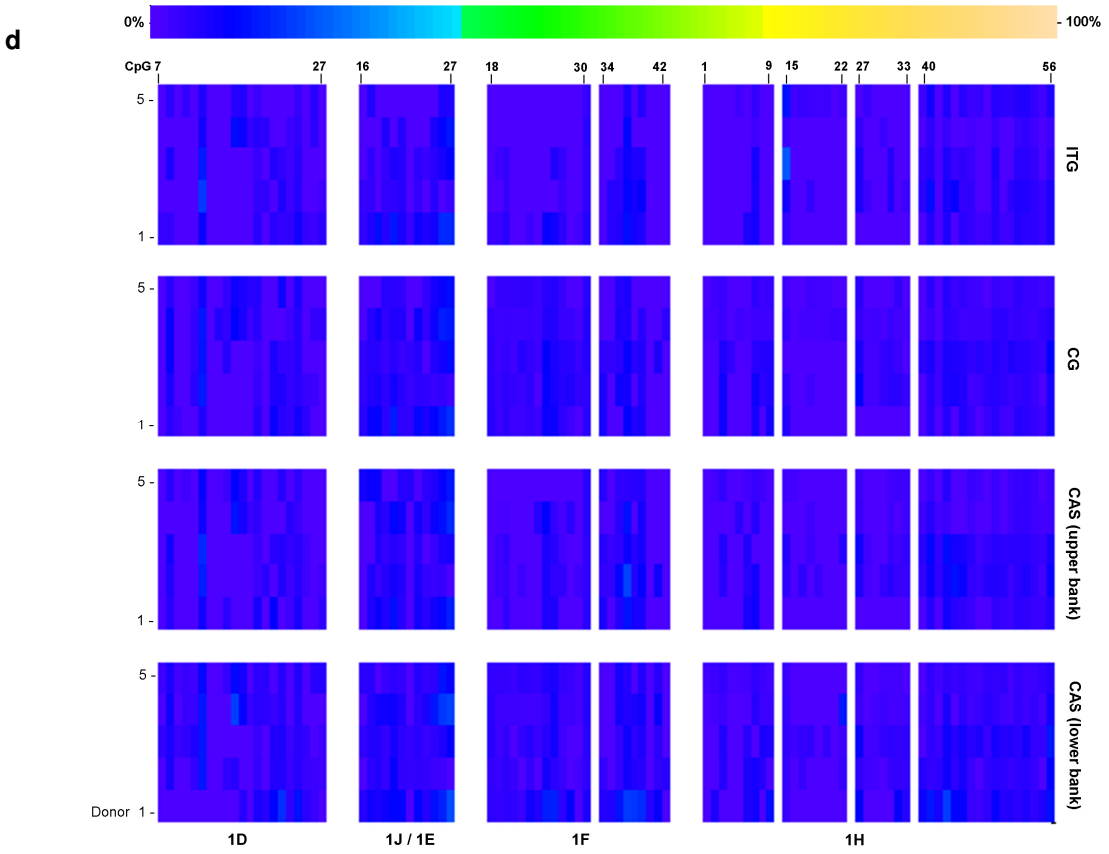
Figure 24. *(pages 101-104)* Heat map of DNA methylation profile from 28 human tissues (a-g). A key for the blue scale is given above the map.

b


0%
100%
CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
5
1
PG
Pit
SFG
MFG
Donor 1
1D
1J / 1E
1F
1H

c


0%
100%
CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
5
1
IFG
SPG
SOG
MTG
Donor 1
1D
1J / 1E
1F
1H


d
0%
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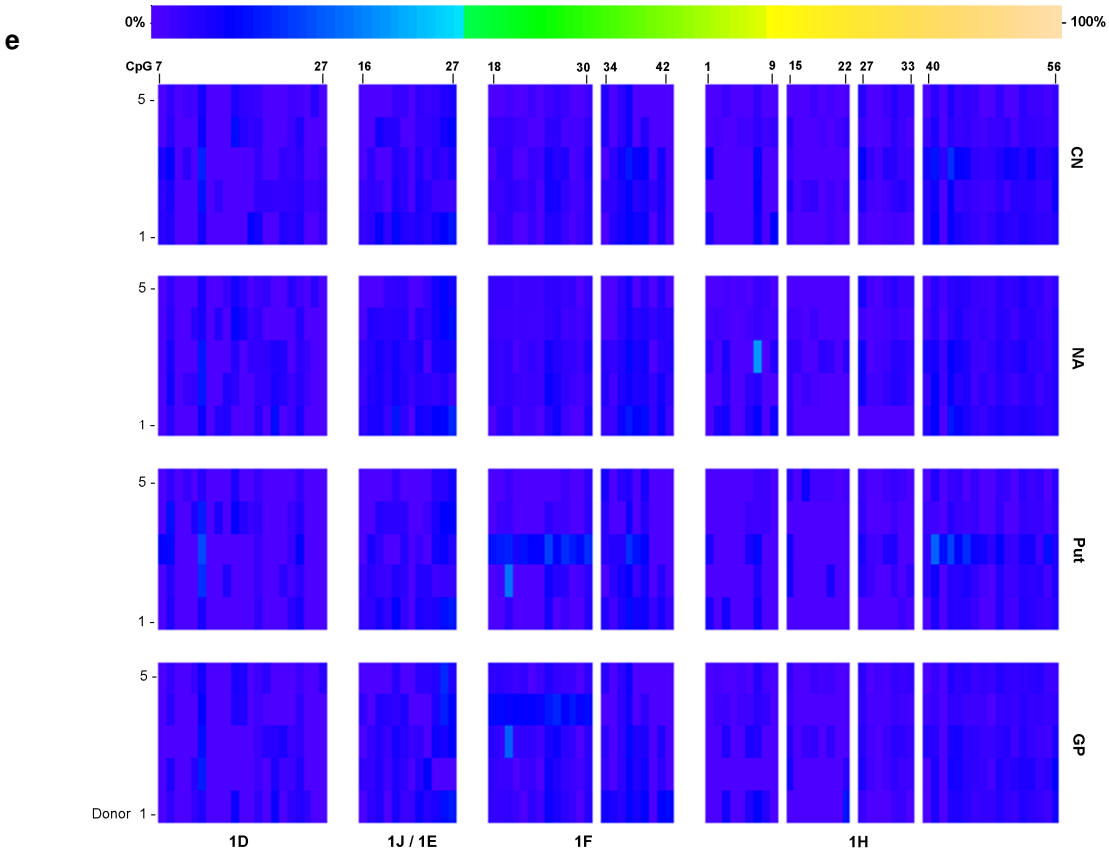
CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
5
1
ITG
CG
CAS (upper bank)
CAS (lower bank)
Donor 1
1D
1J / 1E
1F
1H


e
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CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
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CN
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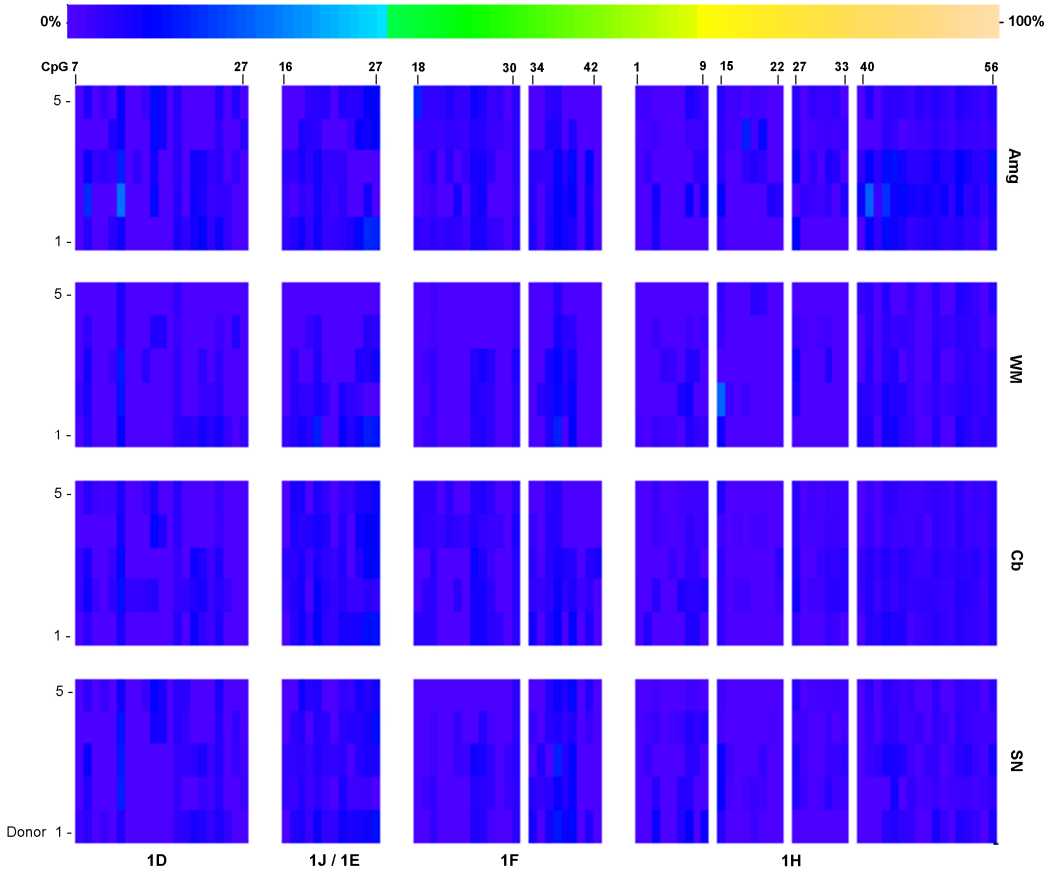
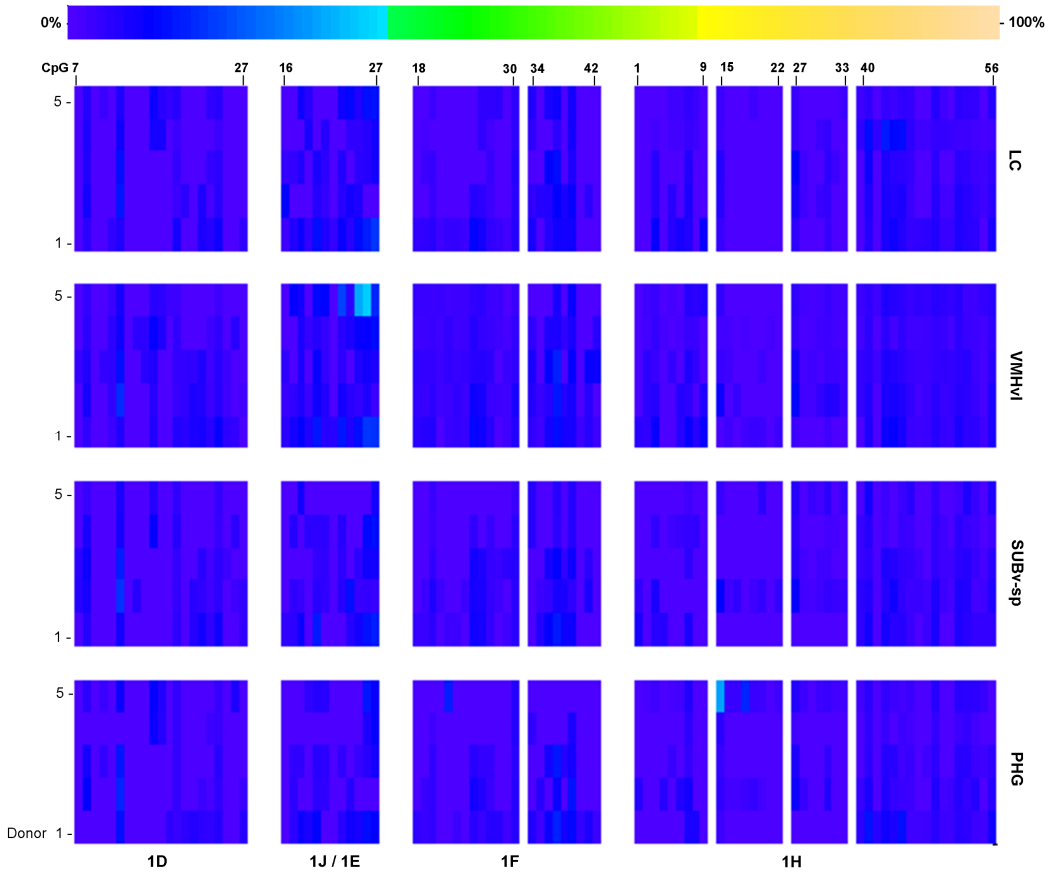
GP
Donor 1
1D
1J / 1E
1F
1H

f


0%
100%
CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
5
1
Amg
WM
Cb
SN
Donor 1
1D
1J / 1E
1F
1H

g


0%
100%
CpG 7 27 16 27 18 30 34 42 1 9 15 22 27 33 40 56
5
1
LC
VMHvl
SUBv-sp
PHG
Donor 1
1D
1J / 1E
1F
1H

