Nuclear receptors: variants and their role in neuro-endocrine-immune regulations

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde durch den Fachbereich I – Psychobiology der Universität Trier

vorgelegt von Dipl. Biol. Andrea Schote-Frese

Gutachter:

Prof. Dr. C. P. Muller

Prof. Dr. D. H. Hellhammer

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under the guidance of

Prof. Dr. Claude P. Muller, Institute of Immunology, National Laboratory of Health, Luxembourg; Departement of Immunology, University of Trier, Germany

and

Prof. Dr. Dirk H. Hellhammer, Department of Theoretical and Clinical Psychobiology, University of Trier, Germany

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List of Abbreviations

ARM	adjacent rank mismatch
AF	activation function
ALL	acute lymphoblastic leukemia
AP-1	activator protein 1
AR	androgen receptor
C/EBP	CCAAT/enhancer binding protein
CAR	constitutive androstane receptor
Coup-TF	chicken ovalbumin upstream promoter-transcription factor
CRE	cAMP response element
CREB	p300/cAMP response element-binding protein
CTE	carboxy-terminal extention
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome gene 1
DBD	DNA binding domain
DRIP	D-vitamin receptor interacting protein
E2	17-beta estradiol
ER	estrogen receptor
ERE	estrogen receptor response element
EST	expressed sequence tag
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GCNF	germ cell nuclear factor
GFP	green fluorescent protein
GR	glucocorticoid receptor
GRE	glucocorticoid receptor response element
GRIP-1	glucocorticoid receptor interacting protein-1
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HC	hydrocortisone
HDAC	histone deacetylase
HNF4α	hepatocyte nuclear factor 4 alpha
HPA	hypothalamic-pituitary adrenal
HRE	hormone response element
HSP	heat shock protein
LBD	ligand binding domain
LXR	liver X receptor
MMTV	Mouse mammary tumor virus
MR	mineralocorticoid receptor
NCoR	nuclear receptor corepressor
NF-1	nuclear factor 1
NFkappaB	Nuclear factor kappa-B
NGFI-A	nerve growth factor-inducible A

NGFI-B	nerve growth factor IB
NR	nuclear receptor
Oct-1	octamer binding transcription factor
PBMC	peripheral blood mononuclear cells
PPAR	peroxisome proliferators-activated receptor
PPIA	peptidylprolyl isomerase A
PPR	putative promoter region
PR	progesterone receptor
PRM	perfect rank match
PXR	pregnane X receptor
RACE	rapid amplification of cDNA ends
RAR	retinoic acid receptor
RE	response element
RNA pol II	RNA polymerase II
ROR	RAR related orphan receptor
RXR	retinoic x receptor
SHR	steroid hormone receptor
SMCC	srb and mediator protein-containing complex
SMRT	silencing mediator of retinoid acid and thyroid hormone receptor
SP-1	specificity protein 1
SRC-1	steriod receptor coactivator-1
TBP	TATA-box binding protein
TF	transcription factor
TR	thyroid hormone receptor
TR2	testicular receptor 2
TR4	testicular receptor 4
TRAP	thyroid receptor-associated protein
UTR	untranslated region
VDR	vitamin D receptor
YY1	Yin Yang 1

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General Abstract

Nuclear receptors (NRs) are important key regulators of a wide variety of different physiological and pathophysiological challenges ranging from inflammation and stress to complex behaviour and disease. They control gene transcription in a ligand dependent manner and are embedded in the huge interaction network of the neuroendocrine and immune system. The best example for the neuroendocrine regulation of immunity is the glucocorticoid receptor (GR). On one hand, GR controls immune related genes such as cytokines and inflammatory mediators, on the other hand, it influences the activity of neurons and impairs the function of the hypothalamic-pituitary adrenal (HPA) axis. The HPA axis controls the basal and stress related homeostasis which is required for health. Disturbance of the GR actions at any level leads to an imbalance of these networks and enhance susceptibility to infection and inflammation as well as stress related and neurodegenerative disorders.

The GR and other members of the NR family are known to undergo alternative splicing to increase transcript and protein diversity which influences NR activities. The GRβ variant, for example, is known as a potent inhibitor of GRα, decreases GRα mediated gene transcription and subsequently increases the susceptibility for, amongst others, major depression. Although, only few data of other NR alternative variants and their role in psychoneuroimmunology are established, these studies revealed some promising findings especially in the field of neurodegenerative diseases.

The research presented in this thesis was undertaken to elucidate the poorly investigated importance of non steroidal NRs in the immune system and to determine the impact of different NR variants. Two receptors, the GR and the chicken ovalbumin upstream promoter-transcription factorII (Coup-TFII), both expressed in the immune system, were investigated regarding possible splice variants and their implication in the control of gene transcription. The discovery of new NR variants is expected to increase the enormous regulatory proteintial and the interaction possibilites with other factors and pathways and to play an important role in psychoneuroimmunology.

Chapter 1:

General Introduction

General Introduction

History of nuclear receptor research

The discovery of estrogen receptors (ER) as mediators between transcriptional control and physiological action of hormones in the early 1960th by Jensen and Jacobson was a milestone in hormone receptor research (Jensen&Jacobson 1962). In 1970, Gustafsson and collaborators purified the rat glucocorticoid receptor (GR) in order to identify the endogenous ligands of GR. They observed that the physiological functions of glucocorticoids (GC) were mediated by a basic GR coupled to multiple associated proteins such as heat shock proteins (HSPs) (Gustafsson 2005). Subsequent cloning of the GR in 1985 by the groups of Evan and Gustafsson lead to the discovery and functional elaboration of the nuclear receptor superfamily (Miesfeld *et al.* 1984; Hollenberg *et al.* 1985; Mangelsdorf *et al.* 1995). Another key finding in the development of the steroid receptor field was the discovery of the three-domain-structure of GR (Wrange&Gustafsson 1978; Carlstedt-Duke *et al.* 1987). These domains dedicate numerous aspects of the nuclear receptor properties. During the latter half of the 1980s, analysis with nuclear magnetic resonance spectroscopy revealed the three dimensional structure of the GR DNA binding domain (DBD) and defined the nowadays classical two zinc finger motifs (Hard *et al.* 1990). The determination of the x-ray crystal structure of an ER-ligand binding domain (LBD) complexed to a ligand, revealed insights into the ligand induced conformational changes of the receptor LBD (Brzozowski *et al.* 1997). 1987 all members of the nuclear receptor (NR) superfamily were cloned based on sequence homologies of DBD and LBD, which resulted 1999 in 48 human and 49 mouse NRs (Nuclear Receptors Nomenclature Committee 1999). Among these, 24 receptors were identified without a ligand and classified as orphan receptors (Laudet&Gronemeyer 2002). Over time scientist put some effort in the identification of orphan ligands and determined that NRs bind to a great spectrum of molecules such as fatty acids, xenobiotics and bile acids involved in nutrition and lipid homeostasis (Gustafsson 2005). In 1988, Chambon *et al.* discovered two distinct transcriptional activation functions (AFs), the ligand-independent AF-1 located in the N-terminal region and the ligand-dependent AF-2 located in the C-terminal region of the ER (Webster *et al.* 1988). They observed that these AFs act in a promoter-context- and cell-specific manner and interact with cell-specific promoter bound transcription factors and other cell specific factors (Bocquel *et al.* 1989). The investigation of proteins interacting

with NRs lead to variety of coactivators and corepressors that influence the multiple functions of NRs such as homo- and heterodimerisation, ligand binding, ligand induced switch from an inactive or repressed condition to a transcriptionally active state (Chambon 2005). Recent studies concentrate on the identification of NR crosstalk, their functions in cell signalling and their implications in health and disease.

Nomenclature for nuclear receptors

The classification of NRs is based on sequence homologies of the DBD and the LBD. Seven subfamilies with different subgroups can be defined. Subfamily 1, the Thyroid Hormone Receptor-like receptors, includes seven major subgroups, group A: Thyroid hormone receptor (TR), group B: Retinoic acid receptor (RAR), group C: Peroxisome proliferator-activated receptor (PPAR), group D: Rev-ErbA, group F: RAR-related orphan receptor (ROR), group H: Liver X receptor (LXR)-like, group I: Vitamin D receptor (VDR)-like. Subfamily 2, Retinoid X Receptor (RXR)-like receptors includes group A: Hepatocyte nuclear factor-4 (HNF4), group B: RXR, group C: Testicular receptor, group E: TLX/PNR and group F: COUP/EAR. Subfamily 3, Estrogen Receptor-like receptors includes Group A: Estrogen Receptor, Group B: Estrogen related receptor and Group C: Steroid receptors. Subfamily 4, Nerve Growth Factor IB (NGFI-B)-like receptors includes just Group A: NGFI-B/NURR1/NOR1. Subfamily 5, Steroidogenic Factor-like receptors includes just Group A: SF1/LRH1. Subfamily 6, Germ Cell Nuclear Factor (GCNF)-like receptors includes just Group A: GCNF and the last Subfamily 0, Miscellaneous includes just Group B: DAX/SHP. The NR Nomenclature Committee defined a gene nomenclature containing the nuclear receptor abbreviation NR followed by subfamily, group and receptor number. In Table 1 all members of the NR superfamily are listed according to their subfamily and group together with the gene and trivial name as well as their accession numbers.

Table 1: Nomenclature for nuclear receptors and commonly used trivial names

Subfamilies and Group	Genes	Trivial Names	Accession numbers
1A	NR1A1	TR*a*, c-erbA-1, THRA	M24748
	NR1A2	TR*b*, c-erbA-2, THRB	X04707
1B	NR1B1	RAR*a*	X06538
	NR1B2	RAR*b*, HAP	Y00291
	NR1B3	RAR*g*, RARD	M57707
	NR1B4	RAR	AF378827
1C	NR1C1	PPAR*a*	L02932
	NR1C2	PPAR*b*, NUC1, PPAR*d*, FAAR	L07592
	NR1C3	PPAR*g*	L40904
1D	NR1D1	REVERB*a*, EAR1, EAR1A	M24898
	NR1D2	REVERB*b*, EAR1*b*, BD73, RVR, HZF2	L31785
	NR1D3	E75	X51548
1E	NR1E1	E78, DR-78	U01087
1F	NR1F1	ROR*a,* RZR*a*	U04897
	NR1F2	ROR*b*, RZR*b*	Y08639
	NR1F3	ROR*g*, TOR	U16997
	NR1F4	HR3, DHR3, MHR3, GHR3	M90806
		CNR3, CHR3	U13075
1G	NR1G1	CNR14	U13074
1H	NR1H1	ECR	M74078
	NR1H2	UR, OR-1, NER1, RIP15, LXR*b*	U07132
	NR1H3	RLD1, LXR, LXR*a*	U22662
	NR1H4	FXR, RIP14, HRR1	U09416
	NR1H5	FXRB	AY094586
1I	NR1I1	VDR	J03258
	NR1I2	ONR1, PXR, SXR, BXR	X75163
	NR1I3	MB67, CAR1, CAR*a*	Z30425
	NR1I4	CAR2, CAR*b*	AF009327
1J	NR1J1	DHR96	U36792
1K	NR1K1	NHR1	U19360
2A	NR2A1	HNF4	X76930
	NR2A2	HNF4G	Z49826
	NR2A3	HNF4B	Z49827
	NR2A4	DHNF4, HNF4D	U70874
2B	NR2B1	RXRA	X52773
	NR2B2	RXRB, H-2RIIBP, RCoR-1	M84820
	NR2B3	RXRG	X66225
	NR2B4	USP, Ultraspiracle, 2C1, CF1, RXR1, RXR2	X52591
2C	NR2C1	TR2, TR2-11	M29960
	NR2C2	TR4, TAK1	L27586
	NR2C3	TR2-4	AF378828
2D	NR2D1	DHR78	U36791

Subfamilies and Group	Genes	Trivial Names	Accession numbers
2E	NR2E1	TLL, TLX, XTLL	S72373
	NR2E2	TLL, Tailless	M34639
	NR2E3	PNR	AF121129
	NR2E4	dissatisfaction	O96680
	NR2E5	fax-1	Q9U4I0
2F	NR2F1	COUP-TFI, COUPTFA, EAR3, SVP44	X12795
	NR2F2	COUP-TFII, COUPTFB, ARP1, SVP40	M64497
	NR2F3	SVP, COUP-TF	M28863
	NR2F4	COUP-TFIII, COUPTFG	X63092
	NR2F5	SVP46	X70300
	NR2F6	EAR2	X12794
	NR2F7	AmNR7	AF323687
2G	NR2G1	HNF, RXR	AJ517420
2H	NR2H1	AmNR4, AmNR8	AF323683
3A	NR3A1	ERa	X03635
	NR3A2	ERb	U57439
3B	NR3B1	ERR1, ERRa	X51416
	NR3B2	ERR2, ERRb	X51417
	NR3B3	ERR3, ERRg	AF094318
	NR3B4	Drosophila ERR	AE003556
3C	NR3C1	GR	X03225
	NR3C2	MR	M16801
	NR3C3	PR	M15716
	NR3C4	AR	M20132
4A	NR4A1	NGFI-B, TR3, N10, NUR77, NAK1	L13740
	NR4A2	NURR1, NOT, RNR1, HZF-3, TINOR	X75918
	NR4A3	NOR1, MINOR	D38530
	NR4A4	DHR38, NGFIB	U36762
		CNR8, C48D5	U13076
5A	NR5A1	SF1, ELP, FTZ-F1, AD4BP	D88155
	NR5A2	LRH1, xFF1rA, xFF1rB, FFLR, PHR, FTF	U93553
	NR5A3	FTZ-F1	M63711
	NR5A4	FF1b	Q9IAI9
5B	NR5B1	DHR39, FTZF1B	L06423
6A	NR6A1	GCNF1, RTR	U14666
	NR6A2	HR4, THR4, GRF	AL035245
0A	NR0A1	KNI, Knirps	X13331
	NR0A2	KNRL, Knirps related	X14153
	NR0A3	EGON, Embryonic gonad, EAGLE	X16631
	NR0A4	ODR7	U16708
	NR0A5	Trithorax	M31617
0B	NR0B1	DAX1, AHCH	S74720
	NR0B2	SHP	L76571

General structure of nuclear receptors

A typical NR is composed of several functional domains. The variable N-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The conserved DBD, or region C, is responsible for the recognition of specific DNA sequences. A variable linker region D connects the DBD to the conserved E/F region that contains the LBD as well as the dimerisation surface. Within the C-terminal portion of the ligand binding domain is the ligand-dependent AF-2 core transactivation domain located (Figure 1).

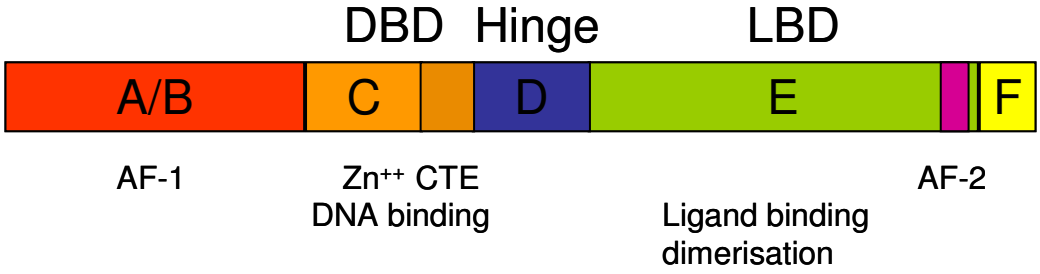

Figure 1: Schematic representation of a nuclear receptor (NR). A typical NR is composed of several functional domains. The variable N-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The conserved DNA-binding domain (DBD), or region C, contains two zinc-finger motives and a carboxy-terminl extention (CTE). The DBD is responsible for the recognition of specific DNA sequences. A variable hinge region D connects the DBD to the conserved E/F region that contains the ligand binding domain (LBD) as well as the dimerisation surface and the ligand-dependent AF-2 core transactivation domain.

The A/B domain

The A/B domain is the most variable region between different NR family members both in sequence and size. In many receptors this domain contains an autonomous transcriptional activation function AF-1 that contributes to constitutive ligand independent receptor activation. Alternative splicing and promoter usage in this region generate multiple isoforms that differ in their A/B domain but are identical in their DBD and LBD (Aranda&Pascual 2001). This region shows promoter and cell type specific activity mediated by the binding of cell type specific factors such as p160 coactivators and components of the D-vitamin receptor interacting protein (DRIP) complex (Warnmark *et al.* 2003; Trotter&Archer 2007). The A/B domain is on the other hand target of posttranslational modifications such as phosphorylations that modulates transcriptional activity (Aranda&Pascual 2001).