For each promoter, average methylation levels at each CpG position for the 28 tissues were highly variable. Average of coefficient of variation in all CpG sites was 1.77 in promoter 1D, 0.81 in promoter 1J/1E, 1.34 in promoter 1F and 1.26 in promoter 1H. In all cases, methylation levels were below 12% (Figure 25a-d). Analysing the sum methylation of all CpG sites throughout each promoter for all 28 tissues (Figure 26), the methylation sum of promoter 1D showed a large variability amongst the tissues (Figure 26a). The inferior temporal gyrus had a significantly higher methylation level than that of the inferior frontal gyrus, cerebellum, locus coeruleus and parahippocampal gyrus. Between superior parietal gyrus and cerebellum, there was a significant difference of promoter 1D methylation degree. In promoter 1J/1E, inferior temporal gyrus was significantly highly methylated than ventromedial hypothalamus (ventrolateral) (Figure 26b). In promoter 1F, superior parietal gyrus showed a significantly higher methylation level compared to calcarine sulcus (lower bank) and cerebellum (Figure 26c). In promote 1H, the level of methylation in pineal gland had a significantly higher level than that of superior frontal gyrus, inferior frontal gyrus and amygdala (Figure 26d). We investigated the total CpG dinucleotides methylation level of the sequenced region, cerebellum showed a significant lower methylation level compared to superior parietal gyrus and inferior temporal gyrus (Figure 26e).

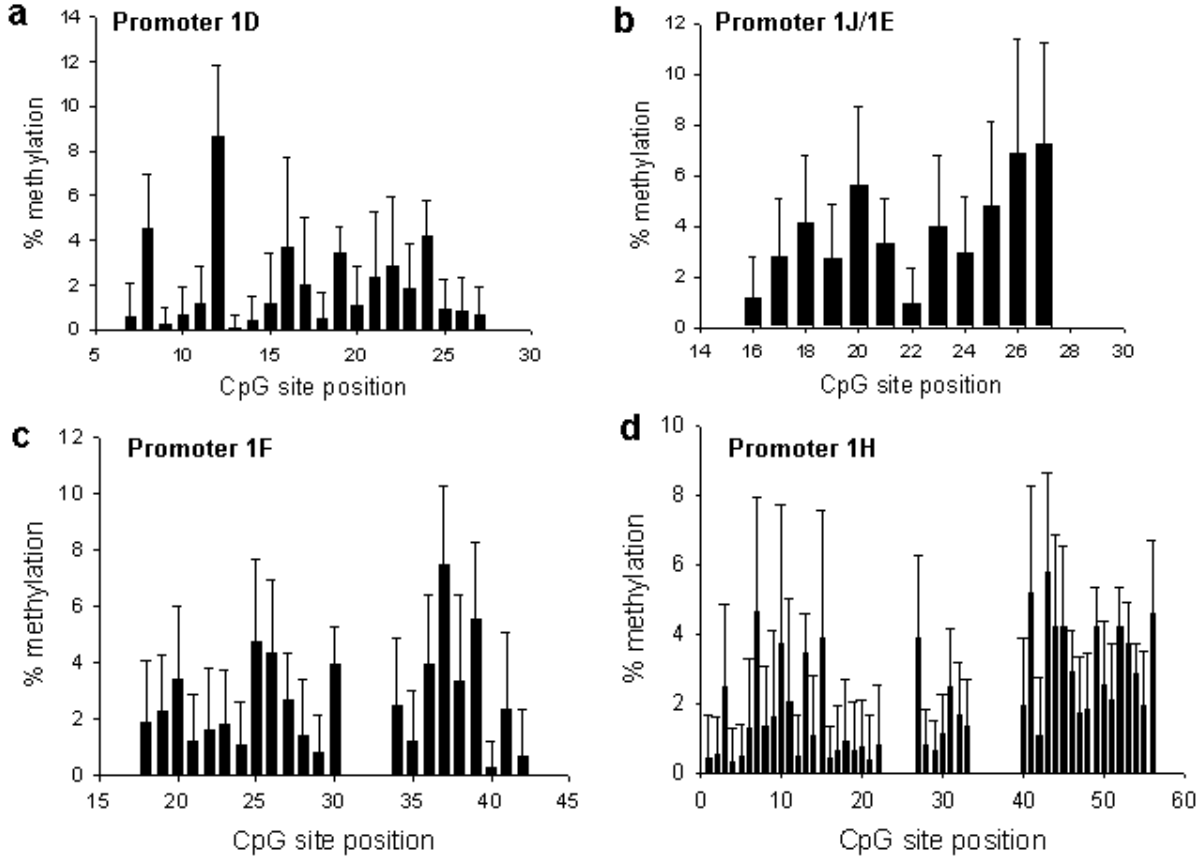


Figure 25. Percentage of the methylation level of individual CpG dinucleotides in 28 human tissues. Results are shown as the mean ± Stdev.

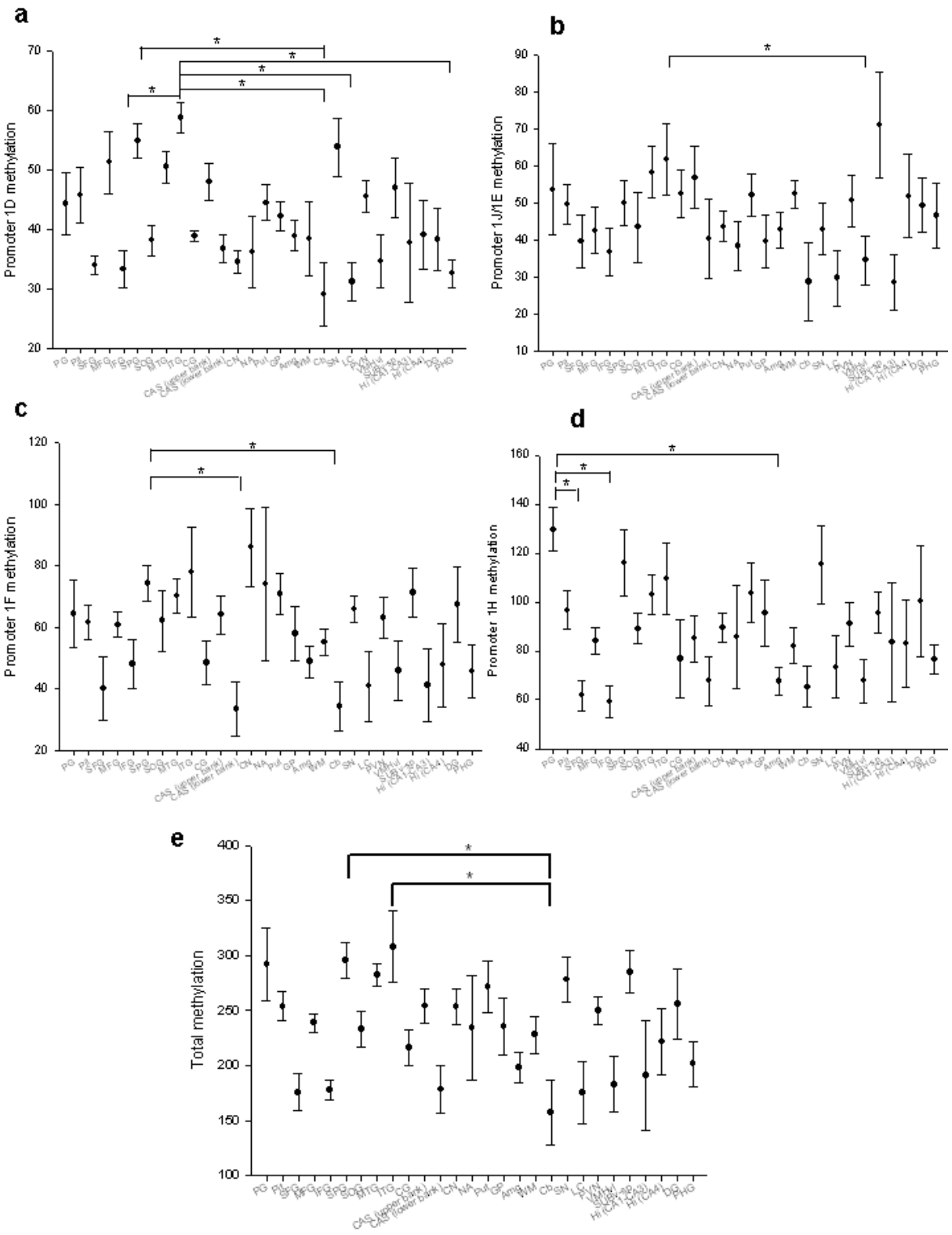


Figure 26. The methylation pattern of the sum of total CpG sites methylation in each promoter and total methylation level in 28 human tissues. * P < 0.05. Results are shown as the mean ± SEM.

5.5 Discussion

The aim of this study was to investigate the *GR* first exon transcripts and *GR* 3' splice variant distribution in human healthy brain regions and the methylation status of their associated promoters. Here, we showed human *GR* first exons do not have a region specific expression pattern, and that *GR* 3' splice variants (GRα and GR-P) were equally distributed throughout all the brain regions. However, the GR/MR ratio showed significant differences, with the highest GR/MR ratio in the pituitary gland. Methylation levels of individual CpG dinucleotide throughout GR promoters were low in different human organs although there was significant heterogeneity between the donors.

The GR is ubiquitously expressed in human brain. None of the *GR* 3' splice variants (GRα, GRβ and GR-P) showed significant difference in any of the tissues investigated here. This is in line with a previous study (Alt et al. 2010) where we observed similar *GR* 3' splice variant expression in five regions of the limbic system *post mortem* (inferior frontal gyrus, cingulated gyrus, nucleus accumbens, amygdale and the hippocampus subfields (CA1-CA3, CA4 and dentate gyrus)) from six non-depressed donors. Here, we observed a similar constant GRα: GR-P ratio and a significant positive correlation between the two splice variants, although the ratio was ~ 1000 time lower than in Alt et al. (2010). Reduced GRα and GRβ ratio has been reported to be associated with mood disorders (Matsubara et al. 2006; Perlman et al. 2004), however, the expression of the GRβ splice variant was very low or undetectable and when it was detected represented <0.01% of the total GR. This concords with observation from several other studies (Alt et al. 2010; de Lange et al. 2001; DeRijk et al. 2003; Russcher et al. 2007), suggesting that GRβ had a limited functional role in human brain.