The DBD

The DBD is the most conserved region of nuclear receptors and composed of two zinc-finger motives and a carboxy-terminal extention (CTE). Each zinc-finger motive contains four invariable cysteine molecules which coordinate the binding of the zinc atom. The cysteine residues together with the zinc atoms are necessary to maintain the three dimensional structure. Its core contains two α-helixes (helix I and II). Both helixes are oriented in right angles to each other and cover different functions. Helix I, also referred to as recognition helix, binds the major groove of DNA making contact with specific hormone response elements (HREs), helix II is involved in receptor dimerisation (Aranda&Pascual 2001; Khorasanizadeh&Rastinejad 2001). The CTE provides both protein-DNA and protein-protein interfaces and is important for monomeric DNA binding (Khorasanizadeh&Rastinejad 2001).

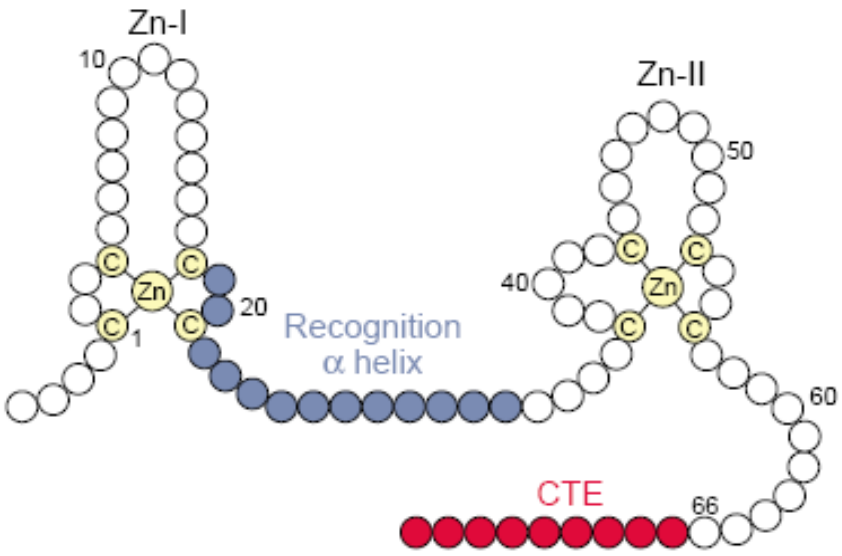

Figure 2: The NR DBD with its two zinc finger motives, the conserved recognition α-helix (shown in blue) and a variable C-terminal extension (CTE). (Khorasanizadeh&Rastinejad 2001)

The hinge region

The hinge region is highly variable and serves as a linker between DBD and LBD. The hinge region is very flexible allowing the DBD a rotation of up to 180°. Due to this flexibility some receptors can bind to direct and inverted HREs. The hinge region harbours nuclear localisation signals and serves as binding site for corepressor proteins (Aranda&Pascual 2001).

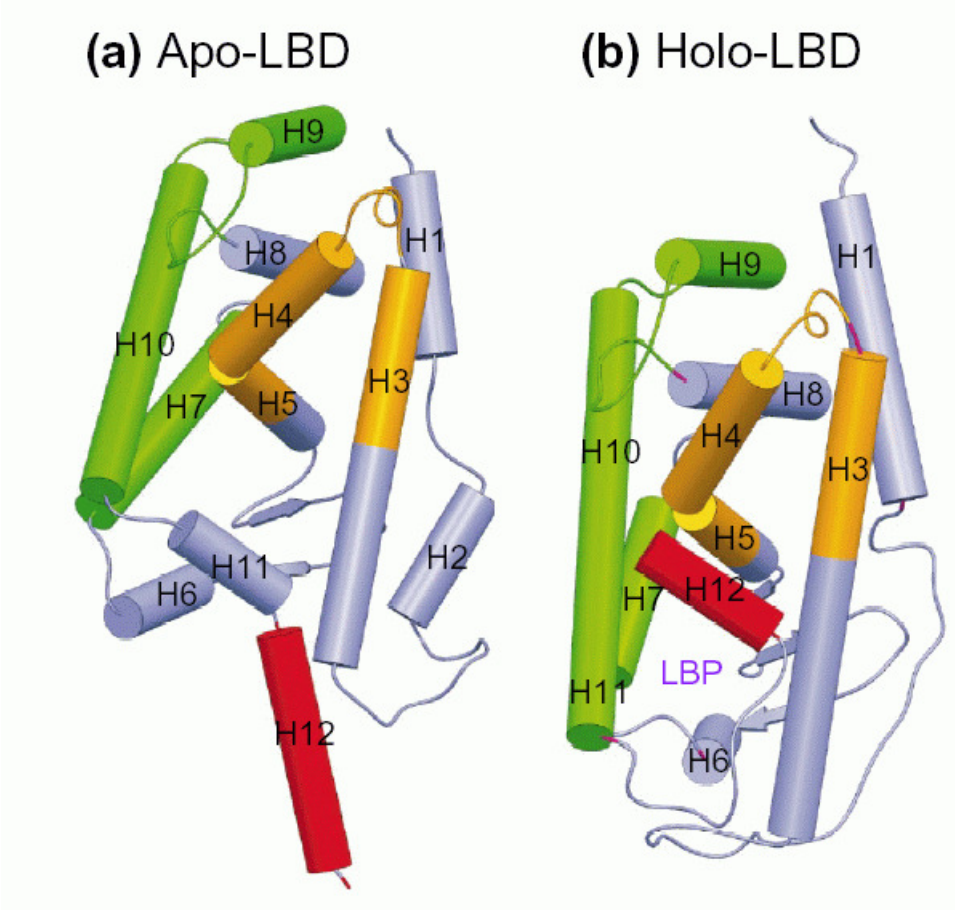

Figure 3: a) Nuclear receptor Apo-LBD (ligand binding domain without a ligand), b) Holo-LBD (ligand binding domain with an agonist bound), taken from: www.cmbi.ru.nl/edu/bioinf4/fri-Prac/nr.shtml