MR was detectable throughout the brain region and pituitary gland, here we showed that GR/MR ratio varied between the different tissues. Klok et al reported that GR/MR ratio had no significant differences in five limbic regions from non-depressed subjects (Klok et al. 2011), a result that we confirmed here. A higher GR/MR ratio was observed in both the pituitary gland and cerebellum. In both cases, GR levels were similar to the surrounding tissues, and MR levels lower, implying a decreased sensitivity to nadir GC levels and to mineralocorticoids. The inferred increase in sensitivity to high GC levels in the pituitary gland agrees with its role in mediating vulnerability to chronic stress (Wagner et al. 2011) and its role in the GC negative feedback loop (Schmidt et al. 2009). High cerebellar GR level has also been reported (Morimoto et al. 1996; Pavlik and Buresova 1984). Here, the lowest GR/MR ratio was observed in the pineal gland, ventral subiculum pyramidal layer and the three hippocampal subfields. MR is known to be highly expressed in the hippocampus (Klok et al.

2011) and the GR/MR ratio is relatively low and here we observed equivalent amounts of GR and MR mRNA. The pineal gland produces the melatonin which is reported to affect sleep, circadian rhythm and seasonal cycles (Macchi and Bruce 2004), the low ratio GR/MR implies that MR is involved in these basic physiological function.

Alterations in the MR/GR balance may be one of the mechanisms by which stress may trigger depressive episodes (de Kloet et al. 1998). Lower MR levels have been found in brain from MDD patients (Klok et al. 2011) and depressed subjects who committed suicide (Klok et al. 2011; Lopez et al. 1998). In our study, MR in the five healthy donors highly correlated with the functional GRα in all 28 human tissues suggesting that both important functions on cortisol in human brain.

All the GR first exon transcripts showed similar expression pattern. As in many tissues and cell lines investigated (Alt et al. 2010; Johnson et al. 2008; Russcher et al. 2007; Turner and Muller 2005), exon 1C was the predominantly expressed exon in the human brain. No significant differences were observed between GR first exons in all 28 human tissues except exon 1A3. There was a striking higher level of exon 1A3 in the pituitary gland than the other tissues. Previously, exon 1A3 expression had been observed in cells of the hematopoietic lineage and in the adrenal gland (Presul et al. 2007; Russcher et al. 2007) and transcripts containing exon 1A3 preferentially translate GR protein from the second ATG codon in the mRNA GR-B (Lu et al. 2007; Pedersen et al. 2004): The GR-B isoform is more active than GR-A and induces a different set of target genes, as such, the higher expression of exon 1A3 in pituitary gland we observed, as well as the adrenal gland would suggest an increased GC sensitivity and a specific genomic response in these tissues. In all 28 human tissues there was no clear region specific alternative GR promoter usage as originally observed for a wide range of tissues (Turner and Muller 2005). The relatively stable expression levels of the GR first exons were mirrored in the low variability in methylation levels between tissues. Methylation of CpG dinucleotides within the individual GR promoters is thought to play a central role in alternative first exon expression as well as influencing total GR levels. However, there was no statistical link between methylation levels of the individual promoters and the expression of their associated first exons. Although the methylation patterns were highly individualised among donors, the highest methylation level of individual CpG site never reached 25%. The low methylation level observed concords with our previous report of such low levels in the limbic region of non-depressed donors (Alt et al. 2010). Overall, this suggests that GR promoters are poorly methylated throughout the brain, and have very little role in controlling alternative first exon usage.

The sum of total CpG sites methylation throughout the complete promoter showed that the methylation levels were significantly different between several tissues, especially the cerebellum, which is involved in both cognition and affect and connects with brain areas associated with emotional processing, including the limbic system (Schmahmann and Pandya 1997) and the prefrontal cortex (Ramnani 2006), concordant with the higher GR/MR ratio observed in cerebellum in our study. However, no significant correlation was found between the total methylation of the CpG island and total GR levels for the 28 tissues. Since we did not sequence all the CpG sites in the CpG island, this prevents from concluding that the overall methylation level does not influence total GR levels, although it does suggest it. In addition, GR gene expression may be influenced by other factors such as gene variation. The weakness of the present study is the limited number of the cohort, we can not further investigate the pattern of age-related changes in GR mRNA levels which has been investigated in several studies (Matthews 1998; Perlman et al. 2007; Pryce 2008).

Concomitant to the lack of region specific expression are the correlations between the alternative first exon levels. Such correlations imply that all of the GR first exons are regulated in a similar manner in these tissues, and that each represents a fixed proportion of the total GR expression.

The associations between promoter usage and 3' splice variants have been investigated in the present study. The GRα expression was associated with expression of exon 1A3, this is in agreement with exon 1A usage was associated with increased translation efficiency of GRα (Pedersen and Vedeckis 2003). Positive significant correlations between GRα and exon 1C have been also proved in human tissues and cell lines (Russcher et al. 2007) suggesting the expression of GRα is preferentially regulated by promoter 1C. GR-P expression was related to usage of exon 1B (Russcher et al. 2007), however, here we only found that GR-P had a positive correlation with exon 1F and exon 1C. Our study is in line with the finding showing that exon 1C was the preferential promoter for GRα and GR-P (Johnson et al. 2008). Interestingly a negative correlation between exon 1C and GR-P was reported in MDD patients (Alt et al. 2010). In contrast, the GR-P expression did not correlated with exon1C in non-depressed control donors.

Whilst there was very little effect of the overall methylation level on the first exon expression, methylation within each promoter was variable. In promoter 1D, the highest methylated CpG was found in position 12 with 8% methylation level average in all tissues. CpG^{16} and CpG^{17} locate in the YY1 binding site had < 25% methylation in agreement with our previous observation in PBMCs (Turner et al. 2008). However, the methylation level of $CpGs^{18\text{-}20}$ was below 10% in all the donors

whilst in PBMCs it was more variable with 3 of 20 donors having 25%-50% methylation. In the sequence of 269 bp covering part of promoter 1J, exon 1J and promoter 1E, CpGs$^{26-27}$ immediately upstream of the exon 1E transcription start site were methylated to a greater extent (~ 7% on average) than the other positions within promoter 1E, although the CpG^{27} was observed in PBMCs with a much higher level (>25%) in at least 25% of donors (Turner et al. 2008). Low methylation level in this region was also observed in non-depression and MDD patients (Alt et al. 2010). Promoter 1F has been well studied due to the susceptibility to methylation (McGowan et al. 2009; Weaver et al. 2004). The human NGFI-A includes two CpG sites (CpG$^{41-42}$), the majority of these CpG sites were unmethylated in all 28 tissues from five donors, fully concording with the previous human brain (Alt et al. 2010; Moser et al. 2007) and PBMC reports (Turner et al. 2008). However, the methylation status in central tissues does not seem to completely reflect the methylation status in peripheral tissues. The highest methylation level of individual CpG dinucleotide was < 25% in promote 1H, the highest methylation level of CpG^{41} reached ~ 20%, however, up to 75% methylation of CpG^{41} was observed in PBMCs (Turner et al. 2008). This difference might be due to the sensitivity of the different techniques used for methylation quantification, but also might be due to the small size of samples because high methylation level were found only in one of the 20 donors (Turner et al. 2008), here we have only five donors. The relationship between methylation of blood and brain DNA is not well known although there were studies suggesting that the catechol-O-methyltransferase promoter methylation status and between blood and brain were similar in rat (Ursini et al. 2011) and human (Murphy et al. 2005).

In summary, the results of the current study indicate that the human GR first exon was not region specific, and the high correlations amongst several first exons were suggest they are co-regulated. The 3' splice variants (GRα and GR-P) were equally distributed in all the brain regions and GRβ expression was low. GR/MR ratio showed significant difference between the 28 tissues with the highest GR/MR ratio in pituitary gland. Methylation levels of individual CpG dinucleotide were low although there was significant heterogeneity between the donors. The sum of total CpG sites methylation in each promoter and total methylation level in each human tissue showed significantly differences, however, no link between methylation level and gene expression was found. To our knowledge, for the first time the current study showed GR first exons expression and overall human brain. Our study adds evidence that a similar GR promoter usage and a similar first exon transcripts throughout the peripheral tissues (Turner and Muller 2005) and brain (Alt et al. 2010).

CHAPTER 6

General Discussion

In this thesis, both genomic and environmental factors linked with human GR expression are covered. As *GR* promoter usage is largely tissue- and cell- type specific, SNPs and methylation levels in promoter regions may have tissue-specific functional consequences. Thus, with each alternative exon having its own promoter, differential promoter methylation and gene variation provided a mechanism for regulating the activity of each promoter individually, and in a cell- and tissue- specific manner.

6.1 Transcription control of GR

GCs regulate a number of developmental and physiological processes by controlling the transcriptional activity of genes in target tissues. GR levels are critical for obtaining the required GC response, and are thought to be controlled at the transcription level. Transcriptional regulation of the GR is thought to depend on the use of alternative first exons and their associated promoters. The *GR* alternative first exons have been explored in several studies. From the initial cloning of the receptor in 1986 until 2005 three first exons (1A, 1B and 1C) were known. In later studies, this was then increased to 9 first exons (1A, 1I, 1D, 1J, 1E, 1B, 1F, 1C and 1H) (Presul et al. 2007; Turner and Muller 2005).

In this thesis, we started exploring the transcriptional control of the GR by investigating the activity of seven regions in the CpG island that were thought to be proximal promoters for the individual first exons (Chapter 2). We confirmed the hypothesis that each of these regions had promoter activity, and that each alternative first exon had its own promoter directly upstream. The promoter activity mirrored the previously reported first exon transcript abundance data. Promoter 1C had the highest promoter activity in almost all the cell lines investigated coinciding with exon 1C having the highest expression in the different tissues and cell lines (Alt et al. 2010; Johnson et al. 2008; Presul et al. 2007; Russcher et al. 2007; Turner and Muller 2005). Although the ten cell lines used in our study did not represent the entire panoply of human tissues, our data reflected the transcription activity of GR first exons in several human organs such as brain, cervix, kidney, liver and blood. Since GR plays a critical role in the HPA negative feedback loop, we expanded this by examining the first exon distribution in 27 brain tissues as well as the pituitary gland from healthy donors (Chapter 5). As expected, the highest expression was found for exon 1C, suggesting the promoter 1C may be considered as constitutively active whereas the other promoters tend to be more tissue-specific. Exon 1C has been reported to preferentially generate expression of GRα which is the most active GR isoform (Johnson et al. 2008;

Russcher et al. 2007). To investigate the relationship between *GR* alternative promoter usage and *GR* 3' splice variants expression in brain, we examined the Pearson correlation between *GR* first exon transcripts and 3' splice variant. The GRα expression was associated with expression of exon 1A3, exon 1C, exon 1B and exon 1F. GR-P had a positive correlation with exon 1F and exon 1C. Our study is in line with the finding showing that exon 1C was the preferential promoter for GRα and GR-P (Johnson et al. 2008). Consistent with the previous finding (Johnson et al. 2008; Russcher et al. 2007) in different tissues and cell lines, our data suggest that *GR* alternative promoter usage is a potential mechanism influencing *GR* 3' splice variant distribution. As already described in chapter 5, the limitation of this study is the number of donors which precludes analysing effects of age on the effect of *GR* first exon and 3' splice variant expression. GR and MR are both involved in the negative feedback loop of the HPA axis. A higher GR/MR ratio was both observed in the pituitary gland and cerebellum, the lowest GR/MR ratio was observed in the pineal gland, ventral subiculum pyramidal layer and the three hippocampal subfields.

6.2 Gene variation on GR

SNPs can influence gene expression by affecting promoter activity, transcription efficiency, gene splicing, mRNA stability and translation efficacy (Kimchi-Sarfaty et al. 2007). In regulatory regions, functional SNPs have been shown to alter the promoter activity and transcription levels. It has been reported that promoter SNPs were found in ~ 35% of genes by screening a sub-set of 16 chromosomes (Hoogendoorn et al. 2003), although for the vast majority of these genes, these were predicted rather than confirmed promoter regions.

Numerous *GR* SNPs are known to alter the physiological stress response and are associated with MDD (DeRijk and de Kloet 2005; Kumsta et al. 2007; Wust et al. 2004). For instance, R23K was associated with MDD in a Swedish cohort (van West et al. 2006). Similarly, the association between the *BclI* G allele and R23K alleles significantly increased risk of MDD (van Rossum et al. 2006). Moreover, Zobel et al (Zobel et al. 2008) discovered that promoter SNPs rs10052957 and rs1866388 were involved in unipolar depression. Therefore, genetic variation in *GR* might be one of the factors which influence HPA axis dysfunction and alter the risk for MDD. There is also now evidence that a specific *GR* haplotype may be involved in the pathogenesis of such diseases (Otte et al. 2009; van

Rossum et al. 2006; van West et al. 2006; Kumsta et al. 2009; Rajeevan et al. 2007; van Winsen et al. 2009; Lahti et al. 2011).

As described in chapter 2, several SNPs have been identified in *GR* in which NR3C1-1 was located in the CpG island promoter regions, whilst *TthIII*I was located outside of the CpG island. We screened the seven *GR* proximal promoters for sequence variants in 221 donors and cloned the SNPs into a luciferase reporter gene system to analyse their ability to drive transcription in a reporter gene system. In our study promoter constructs including SNPs showed a lower reporter gene activity than the wild type, a reduction that was cell- type specific. Thus, SNPs affect the functioning of important promoters, probably by lowering specific transcripts in a tissue-specific manner, potentially leading to increased disease susceptibility. However, the associated levels of first exon transcripts and 3' splice variants as a measure of *in vivo* promoter activity of genotypes with and without the SNPs were unfortunately not quantified from the genotyped donors. Several SNPs were associated with the addition or deletion of *in silico* predicted transcription factor binding sites. In future detailed examinations of these promoters, it would be interesting to analyse the effect of the functional SNPs on DNA-protein interaction by performing techniques such as ChIP, DNA footprinting or gel shift assays, although such techniques would require a large time investment. Two new CpG dinucleotides sites which are potentially susceptible to methylation were introduced by SNPs located in promoters 1C and 1B which are widely expressed. The methylation introduced by these SNPs could alter the putative transcription factor binding efficiency. In addition, methylated DNA can recruit methyl binding domain proteins (MBDs), such as methyl-CpG-binding protein 2 (MeCP2) and facilitate histone deacetylation (Tsankova et al. 2007). Thus, it would be highly interesting to identify the potential methylation status by bisulfite treatment followed by pyrosequencing. However, our data from the brain now leads us to suggest that the introduction of an additional CpG site would have a limited effect on GR levels and function.

We hypothesized that there would be a cumulative effect on this promoter haplotype including several function SNPs in different human tissues based on the alternative first exon usage. We analysed the correlation between this promoter haplotype 4-2 and several phenotypes of 221 donors including salivary and serum cortisol concentration, ACTH responses, dexamethasone suppression test, cortisol awakening response and skin blanching. However, only cortisol awakening response in this haplotype 4-2 showed significant difference in sex (P= 0.03) (Cao-Lei et al, unpublished observation).

6.3 Epigenetic regulation of GR promoters

Apart from gene variation, epigenetic modification plays a critical role in regulating GR expression. Environmental factors including postnatal handling, diet and variations in maternal behaviour such as licking/grooming (LG) that the pups received directly affect the HPA axis responses to stress and behavioural development (Caldji et al. 1998; Francis et al. 1999; Liu et al. 1997; Menard et al. 2004; Roth et al. 2009; Toki et al. 2007; Weaver et al. 2004; Weaver et al. 2005; Zhang et al. 2006). HPA axis changes induced variation in maternal care is associated with altered hippocampal GR expression. Similarly, increased hippocampal GR expression was found after artificial tactile stimulation of rodent pups (Jutapakdeegul et al. 2003). The molecular mechanism involved in this environment effect has been reported by Meaney's group. They demonstrated that maternal care increased serotonin (5-HT) activity in the hippocampus, enhancing the cAMP/PKA signalling pathways, resulting in increased NGFI-A expression, itself increasing GR expression (Laplante et al. 2002; Meaney et al. 2000; Mitchell et al. 1992; Mitchell et al. 1990; Weaver et al. 2007). Weaver et al. demonstrated that the Nr3c1 promoter methylation pattern was dependent on maternal care and the NGFI-A binding site was highly methylated in pups received less maternal care (Weaver et al. 2004). Based on this finding, numerous studies concerning epigenetic modification of the GR promoter subsequently emerged in both humans and rodents. However, several findings were in conflict with the finding of Weaver et al (Weaver et al. 2004). Neither in rats fed with a methyl-supplemented diet (Herbeck et al. 2009) nor maternal separation stressed rats (Daniels et al. 2009) was there a significant effect on *GR* promoter 1_7 methylation levels. Likewise, the NGFI-A binding site in *post mortem* human hippocampal tissue from a wide range of neurological disorders was uniformly unmethylated (Moser et al. 2007), and no methylation of the NGFI-A binding site was found in either brains of patients with MDD or their associated controls (Alt et al. 2010). Thus, the poor correlation observed between promoter methylation and altered exon 1F expression levels suggest that other mechanisms play a role. However, for humans, high methylation levels in an alternative hypothetical NGFI-A binding site was observed in *post mortem* hippocampi of suicide victims and correlated with childhood abuse, suggesting that regulation may occur at different positions within the promoter.

In chapter 3, we focused on the investigation of the role of NGFI-A in the transcriptional regulation of the *GR in vitro*. In the rat, there is a functional NGFI-A binding site immediately upstream of the exon 1_7 transcript start site, however, the functionality of NGFI-A in the human had not been properly

investigated. We showed that NGFI-A poorly activated a human promoter 1F reporter gene and did not induce endogenous 1F transcripts although NGFI-A did bind to the promoter 1F. In chapter 4, after predicting the activity of six transcription factors by *ISPF,* we employed single CpG nucleotide methylation, covering the NGFI-A binding region (orthologous to rat Weaver et al. 2004) as well as the upstream E2F1 sites (identified as NGFI-A by McGowan et al. 2009), together with E2F1 overexpression, we demonstrated that E2F1 was involved in the regulation of promoter 1F. Two *bona fide* E2F1 binding sites were indentified on the GR promoter 1F, and one of the binding sites (containing CpG^{32}) has been previously considered as a potential NGFI-A binding site (McGowan et al. 2009) and was methylated in suicide victims with prior childhood abuse. We demonstrated that E2F1 was a key element of the transcriptional complex critical for the expression of GR 1F transcripts.

In chapter 2, we showed that all the proximal CpG island promoter regions were inactivated by complete CpG dinucleotide methylation *in vitro*, however, 100% methylation of all CpGs in a reporter gene promoter may not represent the physiological situation. Patch methylation of promoter 1F in which a completely methylated promoter was ligated into an unmethylated reporter gene backbone, reduced their activity to ~ 25% of the corresponding unmethylated sequence (McGowan et al. 2009). Weaver *et al* and McGowan *et al* suggested that the methylation of single CpG dinucleotide with NGFI-A binding sites had a critical role on blocking binding of transcription factor and further influenced exon 1F transcription activity. This is particularly interesting because if this is the case, the methylation on single CpG dinucleotide would modulate levels of the 1F transcript. As described above, our findings from single CpG nucleotide methylation experiment showed that neither independent methylation of CpGs nor simultaneous methylation of 2 or 3 CpGs was able to reduce the reporter gene activity. Since E2F1 was an essential transcription factor which plays a pivotal role in regulating the expression of genes involved in cell cycle (Dyson 1998; Fan and Bertino 1997; Inoshita et al. 1999), it would be detrimental if its target genes would be silenced by methylation of a few CpG dinucleotides in its promoter. Therefore, it seems unlikely that single CpG dinucleotide methylation can mediate the inhibition of transcription, although we do not know if high level of methylation at a single CpG dinucleotide methylation with those surrounding being poorly or unmethylated exists *in vivo.* Our data suggests that methylation levels throughout a promoter may be more important.

The methylation status of GR promoter 1F is well documented, however, exon 1F only represents ~ 1% total GR transcripts in the brain (Alt et al. 2010). Therefore, more attention must be paid to the

methylation status of the other *GR* alternative promoter regions. Turner et al (Turner et al. 2008) explored the *GR* promoter methylation status in human PBMCs demonstrating that methylation pattern and level were highly variable between individuals in promoter 1D, 1E, 1J, 1F and 1H. An important but unresolved question is whether methylation status of the peripheral blood reflects that of the brain? There are currently few data concerning the methylation status of GR alternative promoter regions due to the restrictions implicit in the investigation of *post mortem* brains. The level and pattern of methylation in five brain regions concerning the limbic system from six MDD donors and six health controls were reported by Alt et al (Alt et al. 2010). In chapter 5, we investigated the methylation pattern in 27 brain tissues as well as the pituitary gland, demonstrating that methylation patterns were highly individualised among donors and the methylation level was low, the highest methylation level of individual CpG site never reached 25% and suggesting that the GR promoters in CpG island in brain from healthy donors were poorly methylated. Our study currently represented the most complete picture of methylation status of GR alternative promoters in human healthy donors although limited by the small number of the cohort. However, we can not draw a clear conclusion from our study whether the methylation status in human central tissues if reflected by the methylation status of PBMCs. In PBMCs, the majority of positions had >25% methylation level in at least one donor and several CpG sites were over 50% methylation. This high methylation level and variability did not appear in our central tissues although this difference might be due to the sensitivity of the different techniques used for methylation quantification or the number of donors investigated. Further, studies investigating methylation pattern of peripheral tissues as well as distinct brain areas and how they correlate would be interesting and very informative, potentially allowing us to use peripheral levels as a valid readout of central levels.

6.4 Alternative promoters in humans

Since 2000, over 200 papers concerning the identification of alternative promoters have been published. In one data set, 9% of the mouse genes have been found to contain alternative first exons (Okazaki et al. 2002). In humans, large-scale studies to identify promoter regions suggested that 14%-58% of human genes may contain alternative promoters (Carninci et al. 2006; Cooper et al. 2006; Kim et al. 2005; Kimura et al. 2006; Landry et al. 2003; Sharov et al. 2005; Zavolan et al. 2002). Some putative alternative promoters have been shown to be functional (Trinklein et al. 2003). There is

growing evidence that alternative promoters are the key to understanding regulating gene expression and generating the complexity implicit in the elaborate mammalian molecular systems (Landry et al. 2003). High throughput screening techniques have shown that 17% of alternate promoters were detectable as being tissue specific, and the majority were found in CpG islands (Kimura et al. 2006). Recent annotations of the human genome suggested that almost half of the protein-coding genes contained alternative promoters (Davuluri et al. 2008), although the prevalence of alternative promoters within the genome, their biological roles and the regulatory mechanisms are still not clear.