The LBD

The C-terminal LBD is a multifunctional domain that mediates ligand binding, homo- and heterodimerisation, interaction with HSPs, corepressors and coactivators, nuclear localisation and therewith transcriptional activity. The LBDs are formed by 12 α-helical regions, numbered from H1 to H12 and a conserved turn between H5 and H6 (Aranda&Pascual 2001). The three dimensional structures and biochemical analyses indicate that LBDs are able to form two distinct structures depending on the ligand binding state of the receptor, the apo receptor without a ligand and the holo receptor with a ligand. The binding of a ligand induces conformational changes that disrupts the binding of corepressors and generates surfaces that allow interaction with coactivator molecules. In this mouse trap model, receptor agonists induce transcriptional competent interfaces while receptor antagonists build a non-functional interface preventing the interaction between NR and coactivators (Brzozowski *et al.* 1997).

Nuclear receptors are transcription factors

The expression of genes can be regulated at several levels, such as transcription, translation, RNA processing and post-translation. NRs act as transcription factors (TFs) and regulate gene expression on the transcriptional level. The transcriptional activity of a gene can be controlled epigenetically via methylation, at the level of chromatin remodelling and at the level of assembly and activity of the initiation and elongation of polymerase complexes. NRs are able to regulate genes in a positive and negative manner and may be classified into two broad classes (class I and class II NR) according to their mechanism of action and subcellular distribution in the absence of their ligands (Giguere 1999; Aranda&Pascual 2001; Laudet&Gronemeyer 2002; Rochette-Egly 2005; Trotter&Archer 2007).

Molecular mechanisms of nuclear receptor actions

Class I NRs (includes members of the NR subfamily 3) are in the absence of ligands located in the cytosol. Hormone binding to these NRs leads to conformational change resulting in the dissociation of HSP. Translocation to the nucleus is followed by steroid receptor homodimerisation and binding to a specific HRE. The NR-DNA-complex in turn recruits other proteins that are responsible for transcription of downstream DNA into mRNA. Subsequent translation into protein results in changes of cellular functions. Figure 4A shows the molecular mechanism of class I NR.

Class II NRs (principally NR subfamily 1) are regardless of their ligand binding status located in the nucleus and there bound to DNA. The NR shown in Figure 4B is the TR heterodimerised to the RXR. In the absence of ligand, the TR is bound to corepressor proteins. Ligand binding to TR causes the dissociation of corepressors and recruitment of coactivator proteins which in turn recruit additional proteins such as RNA polymerase. This complex is responsible for translation of downstream DNA into RNA and eventually protein which results in a change in cell function (Figure 4B).

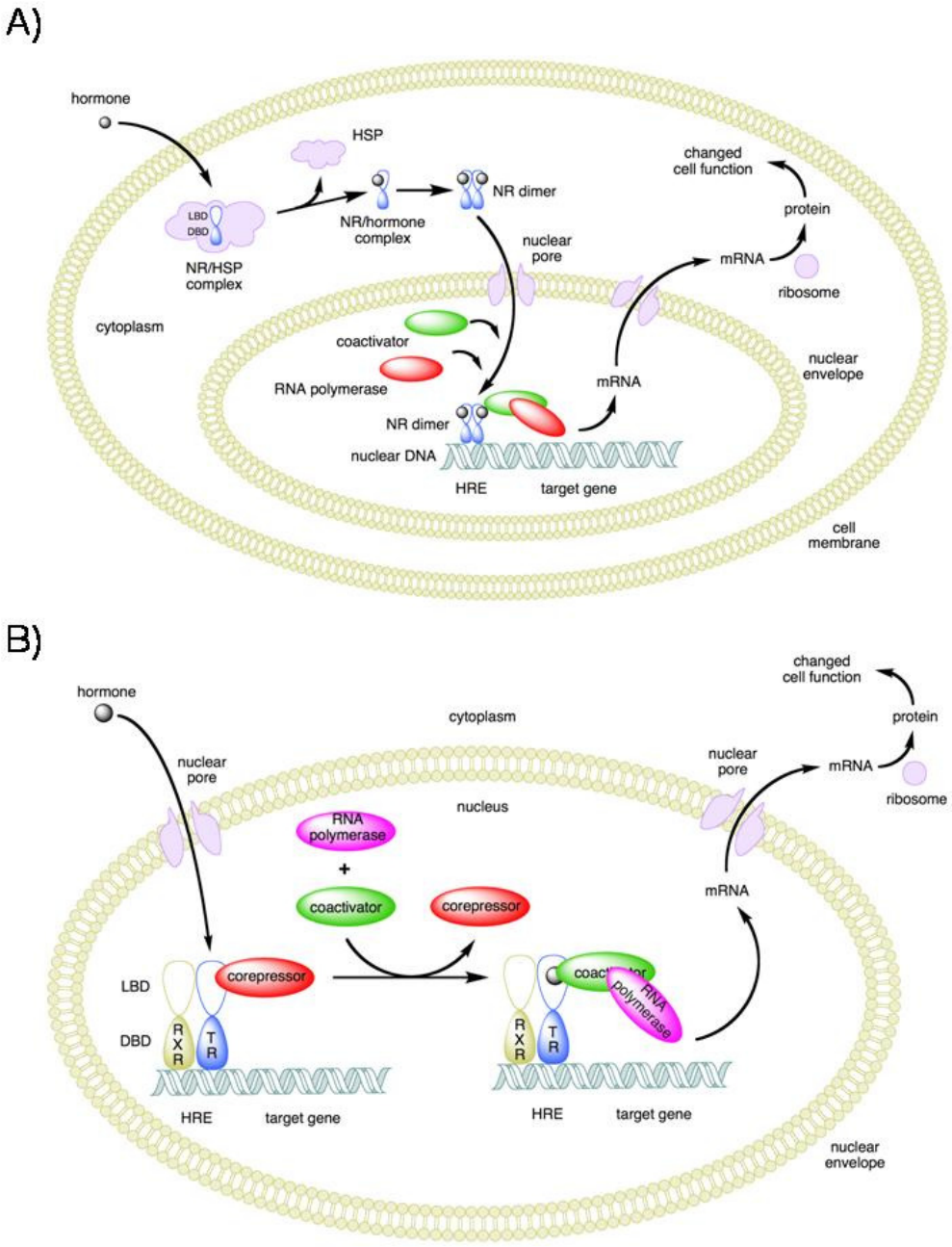

Figure 4: Molecular mechanism of class I and class II NRs. A) Typical mechanism of action of class I NRs, e.g. steroid receptors such as GR that are, in the absence of a ligand, associated with a heat shock protein (HSP) complex in the cytosol, after ligand binding conformational changes leads to the release of the HSP and to the nuclear translocalisation. In the nucleus the dimerised receptors bind to hormone response elements (HRE) and activate gene transcription. B) Typical mechanism of action of a class II NR, e.g. TR that are located in the nucleus and as heterodimers with RXR bound to the DNA. The association with corepressors is changed after ligand binding to a binding of coactivators which than triggers gene transcription. Taken from: http://en.wikipedia.org/wiki/Image:Nuclear_receptor_action.png and http://en.wikipedia.org/wiki/Image:Type_ii_nuclear_receptor_action.png

Chromatin remodelling and transcriptional regulation through nuclear receptors

Eukaryotic DNA is ordinarily refractory to transcription due to its organisation in nucleosomes. Nucleosomes are composed of two molecules of each of the four core histones (H2A, H2B, H3, H4), about 180 bp of DNA and a single molecule of a linker histone H1 (Kornberg&Lorch 1999). The DNA is wrapped around this histone octamer which interferes with many DNA transactions. Nucleosomes thus serve as general gene repressors and help to assure the inactivity of all genes that are not specifically positively regulated. Active promoters are associated with histones that are modified in various ways, including acetylation, phosphorylation and methylation and subsequently, nucleosomes are removed from these promoters (Boeger *et al.* 2005). Due to the loss of nucleosomes from transcriptionally active promoters, the RNA polymerase II (RNA pol II) transcription initiation complex is assembled on a naked DNA molecule. The RNA pol II transcription machinery consists of three components: a 12-subunit polymerase, capable of RNA synthesis and proofreading the transcript; a set of five general TFs, TFIIB, -D, -E, -F and –H responsible for promoter recognition and unwinding the promoter DNA; and a 20-subunit Mediator, which mediates regulatory information from activator and repressors proteins to the RNA pol II (Boeger *et al.* 2005).

NRs have a unique ability to bind to DNA although it is wrapped around the histone core. Unligated, DNA bound NRs interact in the nucleus with corepressors, that recruit proteins with histone deacetylase activities (HDAC) (Figure 5A). Deacetylated histones are associated with silenced regions of the genome through chromatin condensation resulting in transcription repression (Horwitz *et al.* 1996; Xu *et al.* 1999; Chen 2000; Laudet&Gronemeyer 2002; Chambon 2005; Trotter&Archer 2007). Ligand binding to the NR starts the series of events that arrives at the releasing of nucleosomal repression and the initiation of gene transcription. Coactivators are recruited to the DNA bound nuclear receptor and form a complex with histone acetyltransferase (HAT) activity. Acetylation of histones decondensate the chromatin, a necessary but not sufficient step for target gene transcription (Figure 5B). The modification of the chromatin environment of target genes provide access for the RNA Pol II transcription machinery (Horwitz *et al.* 1996; Xu *et al.* 1999; Laudet&Gronemeyer 2002; Chambon 2005; Trotter&Archer 2007). Subsequently, transcription activation is realised via the formation of a mediator SMCC/DRIP/TRAP complex with the NR and an interaction of that complex with general TFs, such as TATA-

box binding protein (TBP) and TFII (Horwitz *et al.* 1996; Xu *et al.* 1999; Laudet&Gronemeyer 2002; Chambon 2005; Trotter&Archer 2007). The assembly of the complete RNA pol II subsequently initiates target gene transcription (Figure 5C). Taken together, allosteric changes in the ligand bound nuclear receptor affect protein-protein-interaction and the recruitment cascades that lead to formation of complexes. This in the end can remodel the chromatin structure, modify recruitment and formation of transcription initiation complexes at target gene promoters and therewith positively or negatively regulate target gene expression.