In this thesis, transcriptional regulation of GR and its physiological role in the regulation of stress were studied in detail. Thus, the GR as a prototypical example may partly help us to understand the complex regulation mechanism of this phenomenon.

6.5 Integration of transcriptional, methylation and variant data- A future method for analysis complex traits

In this thesis, several layers of complexity in GR transcriptional regulation have been discussed. The GR alternative promoter usage was involved in regulating gene expression in a tissue- and cell- type specific manner, producing an array of different 5' splice variants, all encoding the same protein. The activity of the individual promoters was also modulated by sequence variants (SNPs) and methylation, both with tissues specific effects; SNPs because promoters are used in a tissue specific manner, and DNA methylation since it is tissue specific and the methylated promoters are used in a tissue- specific manner.

This combination of alternative promoters and their epigenetic regulation may in part explain why the haplotype 4-2 observed, with several promoter SNPs, only represents a small increase in risk for psychological disorders. On a genome wide scale, it is accepted that genetic variants identified in genome-wide association studies (GWAS) are generally poor predictors of complex traits (Ruiz-Narvaez 2011). The complex interplay of alternative first exons with the epigenetic regulation further complicates traditional functional locus analysis. In the absence of data on the inter- and intra-individual variability in first exon / promoter usage, and the ability of individuals to compensate for SNPs or methylation reducing the activity of one promoter by upregulating another, we are unable to correctly assess the importance of single SNPs. This may be part of the explanation for the poor performance of GWAS, in identifying complex traits, suggesting that as the number of genes with

alternate first exons continues to increase, the integration of expression data with allele data may be necessary for valid GWAS. Our data suggests that the GR represents a suitable candidate to attempt such integration, using complex traits such as MDD as a model.

In summary, data in this thesis covers the transcriptional regulation of the GR at several levels. Elements controlling GR transcription are identified, and our data suggest that, as the number of genes with known / identified alternative first exon increases we need to integrate data on alternative promoter usage together with SNP functionality to be able to understand complex traits.

6.6 Perspectives

The work presented in this thesis, as well as describing in detail promoter 1F, and more generally the *GR* CpG island has also raised several questions, and points for further investigation. Our data suggest that for genes with many alternative first exons, it is necessary to integrate alternative first exon expression, DNA methylation data and SNP frequency before trying to establish an association with a disease. This represents an interesting but not insurmountable challenge. It would be interesting, using model genes like the *GR* to build a mathematical expression model incorporating these elements. Such a model should be able to predict tissue specific effects of any of the parameters investigated. Such investigation should improve significantly the validity of association studies for complex traits or diseases.

Data presented in this thesis brings into question the exact role of DNA methylation in the transcriptional control of the *GR*. We have shown that single CpG dinucleotide methylation does not have a measurable effect on promoter activity. Similarly, promoter methylation levels (sum or individual CpG within the promoter) did not correlate with gene expression levels in the brain, however, all the reports to date show reliably low methylation levels in all the human tissues investigated. This raises the question of whether high methylation levels may be found for the GR, and if so, where, and under which conditions. If found, do they have measurable effects on GR transcription? If they have no effect then it is necessary to identify the exact role of methylation, and potentially investigate the role of other epigenetic markers instead.

Transcriptional control of the GR is not limited to the mechanisms described in this thesis. In the future, it will be necessary to build on and integrate into this work after mechanisms: such as microRNA control of transcription and translation. MicroRNAs have been reported to be involved in almost

cellular process investigated (Bushati and Cohen 2007; Kloosterman and Plasterk 2006). The 3' UTR of the GR contains predicted numerous seed regions (de Kloet et al. 2009) and MicroR-124a was reported to be able to bind in this region resulting decreased GR activity (Vreugdenhil et al. 2009). However, little is known about miRNAs targeting the 5' UTR of GR mRNA although a number of the miRNA binding sites were predicted within the GR first exons (Turner et al. 2010). Therefore, to indentify the miRNA targeting GR first exons would be helpful to understand the complex regulation mechanism of GR.

References

Alt SR, Turner JD, Klok MD, Meijer OC, Lakke EA, Derijk RH, Muller CP (2010) Differential expression of glucocorticoid receptor transcripts in major depressive disorder is not epigenetically programmed. Psychoneuroendocrinology 35: 544-556

Altshuler DM, Gibbs RA, Peltonen L et al. (2010) Integrating common and rare genetic variation in diverse human populations. Nature 467: 52-58

Auboeuf D, Dowhan DH, Kang YK, Larkin K, Lee JW, Berget SM, O'Malley BW (2004) Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. Proc Natl Acad Sci U S A 101: 2270-4

Bamberger CM, Schulte HM, Chrousos GP (1996) Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev 17: 245-61

Barrett TJ, Vig E, Vedeckis WV (1996) Coordinate regulation of glucocorticoid receptor and c-jun gene expression is cell type-specific and exhibits differential hormonal sensitivity for down- and up-regulation. Biochemistry 35: 9746-53

Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A, Buttgereit F (2004) Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. Faseb J 18: 70-80

Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16: 6-21

Bird A (2007) Perceptions of epigenetics. Nature 447: 396-8

Blank MC, Stefanescu RN, Masuda E, Marti F, King PD, Redecha PB, Wurzburger RJ, Peterson MG, Tanaka S, Pricop L (2005) Decreased transcription of the human FCGR2B gene mediated by the -343 G/C promoter polymorphism and association with systemic lupus erythematosus. Hum Genet 117: 220-7

Bockmuhl Y, Murgatroyd CA, Kuczynska A, Adcock IM, Almeida OF, Spengler D (2011) Differential Regulation and Function of 5'-Untranslated GR-Exon 1 Transcripts. Mol Endocrinol 25: 1100-10

Bortoluzzi S, Coppe A, Bisognin A, Pizzi C, Danieli GA (2005) A multistep bioinformatic approach detects putative regulatory elements in gene promoters. BMC Bioinformatics 6:121

Bredy TW, Brown RE, Meaney MJ (2007) Effect of resource availability on biparental care, and offspring neural and behavioral development in the California mouse (Peromyscus californicus). Eur J Neurosci 25: 567-75

Breslin MB, Geng CD, Vedeckis WV (2001) Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol 15: 1381-95

Breslin MB, vedeckis WV (1996) The Glucocorticoid Receptor and c-jun Promoters Contain AP-1 Sites That Bind Different AP-1 Transcription factors. Endocrine 5: 15-22

Breslin MB, Vedeckis WV (1998) The human glucocorticoid receptor promoter upstream sequences contain binding sites for the ubiquitous transcription factor, Yin Yang 1. J Steroid Biochem Mol Biol 67: 369-81

Brown SE, Fraga MF, Weaver IC, Berdasco M, Szyf M (2007) Variations in DNA methylation patterns during the cell cycle of HeLa cells. Epigenetics 2: 54-65

Bryans M, Kass S, Seivwright C, Adams RL (1992) Vector methylation inhibits transcription from the SV40 early promoter. FEBS Lett 309:97-102

Buijs RM, van Eden CG, Goncharuk VD, Kalsbeek A (2003) The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. J Endocrinol 177: 17-26

Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop KA (2007) Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? Br J Nutr 97: 1036-46

Bushati N, Cohen SM (2007) microRNA functions. Annu Rev Cell Dev Biol 23: 175-205

Caldji C, Tannenbaum B, Sharma S, Francis D, Plotsky PM, Meaney MJ (1998) Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc Natl Acad Sci U S A 95: 5335-40

Cao-Lei L, Leija SC, Kumsta R, Wust S, Meyer J, Turner JD, Muller CP (2011) Transcriptional control of the human glucocorticoid receptor: identification and analysis of alternative promoter regions. Hum Genet 129: 533-43

Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engstrom PG, Frith MC, Forrest AR, Alkema WB, Tan SL, Plessy C, Kodzius R, Ravasi T, Kasukawa T, Fukuda S, Kanamori-Katayama M, Kitazume Y, Kawaji H, Kai C, Nakamura M, Konno H, Nakano K, Mottagui-Tabar S, Arner P, Chesi A, Gustincich S, Persichetti F, Suzuki

H, Grimmond SM, Wells CA, Orlando V, Wahlestedt C, Liu ET, Harbers M, Kawai J, Bajic VB, Hume DA, Hayashizaki Y (2006) Genome-wide analysis of mammalian promoter architecture and evolution. Nat Genet 38: 626-35

Chen F, Watson CS, Gametchu B (1999) Association of the glucocorticoid receptor alternatively-spliced transcript 1A with the presence of the high molecular weight membrane glucocorticoid receptor in mouse lymphoma cells. J Cell Biochem 74: 430-46

Conway-Campbell BL, McKenna MA, Wiles CC, Atkinson HC, de Kloet ER, Lightman SL (2007) Proteasome-dependent down-regulation of activated nuclear hippocampal glucocorticoid receptors determines dynamic responses to corticosterone. Endocrinology 148: 5470-7

Cooper DN, Taggart MH, Bird AP (1983) Unmethylated domains in vertebrate DNA. Nucleic Acids Res 11: 647-58

Cooper-Kuhn CM, Vroemen M, Brown J, Ye H, Thompson MA, Winkler J, Kuhn HG (2002) Impaired adult neurogenesis in mice lacking the transcription factor E2F1. Mol Cell Neurosci 21: 312-23

Cooper SJ, Trinklein ND, Anton ED, Nguyen L, Myers RM (2006) Comprehensive analysis of transcriptional promoter structure and function in 1% of the human genome. Genome Res 16: 1-10

Daniels WM, Fairbairn LR, van Tilburg G, McEvoy CR, Zigmond MJ, Russell VA, Stein DJ (2009) Maternal separation alters nerve growth factor and corticosterone levels but not the DNA methylation status of the exon 1(7) glucocorticoid receptor promoter region. Metab Brain Dis 24: 615-27

Davis RL, Turner DL (2001) Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene 20: 8342-57

Davuluri RV, Suzuki Y, Sugano S, Plass C, Huang TH (2008) The functional consequences of alternative promoter use in mammalian genomes. Trends Genet 24: 167-77

DeGregori J, Kowalik T, Nevins JR (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol Cell Biol 15: 4215-24

de Kloet ER, Fitzsimons CP, Datson NA, Meijer OC, Vreugdenhil E (2009) Glucocorticoid signaling and stress-related limbic susceptibility pathway: about receptors, transcription machinery and microRNA. Brain Res 1293: 129-41

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6: 463-75

de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev 19: 269-301

Dekker MJ, van den Akker EL, Koper JW, Manenschijn L, Geleijns K, Ruts L, van Rijs W, Tio-Gillen AP, van Doorn PA, Lamberts SW, Jacobs BC (2009) Effect of glucocorticoid receptor gene polymorphisms in Guillain-Barre syndrome. J Peripher Nerv Syst 14: 75-83

de Lange P, Segeren CM, Koper JW, Wiemer E, Sonneveld P, Brinkmann AO, White A, Brogan IJ, de Jong FH, Lamberts SW (2001) Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res 61: 3937-41

Derijk RH (2009) Single nucleotide polymorphisms related to HPA axis reactivity. Neuroimmunomodulation 16: 340-52

DeRijk R, de Kloet ER (2005) Corticosteroid receptor genetic polymorphisms and stress responsivity. Endocrine 28: 263-70

Derijk RH, de Kloet ER (2008) Corticosteroid receptor polymorphisms: determinants of vulnerability and resilience. Eur J Pharmacol 583: 303-11