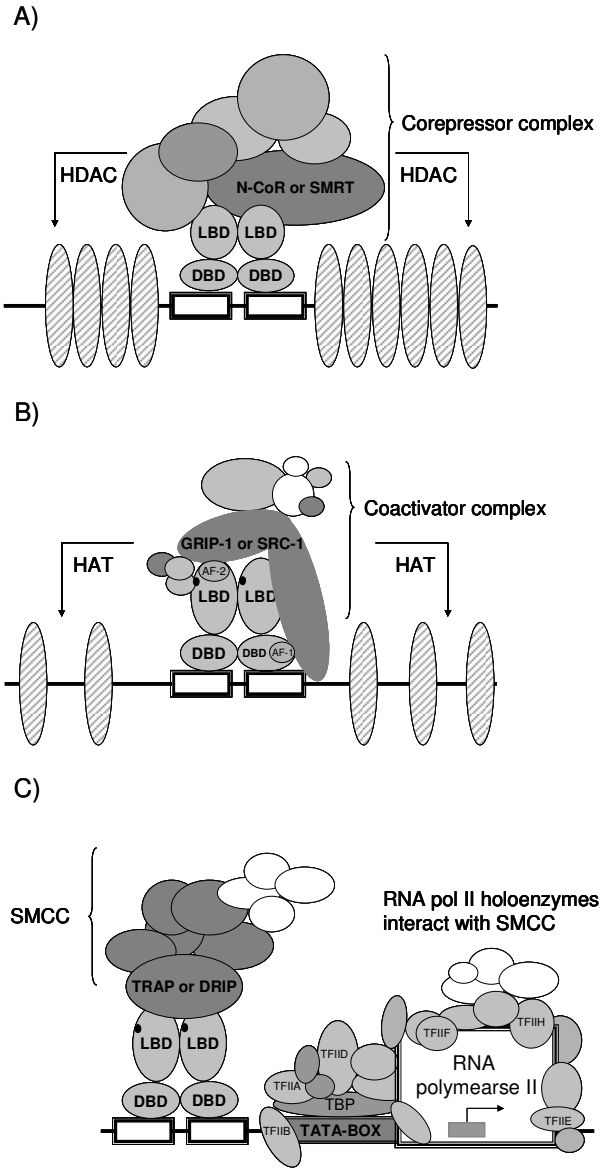

Figure 5: Molecular mechanisms of NR actions A) Transcriptional repression due to recruitment of corepressor complex with histone deacetylase (HDAC) activity. B) Transcriptional derepression due to recruitment of coactivator complex with histone acetyltransferase (HAT) activity. C) Transcription activation due to assembling of Srb and mediator protein-containing complex (SMCC) and interaction with RNA polymerase II (RNA pol II).

Hormone response elements

NRs regulate transcription by binding to HREs. These elements are often located in the 5'-flanking region of target genes close to the core promoter but in some cases they are present in enhancer regions several kilobases upstream of the transcription start site. All NRs recognise derivates of hexameric DNA core motives, 5´-AGAACA for steroid receptors or 5´-AGG/TTCA for remaining members of the superfamily. Due to optimisation of their interaction with this sequence, NR evolutionally either modifies residues or generates homo- or heterodimerisation interfaces (Laudet&Gronemeyer 2002). For dimeric HREs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IP) or direct repeats (DR). These configurations influence the receptor dimerisation pattern on an HRE (Figure 6A). Steroid receptors, for example, form symmetrical head to head arranged homodimers on palindromic HREs whereas non-steroidal receptors recognise HREs with different configurations, Pal, IP and DR (Aranda&Pascual 2001). The spacer length between two recognition sites can vary between one and five nucleotides and determine selectivity and specificity. Additionally, small differences in the half-site sequence and the sequence of the flanking extension of HREs also appear to be important for the determination of receptor binding efficiency (Mader *et al.* 1993).

Monomers, homodimers and heterodimers

Several orphan receptors such as RevErb, ROR, TLX and NGFI-B can bind as monomers with high affinity to HREs (Figure 6A). Monomeric HREs consist of a single copy of a core recognition sequence, mainly AGG/TTCA half site that is preceded by a 5'-flanking A/T-rich sequence (Glass 1994; Giguere 1999; Aranda&Pascual 2001). Monomeric NRs use the CTE to recognise their HRE. The CTE makes extensive contacts with the minor groove of DNA and effectively extend the contact surface of the DBD to HRE surrounding sequences.

Steroid receptors bind preferentially as homodimers to their HRE (Figure 6A). Two steroid receptor monomers bind cooperatively to their response elements (REs) and dimerise via interfaces localised in both DBD and LBD. Palindromic DNA repeats impose a symmetrical structure that results in a head-to-head arrangement of the DBDs with each DBD of the homodimer making analogous contacts with one half-site. Crystallographic analysis of the DBD of the GR and ER identified 2-3 residues in the N-terminal zinc-

finger, commonly referred to as P-box, that are involved in the direct contact with the DNA and responsible for the ER response element (ERE) *vs* GR response element (GRE) recognition (Mader *et al.* 1989). A second region located in the second zinc-finger, the D-box, was found to be important in the differentiation between the binding to a 3bp and a 0bp-spaced palindromic HRE and contribute to the DBD dimerisation interface (Yen *et al.* 1994; Laudet&Gronemeyer 2002).