DeRijk RH, Schaaf M, Stam FJ, de Jong IE, Swaab DF, Ravid R, Vreugdenhil E, Cidlowski JA, de Kloet ER, Lucassen PJ (2003) Very low levels of the glucocorticoid receptor beta isoform in the human hippocampus as shown by Taqman RT-PCR and immunocytochemistry. Brain Res Mol Brain Res 116: 17-26

Derijk RH, Schaaf MJ, Turner G, Datson NA, Vreugdenhil E, Cidlowski J, de Kloet ER, Emery P, Sternberg EM, Detera-Wadleigh SD (2001) A human glucocorticoid receptor gene variant that increases the stability of the glucocorticoid receptor beta-isoform mRNA is associated with rheumatoid arthritis. J Rheumatol 28: 2383-8

Detera-Wadleigh SD, Encio IJ, Rollins DY, Coffman D, Wiesch D (1991) A TthIII1 polymorphism on the 5' flanking region of the glucocorticoid receptor gene (GRL). Nucleic Acids Res 19: 1960

Diba F, Watson CS, Gametchu B (2001) 5'UTR sequences of the glucocorticoid receptor 1A transcript encode a peptide associated with translational regulation of the glucocorticoid receptor. J Cell Biochem 81: 149-61

Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12: 2245-62

Ehrlich M (2003) Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 88: 899-910
Ernst C, McGowan PO, Deleva V, Meaney MJ, Szyf M, Turecki G (2008) The effects of pH on DNA methylation state: In vitro and post-mortem brain studies. J Neurosci Methods 174: 123-5
Fan J, Bertino JR (1997) Functional roles of E2F in cell cycle regulation. Oncogene 14: 1191-200Fei Y, Hu J, Liu S, Liu X, Wang F, Gong J (2010) E-cadherin-160 C/A promoter polymorphism and risk of pancreatic carcinoma in Chinese population. Cancer Genet Cytogenet 197: 25-31
Filiberto AC, Maccani MA, Koestler D, Wilhelm-Benartzi C, Avissar-Whiting M, Banister CE, Gagne LA, Marsit CJ (2011) Birthweight is associated with DNA promoter methylation of the glucocorticoid receptor in human placenta. Epigenetics 6: 566-72
Fischer A, Gessler M (2007) Delta-Notch--and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res 35: 4583-96
Francis D, Diorio J, Liu D, Meaney MJ (1999) Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286: 1155-8
Gametchu B, Watson CS (2002) Correlation of membrane glucocorticoid receptor levels with glucocorticoid-induced apoptotic competence using mutant leukemic and lymphoma cells lines. J Cell Biochem 87: 133-46
Gametchu B, Watson CS, Shih CC, Dashew B (1991) Studies on the arrangement of glucocorticoid receptors in the plasma membrane of S-49 lymphoma cells. Steroids 56: 411-9
Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. J Mol Biol 196: 261-82
Geng CD, Pedersen KB, Nunez BS, Vedeckis WV (2005) Human glucocorticoid receptor alpha transcript splice variants with exon 2 deletions: evidence for tissue- and cell type-specific functions. Biochemistry 44: 7395-405
Geng CD, Schwartz JR, Vedeckis WV (2008) A conserved molecular mechanism is responsible for the auto-up-regulation of glucocorticoid receptor gene promoters. Mol Endocrinol 22: 2624-42
Geng CD, Vedeckis WV (2004) Steroid-responsive sequences in the human glucocorticoid receptor gene 1A promoter. Mol Endocrinol 18: 912-24
Gross KL, Cidlowski JA (2008) Tissue-specific glucocorticoid action: a family affair. Trends Endocrinol Metab 19: 331-9
Gruenbaum Y, Stein R, Cedar H, Razin A (1981) Methylation of CpG sequences in eukaryotic DNA. FEBS Lett 124: 67-71
Harrington MA, Jones PA, Imagawa M, Karin M (1988) Cytosine methylation does not affect binding of transcription factor Sp1. Proc Natl Acad Sci U S A 85: 2066-70
Harrison P, Pointon JJ, Chapman K, Roddam A, Wordsworth BP (2008) Interleukin-1 promoter region polymorphism role in rheumatoid arthritis: a meta-analysis of IL-1B-511A/G variant reveals association with rheumatoid arthritis. Rheumatology (Oxford) 47: 1768-70
Hartman J, Muller P, Foster JS, Wimalasena J, Gustafsson JA, Strom A (2004) HES-1 inhibits 17beta-estradiol and heregulin-beta1-mediated upregulation of E2F-1. Oncogene 23: 8826-33
Hayashi R, Wada H, Ito K, Adcock IM (2004) Effects of glucocorticoids on gene transcription. Eur J Pharmacol 500: 51-62
Hay CW, Docherty K (2006) Comparative analysis of insulin gene promoters: implications for diabetes research. Diabetes 55:3201-3213
Herbeck YE, Gulevich RG, Amelkina OA, Plyusnina IZ, Oskina IN (2009) Conserved methylation of the glucocorticoid receptor gene exon 1(7) promoter in rats subjected to a maternal methyl-supplemented diet. Int J Dev Neurosci 28: 9-12
Henke RM, Meredith DM, Borromeo MD, Savage TK, Johnson JE (2009) Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. Dev Biol 328: 529-40
Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM (1985) Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318: 635-41
Holler M, Westin G, Jiricny J, Schaffner W (1988) Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev 2: 1127-35
Hoogendoorn B, Coleman SL, Guy CA, Smith K, Bowen T, Buckland PR, O'Donovan MC (2003) Functional analysis of human promoter polymorphisms. Hum Mol Genet 12: 2249-54
Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, Grobbee DE, Brinkmann AO, De Jong FH, Lamberts SW (1998) A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 83: 144-51

Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, Jackson D, Clee C, Plumb R, Rogers J, Humphray S, Cox T, Langford C, Bird A (2008) A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol 6: e22

Illingworth RS, Bird AP (2009) CpG islands--'a rough guide'. FEBS Lett 583: 1713-20

Inoshita S, Terada Y, Nakashima O, Kuwahara M, Sasaki S, Marumo F (1999) Regulation of the G1/S transition phase in mesangial cells by E2F1. Kidney Int 56: 1238-41

Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12: 118-34

Johnson RF, Rennie N, Murphy V, Zakar T, Clifton V, Smith R (2008) Expression of glucocorticoid receptor messenger ribonucleic acid transcripts in the human placenta at term. J Clin Endocrinol Metab 93: 4887-93

Juruena MF, Cleare AJ, Pariante CM (2004) [The hypothalamic pituitary adrenal axis, glucocorticoid receptor function and relevance to depression]. Rev Bras Psiquiatr 26: 189-201

Jutapakdeegul N, Casalotti SO, Govitrapong P, Kotchabhakdi N (2003) Postnatal touch stimulation acutely alters corticosterone levels and glucocorticoid receptor gene expression in the neonatal rat. Dev Neurosci 25: 26-33

Kadonaga JT (1998) Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell 92: 307-13

Kageyama R, Ohtsuka T, Kobayashi T (2007) The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 134: 1243-51

Kalinyak JE, Dorin RI, Hoffman AR, Perlman AJ (1987) Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J Biol Chem 262: 10441-4

Ke X, Schober ME, McKnight RA, O'Grady S, Caprau D, Yu X, Callaway CW, Lane RH (2010) Intrauterine growth retardation affects expression and epigenetic characteristics of the rat hippocampal glucocorticoid receptor gene. Physiol Genomics 42: 177-89

Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315: 525-8

Kim TH, Barrera LO, Zheng M, Qu C, Singer MA, Richmond TA, Wu Y, Green RD, Ren B (2005) A high-resolution map of active promoters in the human genome. Nature 436: 876-80

Kimura K, Wakamatsu A, Suzuki Y, Ota T, Nishikawa T, Yamashita R, Yamamoto J, Sekine M, Tsuritani K, Wakaguri H, Ishii S, Sugiyama T, Saito K, Isono Y, Irie R, Kushida N, Yoneyama T, Otsuka R, Kanda K, Yokoi T, Kondo H, Wagatsuma M, Murakawa K, Ishida S, Ishibashi T, Takahashi-Fujii A, Tanase T, Nagai K, Kikuchi H, Nakai K, Isogai T, Sugano S (2006) Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes. Genome Res 16: 55-65

Klok MD, Alt SR, Irurzun Lafitte AJ, Turner JD, Lakke EA, Huitinga I, Muller CP, Zitman FG, de Kloet ER, Derijk RH (2011) Decreased expression of mineralocorticoid receptor mRNA and its splice variants in postmortem brain regions of patients with major depressive disorder. J Psychiatr Res 45: 871-8

Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. Dev Cell 11: 441-50

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, Grobbee DE, Karl M, de Jong FH, Brinkmann AO, Lamberts SW (1997) Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Hum Genet 99: 663-8

Kornblihtt AR (2005) Promoter usage and alternative splicing. Curr Opin Cell Biol 17: 262-8

Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST (1995) A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res 55: 2727-9

Kretz O, Schmid W, Berger S, Gass P (2001) The mineralocorticoid receptor expression in the mouse CNS is conserved during development. Neuroreport 12: 1133-7

Kumsta R, Entringer S, Hellhammer DH, Wust S (2007) Cortisol and ACTH responses to psychosocial stress are modulated by corticosteroid binding globulin levels. Psychoneuroendocrinology 32: 1153-7

Kumsta R, Entringer S, Koper JW, van Rossum EF, Hellhammer DH, Wust S (2007) Sex specific associations between common glucocorticoid receptor gene variants and hypothalamus-pituitary-adrenal axis responses to psychosocial stress. Biol Psychiatry 62: 863-9

Kumsta R, Entringer S, Koper JW, van Rossum EF, Hellhammer DH, Wust S (2008) Glucocorticoid receptor gene polymorphisms and glucocorticoid sensitivity of subdermal blood vessels and leukocytes. Biol Psychol

Kumsta R, Moser D, Streit F, Koper JW, Meyer J, Wust S (2009) Characterization of a glucocorticoid receptor gene (GR, NR3C1) promoter polymorphism reveals functionality and extends a haplotype with putative clinical relevance. Am J Med Genet B Neuropsychiatr Genet

Labuda M, Gahier A, Gagne V, Moghrabi A, Sinnett D, Krajinovic M (2010) Polymorphisms in glucocorticoid receptor gene and the outcome of childhood acute lymphoblastic leukemia (ALL). Leuk Res 34(4):492-7

Lahti J, Raikkonen K, Bruce S, Heinonen K, Pesonen AK, Rautanen A, Wahlbeck K, Kere J, Kajantie E, Eriksson JG (2011) Glucocorticoid receptor gene haplotype predicts increased risk of hospital admission for depressive disorders in the Helsinki birth cohort study. J Psychiatr Res 45: 1160-4

Lander ES, Linton LM, Birren B et al. (2001) Initial sequencing and analysis of the human genome. Nature 409: 860-921

Landry JR, Mager DL, Wilhelm BT (2003) Complex controls: the role of alternative promoters in mammalian genomes. Trends Genet 19: 640-8

Laplante P, Diorio J, Meaney MJ (2002) Serotonin regulates hippocampal glucocorticoid receptor expression via a 5-HT7 receptor. Brain Res Dev Brain Res 139: 199-203

Larsen F, Gundersen G, Lopez R, Prydz H (1992) CpG islands as gene markers in the human genome. Genomics 13: 1095-107

LeClerc S, Palaniswami R, Xie BX, Govindan MV (1991a) Molecular cloning and characterization of a factor that binds the human glucocorticoid receptor gene and represses its expression. J Biol Chem 266: 17333-40

LeClerc S, Xie BX, Roy R, Govindan MV (1991b) Purification of a human glucocorticoid receptor gene promoter-binding protein. Production of polyclonal antibodies against the purified factor. J Biol Chem 266: 8711-9

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ (1997) Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277: 1659-62

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-8

Lopez JF, Chalmers DT, Little KY, Watson SJ (1998) A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biol Psychiatry 43: 547-73

Lu NZ, Cidlowski JA (2005) Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell 18: 331-42

Lu NZ, Collins JB, Grissom SF, Cidlowski JA (2007) Selective regulation of bone cell apoptosis by translational isoforms of the glucocorticoid receptor. Mol Cell Biol 27: 7143-60

Macchi MM, Bruce JN (2004) Human pineal physiology and functional significance of melatonin. Front Neuroendocrinol 25: 177-95

Matsubara T, Funato H, Kobayashi A, Nobumoto M, Watanabe Y (2006) Reduced Glucocorticoid Receptor alpha Expression in Mood Disorder Patients and First-Degree Relatives. Biol Psychiatry 59: 689-95

Matthews SG (1998) Dynamic changes in glucocorticoid and mineralocorticoid receptor mRNA in the developing guinea pig brain. Brain Res Dev Brain Res 107: 123-32

McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, Weaver S, Ester W, Yau JL, Meaney MJ, Seckl JR, Chapman KE (2000) 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. Mol Endocrinol 14: 506-17

McEwen BS (2003) Mood disorders and allostatic load. Biol Psychiatry 54: 200-7

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, Turecki G, Meaney MJ (2009) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 12: 342-8

McGowan PO, Suderman M, Sasaki A, Huang TC, Hallett M, Meaney MJ, Szyf M (2011) Broad epigenetic signature of maternal care in the brain of adult rats. PLoS One 6: e14739

Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K, Seckl JR (2000) Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. J Neurosci 20: 3926-35

Medeiros RB, Papenfuss KJ, Hoium B, Coley K, Jadrich J, Goh SK, Elayaperumal A, Herrera JE, Resnik E, Ni HT (2009) Novel sequential ChIP and simplified basic ChIP protocols for promoter co-occupancy and target gene identification in human embryonic stem cells. BMC Biotechnol 9: 59

Menard JL, Champagne DL, Meaney MJ (2004) Variations of maternal care differentially influence 'fear' reactivity and regional patterns of cFos immunoreactivity in response to the shock-probe burying test. Neuroscience 129: 297-308

Mendel CM (1989) The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 10: 232-74

Mendel CM, Kuhn RW, Weisiger RA, Cavalieri RR, Siiteri PK, Cunha GR, Murai JT (1989) Uptake of cortisol by the perfused rat liver: validity of the free hormone hypothesis applied to cortisol. Endocrinology 124: 468-76

Mitchell JB, Betito K, Rowe W, Boksa P, Meaney MJ (1992) Serotonergic regulation of type II corticosteroid receptor binding in hippocampal cell cultures: evidence for the importance of serotonin-induced changes in cAMP levels. Neuroscience 48: 631-9

Mitchell JB, Iny LJ, Meaney MJ (1990) The role of serotonin in the development and environmental regulation of type II corticosteroid receptor binding in rat hippocampus. Brain Res Dev Brain Res 55: 231-5

Morimoto M, Morita N, Ozawa H, Yokoyama K, Kawata M (1996) Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunohistochemical and in situ hybridization study. Neurosci Res 26: 235-69

Moser D, Molitor A, Kumsta R, Tatschner T, Riederer P, Meyer J (2007) The glucocorticoid receptor gene exon 1-F promoter is not methylated at the NGFI-A binding site in human hippocampus. World J Biol Psychiatry 8: 262-8

Muller-Myhsok B (2005) The usefulness of single nucleotide polymorphisms (SNPs) for genetic epidemiological investigation of complex psychiatric diseases. Prog Neuropsychopharmacol Biol Psychiatry 29: 1017-20

Murphy BC, O'Reilly RL, Singh SM (2005) Site-specific cytosine methylation in S-COMT promoter in 31 brain regions with implications for studies involving schizophrenia. Am J Med Genet B Neuropsychiatr Genet 133B: 37-42

Murray JC, Smith RF, Ardinger HA, Weinberger C (1987) RFLP for the glucocorticoid receptor (GRL) located at 5q11-5q13. Nucleic Acids Res 15: 6765

Musunuru K, Kathiresan S (2008) HapMap and mapping genes for cardiovascular disease. Circ Cardiovasc Genet 1: 66-71

Myers RL, Airey DC, Manier DH, Shelton RC, Sanders-Bush E (2007) Polymorphisms in the regulatory region of the human serotonin 5-HT2A receptor gene (HTR2A) influence gene expression. Biol Psychiatry 61: 167-73

Nemeroff CB (1996) The corticotropin-releasing factor (CRF) hypothesis of depression: new findings and new directions. Mol Psychiatry 1: 336-42

Niu N, Manickam V, Kalari KR, Moon I, Pelleymounter LL, Eckloff BW, Wieben ED, Schaid DJ, Wang L (2009) Human glucocorticoid receptor alpha gene (NR3C1) pharmacogenomics: gene resequencing and functional genomics. J Clin Endocrinol Metab 94: 3072-84

Nobukuni Y, Smith CL, Hager GL, Detera-Wadleigh SD (1995) Characterization of the human glucocorticoid receptor promoter. Biochemistry 34: 8207-14

Nunez BS, Geng CD, Pedersen KB, Millro-Macklin CD, Vedeckis WV (2005) Interaction between the Interferon Signaling Pathway and the Human Glucocorticoid Receptor Gene 1A Promoter. Endocrinology 146: 1449-57

Nunez BS, Vedeckis WV (2002) Characterization of promoter 1B in the human glucocorticoid receptor gene. Mol Cell Endocrinol 189: 191-9

Oakley RH, Sar M, Cidlowski JA (1996) The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. J Biol Chem 271: 9550-9

Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 3: 97-106

Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 3: 97-106

Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 3: 97-106

Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, Yagi K, Tomaru Y, Hasegawa Y, Nogami A, Schonbach C, Gojobori T, Baldarelli R, Hill DP, Bult C, Hume DA, Quackenbush J, Schriml LM, Kanapin A, Matsuda H, Batalov S, Beisel KW, Blake JA, Bradt D, Brusic V, Chothia C, Corbani LE, Cousins S, Dalla E,

Dragani TA, Fletcher CF, Forrest A, Frazer KS, Gaasterland T, Gariboldi M, Gissi C, Godzik A, Gough J, Grimmond S, Gustincich S, Hirokawa N, Jackson IJ, Jarvis ED, Kanai A, Kawaji H, Kawasawa Y, Kedzierski RM, King BL, Konagaya A, Kurochkin IV, Lee Y, Lenhard B, Lyons PA, Maglott DR, Maltais L, Marchionni L, McKenzie L, Miki H, Nagashima T, Numata K, Okido T, Pavan WJ, Pertea G, Pesole G, Petrovsky N, Pillai R, Pontius JU, Qi D, Ramachandran S, Ravasi T, Reed JC, Reed DJ, Reid J, Ring BZ, Ringwald M, Sandelin A, Schneider C, Semple CA, Setou M, Shimada K, Sultana R, Takenaka Y, Taylor MS, Teasdale RD, Tomita M, Verardo R, Wagner L, Wahlestedt C, Wang Y, Watanabe Y, Wells C, Wilming LG, Wynshaw-Boris A, Yanagisawa M, et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420: 563-73

Otte C, Wust S, Zhao S, Pawlikowska L, Kwok PY, Whooley MA (2009) Glucocorticoid receptor gene and depression in patients with coronary heart disease: the Heart and Soul Study-2009 Curt Richter Award Winner. Psychoneuroendocrinology 34: 1574-81

Pavlik A, Buresova M (1984) The neonatal cerebellum: the highest level of glucocorticoid receptors in the brain. Brain Res 314: 13-20

Pedersen KB, Geng CD, Vedeckis WV (2004) Three mechanisms are involved in glucocorticoid receptor autoregulation in a human T-lymphoblast cell line. Biochemistry 43: 10851-8

Pedersen KB, Vedeckis WV (2003) Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. Biochemistry 42: 10978-90

Perlman WR, Webster MJ, Herman MM, Kleinman JE, Weickert CS (2007) Age-related differences in glucocorticoid receptor mRNA levels in the human brain. Neurobiol Aging 28: 447-58

Perlman WR, Webster MJ, Kleinman JE, Weickert CS (2004) Reduced glucocorticoid and estrogen receptor alpha messenger ribonucleic acid levels in the amygdala of patients with major mental illness. Biol Psychiatry 56: 844-52

Pickering BM, Willis AE (2005) The implications of structured 5' untranslated regions on translation and disease. Semin Cell Dev Biol 16: 39-47

Pratt WB, Morishima Y, Murphy M, Harrell M (2006) Chaperoning of glucocorticoid receptors. Handb Exp Pharmacol: 111-38

Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228: 111-33

Prescott NJ, Dominy KM, Kubo M, Lewis CM, Fisher SA, Redon R, Huang N, Stranger BE, Blaszczyk K, Hudspith B, Parkes G, Hosono N, Yamazaki K, Onnie CM, Forbes A, Dermitzakis ET, Nakamura Y, Mansfield JC, Sanderson J, Hurles ME, Roberts RG, Mathew CG (2010) Independent and population-specific association of risk variants at the IRGM locus with Crohn's disease. Hum Mol Genet 19(9):1828-39

Presul E, Schmidt S, Kofler R, Helmberg A (2007) Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. J Mol Endocrinol 38: 79-90

Pryce CR (2008) Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences. Brain Res Rev 57: 596-605

Rajeevan MS, Smith AK, Dimulescu I, Unger ER, Vernon SD, Heim C, Reeves WC (2007) Glucocorticoid receptor polymorphisms and haplotypes associated with chronic fatigue syndrome. Genes Brain Behav 6: 167-76

Ramnani N (2006) The primate cortico-cerebellar system: anatomy and function. Nat Rev Neurosci 7: 511-22

Ramos L, Paabo S, Rebhan M, Schubeler D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39: 457-66

Ratajczak T, Ward BK, Minchin RF (2003) Immunophilin chaperones in steroid receptor signalling. Curr Top Med Chem 3: 1348-57

Razin A, Webb C, Szyf M, Yisraeli J, Rosenthal A, Naveh-Many T, Sciaky-Gallili N, Cedar H (1984) Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. Proc Natl Acad Sci U S A 81: 2275-9

Reul JM, de Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117: 2505-11

Robertson KD, Wolffe AP (2000) DNA methylation in health and disease. Nat Rev Genet 1: 11-9

Rosmond R, Chagnon YC, Chagnon M, Perusse L, Bouchard C, Bjorntorp P (2000) A polymorphism of the 5'-flanking region of the glucocorticoid receptor gene locus is associated with basal cortisol secretion in men. Metabolism 49: 1197-9

Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 65: 760-9

Ruiz-Narvaez EA (2011) What is a functional locus? Understanding the genetic basis of complex phenotypic traits. Med Hypotheses 76: 638-42

Russcher H, Dalm VA, de Jong FH, Brinkmann AO, Hofland LJ, Lamberts SW, Koper JW (2007) Associations between promoter usage and alternative splicing of the glucocorticoid receptor gene. J Mol Endocrinol 38: 91-8

Russcher H, Smit P, van den Akker EL, van Rossum EF, Brinkmann AO, de Jong FH, Lamberts SW, Koper JW (2005a) Two polymorphisms in the glucocorticoid receptor gene directly affect glucocorticoid-regulated gene expression. J Clin Endocrinol Metab 90: 5804-10

Russcher H, van Rossum EF, de Jong FH, Brinkmann AO, Lamberts SW, Koper JW (2005b) Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. Mol Endocrinol 19: 1687-96

Saxonov S, Berg P, Brutlag DL (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A 103: 1412-7

Schmahmann JD, Pandya DN (1997) The cerebrocerebellar system. Int Rev Neurobiol 41: 31-60

Schmidt MV, Sterlemann V, Wagner K, Niederleitner B, Ganea K, Liebl C, Deussing JM, Berger S, Schutz G, Holsboer F, Muller MB (2009) Postnatal glucocorticoid excess due to pituitary glucocorticoid receptor deficiency: differential short- and long-term consequences. Endocrinology 150: 2709-16Shrivastava A, Calame K (1994) An analysis of genes regulated by the multi-functional transcriptional regulator Yin Yang-1. Nucleic Acids Res 22: 5151-5

Sharov AA, Dudekula DB, Ko MS (2005) Genome-wide assembly and analysis of alternative transcripts in mouse. Genome Res 15: 748-54

Song F, Smith JF, Kimura MT, Morrow AD, Matsuyama T, Nagase H, Held WA (2005) Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. Proc Natl Acad Sci U S A 102: 3336-41

Spies CM, Schaumann DH, Berki T, Mayer K, Jakstadt M, Huscher D, Wunder C, Burmester GR, Radbruch A, Lauster R, Scheffold A, Buttgereit F (2006) Membrane glucocorticoid receptors are down regulated by glucocorticoids in patients with systemic lupus erythematosus and use a caveolin-1-independent expression pathway. Ann Rheum Dis 65: 1139-46

Strahle U, Schmidt A, Kelsey G, Stewart AF, Cole TJ, Schmid W, Schutz G (1992) At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc Natl Acad Sci U S A 89: 6731-5

Szyf M (2009) The early life environment and the epigenome. Biochim Biophys Acta 1790: 878-85

Szyf M, Weaver IC, Champagne FA, Diorio J, Meaney MJ (2005) Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. Front Neuroendocrinol 26: 139-62

Takahashi K, Hayashi N, Shimokawa T, Umehara N, Kaminogawa S, Ra C (2008) Cooperative regulation of Fc receptor gamma-chain gene expression by multiple transcription factors, including Sp1, GABP, and Elf-1. J Biol Chem 283: 15134-41

Tissing WJ, Meijerink JP, Brinkhof B, Broekhuis MJ, Menezes RX, den Boer ML, Pieters R (2006) Glucocorticoid-induced glucocorticoid receptor expression and promoter-usage is not linked to glucocorticoid resistance in childhood ALL. Blood

Toki S, Morinobu S, Imanaka A, Yamamoto S, Yamawaki S, Honma K (2007) Importance of early lighting conditions in maternal care by dam as well as anxiety and memory later in life of offspring. Eur J Neurosci 25: 815-29

Torpy DJ, Ho JT (2007) Corticosteroid-binding globulin gene polymorphisms: clinical implications and links to idiopathic chronic fatigue disorders. Clin Endocrinol (Oxf) 67: 161-7

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4

Trinklein ND, Aldred SJ, Saldanha AJ, Myers RM (2003) Identification and functional analysis of human transcriptional promoters. Genome Res 13: 308-12

Tsankova N, Renthal W, Kumar A, Nestler EJ (2007) Epigenetic regulation in psychiatric disorders. Nat Rev Neurosci 8: 355-67

Turner JD, Alt SR, Cao L, Vernocchi S, Trifonova S, Battello N, Muller CP (2010) Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem Pharmacol 80: 1860-8

Turner JD, Muller CP (2005) Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. J Mol Endocrinol 35: 283-92

Turner JD, Pelascini LP, Macedo JA, Muller CP (2008) Highly individual methylation patterns of alternative glucocorticoid receptor promoters suggest individualized epigenetic regulatory mechanisms. Nucleic Acids Res 36: 7207-7218

Turner JD, Schote AB, Macedo JA, Pelascini LP, Muller CP (2006) Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage? Biochem Pharmacol 72: 1529-1537

Ursini G, Bollati V, Fazio L, Porcelli A, Iacovelli L, Catalani A, Sinibaldi L, Gelao B, Romano R, Rampino A, Taurisano P, Mancini M, Di Giorgio A, Popolizio T, Baccarelli A, De Blasi A, Blasi G, Bertolino A (2011) Stress-related methylation of the catechol-O-methyltransferase Val 158 allele predicts human prefrontal cognition and activity. J Neurosci 31: 6692-8

van Rossum EF, Binder EB, Majer M, Koper JW, Ising M, Modell S, Salyakina D, Lamberts SW, Holsboer F (2006) Polymorphisms of the glucocorticoid receptor gene and major depression. Biol Psychiatry 59: 681-8

van Rossum EF, Koper JW, van den Beld AW, Uitterlinden AG, Arp P, Ester W, Janssen JA, Brinkmann AO, de Jong FH, Grobbee DE, Pols HA, Lamberts SW (2003) Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. Clin Endocrinol (Oxf) 59: 585-92

van Rossum EF, Lamberts SW (2004a) Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res 59: 333-57

van Rossum EF, Roks PH, de Jong FH, Brinkmann AO, Pols HA, Koper JW, Lamberts SW (2004b) Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms. Clin Endocrinol (Oxf) 61: 573-81

van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, Sluijs S, Adolfsson R, Mendlewicz J, Deboutte D, Van Broeckhoven C, Claes S (2006) Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. Neuropsychopharmacology 31: 620-7

van Winsen LM, Hooper-van Veen T, van Rossum EF, Koper JW, Barkhof F, Polman CH, Uitdehaag BM (2007) Glucocorticoid receptor gene polymorphisms associated with more aggressive disease phenotype in MS. J Neuroimmunol 186: 150-5

van Winsen LM, Manenschijn L, van Rossum EF, Crusius JB, Koper JW, Polman CH, Uitdehaag BM (2009) A glucocorticoid receptor gene haplotype (TthIII1/ER22/23EK/9beta) is associated with a more aggressive disease course in multiple sclerosis. J Clin Endocrinol Metab 94: 2110-4

Veldhuis JD, Iranmanesh A, Lizarralde G, Johnson ML (1989) Amplitude modulation of a burstlike mode of cortisol secretion subserves the circadian glucocorticoid rhythm. Am J Physiol 257: E6-14

Vreugdenhil E, Verissimo CS, Mariman R, Kamphorst JT, Barbosa JS, Zweers T, Champagne DL, Schouten T, Meijer OC, de Kloet ER, Fitzsimons CP (2009) MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain. Endocrinology 150: 2220-8

Wagner KV, Wang XD, Liebl C, Scharf SH, Muller MB, Schmidt MV (2011) Pituitary glucocorticoid receptor deletion reduces vulnerability to chronic stress. Psychoneuroendocrinology 36: 579-87

Warriar N, Page N, Govindan MV (1996) Expression of human glucocorticoid receptor gene and interaction of nuclear proteins with the transcriptional control element. J Biol Chem 271: 18662-71

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7: 847-54

Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, Szyf M (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 25: 11045-54

Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S, Szyf M, Meaney MJ (2007) The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. J Neurosci 27: 1756-68

Webster JI, Sternberg EM (2004) Role of the hypothalamic-pituitary-adrenal axis, glucocorticoids and glucocorticoid receptors in toxic sequelae of exposure to bacterial and viral products. J Endocrinol 181: 207-21

Wei P, Vedeckis WV (1997) Regulation of the glucocorticoid receptor gene by the AP-1 transcription factor. Endocrine 7: 303-10

Weinmann AS, Bartley SM, Zhang T, Zhang MQ, Farnham PJ (2001) Use of chromatin immunoprecipitation to clone novel E2F target promoters. Mol Cell Biol 21: 6820-32

Werner T (1999) Models for prediction and recognition of eukaryotic promoters. Mamm Genome 10:168-175

Wust S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH (2004) Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. J Clin Endocrinol Metab 89: 565-73

Yudt MR, Cidlowski JA (2001) Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Mol Endocrinol 15: 1093-103

Zavolan M, van Nimwegen E, Gaasterland T (2002) Splice variation in mouse full-length cDNAs identified by mapping to the mouse genome. Genome Res 12: 1377-85

Zhang TY, Bagot R, Parent C, Nesbitt C, Bredy TW, Caldji C, Fish E, Anisman H, Szyf M, Meaney MJ (2006) Maternal programming of defensive responses through sustained effects on gene expression. Biol Psychol 73: 72-89

Zhou J, Cidlowski JA (2005) The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids 70: 407-17

Zobel A, Jessen F, von Widdern O, Schuhmacher A, Hofels S, Metten M, Rietschel M, Scheef L, Block W, Becker T, Schild HH, Maier W, Schwab SG (2008) Unipolar depression and hippocampal volume: impact of DNA sequence variants of the glucocorticoid receptor gene. Am J Med Genet B Neuropsychiatr Genet 147B: 836-43

Zong J, Ashraf J, Thompson EB (1990) The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. Mol Cell Biol 10: 5580-5

Presentation and Meetings participation

2008

--- International Research Training Group (IRTG) Summer School "Psychobiology of Stress, Steroid Hormone Actions", University Leiden, Leiden/Amsterdam Centre for Drug Research (LACDR), 08-11.06.2008
Poster: human GR promoter regions

---39th Annual Meeting of the International Society of Psychoneuroendocrinology (ISPNE), Dresden Germany, 17-20.07.2008
Poster: human GR promoter regions

2009

---Symposium "50th Doctoral Student at the Department of Immunology", National meeting organized by the Institute of Immunology with financial contribution of the FNR, Abbaye Neumünster, Luxembourg, 15.05.2009
Poster: Transcriptional control of human GR

--- IRTG Autumn School "Stress to Pathology, Mechanisms to Action", Remich, Luxemburg, 08-11.11.2009
Talk: Identification and analysis of alternative promoter regions

2010

---Inflammation 2010: Inflammatory cell signalling mechanisms, New Conference Centre Kirchberg, Luxembourg, 28.01.2010

---7th World Congress on stress, Leiden, Netherland, 25-27.08.2010
Talk: Identification and analysis of alternative promoter regions

--- PhD Student Day, University of Luxembourg, Luxembourg, 16.09.2010
Poster: Transcriptional control of the human GR

2011

---10th Symposium on Catecholamines and Other Neurotransmitters in Stress, Slovak, 25-30.06.2011
Poster: Transcriptional regulation and epigenetic sensitivity of the human glucocorticoid receptor transcript 1F

---Life Science PhD Days, University of Luxembourg, Luxembourg, 13-14.09.2011
Poster: Transcriptional regulation and epigenetic sensitivity of the human glucocorticoid receptor transcript 1F

Publications

Turner JD, Alt SR, **Cao L**, Vernocchi S, Trifonova S, Battello N, Muller CP (2010) Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more.
Biochem Pharmacol 80: 1860-1868.

Cao-Lei L, Leija SC, Kumsta R, Wust S, Meyer J, Turner JD, Muller CP (2011) Transcriptional control of the human glucocorticoid receptor: identification and analysis of alternative promoter regions.
Hum Genet 129: 533-43

Cao-Lei L, Alt SR, Leija SC, Mériaux SB, Meijer OC, Turner JD, Muller CP (2010) Transcriptional regulation of the glucocorticoid receptor transcript 1F: NGFI-A or E2F1?
Submitted.

Cao-Lei L, Mériaux SB, Turner JD, Muller CP (2011) Transcriptional regulation and epigenetic sensitivity of the human glucocorticoid receptor transcript 1F.
Submitted.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertationsschrift selbständig verfasst und keine anderen als die angegebenen Hilfsquellen verwendet habe. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Lei Cao