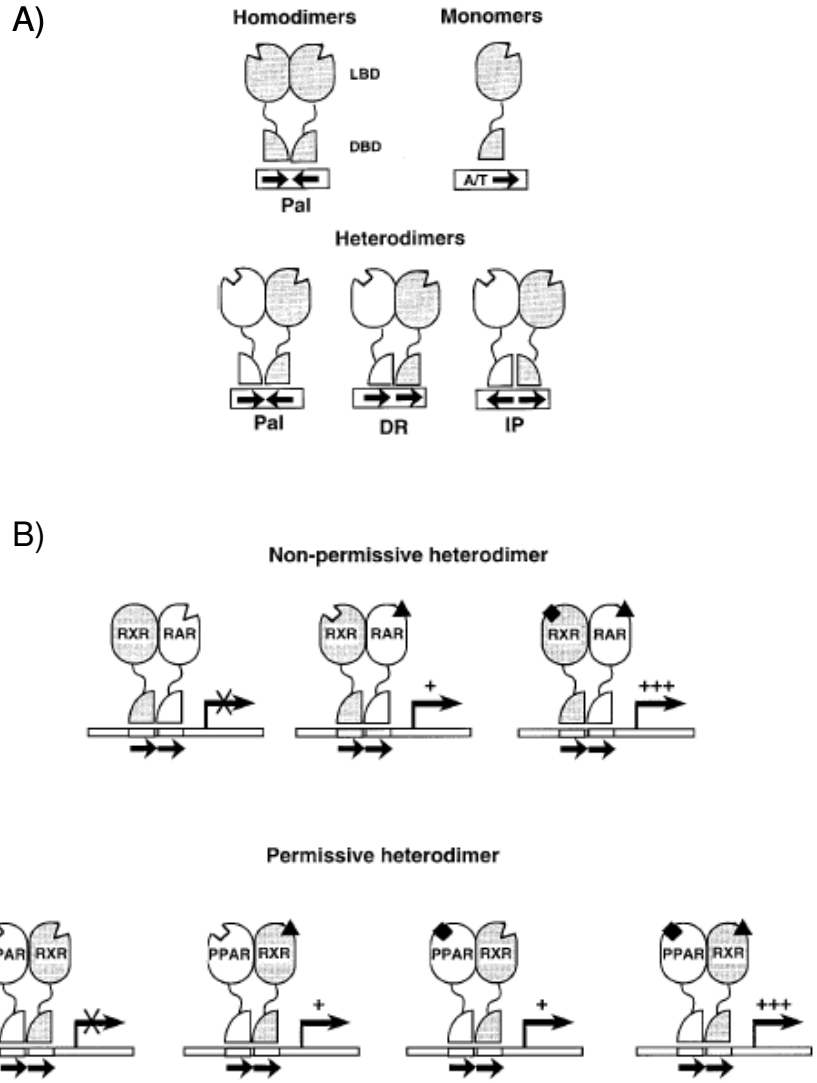

Figure 6: NR binding to HREs. A) Binding as monomers to half core motives flanked by a A/T rich sequence, homodimers to palindromic sequences and heterodimers to palindromic (Pal), direct repeat (DR) and inverted repeats (IP). B) Permissive and non-permissive heterodimers. In non-permissive heterodimers, such as RXR/RAR, heterodimerisation precludes binding of the RXR ligand. Binding of ligand to the RAR moiety causes receptor activation and allows binding of the RXR ligand resulting in synergism. Permissive heterodimers, such as PPAR/RXR, can be activated by ligands of either RXR or its partner receptor and are synergistically activated in the presence of both ligands. (Aranda&Pascual 2001)

Although several non steroidal receptors also bind as homodimers, they preferentially bind to heterodimeric HREs (Figure 6A). RXR function in these cases as a promiscuous partner for different receptors. Typical heterodimeric receptors such as TR, VDR, LXR, CAR, PPAR and RAR can bind to their REs as homodimers but heterodimerisation with RXR strongly increase the efficiency of DNA binding and transcriptional activity (Kliewer *et al.* 1992a; Kliewer *et al.* 1992b; Cooney *et al.* 1993; Mangelsdorf&Evans 1995; Tzameli *et al.* 2003). Some monomeric receptors such as NGFI-B and homodimeric receptors such as chicken ovalbumin upstream promoter-tramscription factor (Coup-TF) can also form heterodimers with RXR resulting in changes in the transcriptional activity (Kliewer *et al.* 1992a; Glass 1994; Giguere 1999). Theoretically, four different states of heterodimer occupancy by ligands can be predicted: both partners unoccupied, only RXR occupied, only the partner receptor occupied and both partners occupied. However, three types of heterodimeric complexes exist: non occupied heterodimers, nonpermissive heterodimers that can be activated only by the partner not by RXR and permissive heterodimers. The last complex can be activated by ligands of either RXR or the partner receptor and are synergistically activated in the present of both ligands. Figure 6B demonstrate the different RXR heterodimers with their respective transcriptional activation (Glass 1994; Aranda&Pascual 2001).

Although RXR is the most prominent heterodimerisation partner, heterodimerisation was also shown for TR/RAR, TR/Coup-TF, RAR/Coup-TF (Berrodin *et al.* 1992; Glass 1994) and for some steroid receptor such as GR/MR (mineralocorticoid receptor) (Liu *et al.* 1995), GR/AR (androgen receptor) (Chen *et al.* 1997) as well as Coup-TF/GR (De Martino *et al.* 2004b) and Coup-TF/ER (Metivier *et al.* 2002). All of these heterodimeric interactions result in transcriptional modifications that are different from the action caused by the receptor homodimer. For example, GR homodimers enhance the transcription of GR target genes whereas the Coup-TF/GR heterodimer leads to a suppression of GR induced gene activation. Additionally, it was shown that only GRα but not the other splice variant GRβ enhanced Coup-TFII induced transactivation of a Coup-TFII target gene (De Martino *et al.* 2004b). This inverted effect of heterodimers was also shown by other studies investigating the heterodimeric functions of GR/MR (Ou *et al.* 2001) and Coup-TF/ER (Metivier *et al.* 2002).

Interaction of nuclear receptor with other factors

NR interactions are complex and occur with a variety of proteins such as i) general transcription factors, ii) sequence specific transcription factors, ii) coactivators and iii) corepressors.

General transcription factors

General TFs interact with the core promoter elements such as the TATA-box and are recognised by RNA pol II. NRs can directly interact with these factors and other components of the transcription machinery. It has been shown that the TBP interact with several nuclear receptors and that overexpression of TBP enhances ligand-dependent transactivation (Sadovsky *et al.* 1995; Schulman *et al.* 1995; Fondell *et al.* 1996; Wu *et al.* 1999; Warnmark *et al.* 2001; Kumar *et al.* 2004a; Copik *et al.* 2006). These interactions initiate recruitment of basal components of the transcription initiation complex to the promoter and subsequently enhance gene transcription. In addition, investigations of TBP and GR interactions described that their crosstalk is directly realised via the GR AF-1 domain. Endogenous GR regulated genes and genes with the mouse mammary tumor virus (MMTV) promoter are induced in an TBP dependent manner without recruitment of other transcription machinery factors (Copik *et al.* 2006).

Sequence specific transcription factors

Sequence specific TFs such as CREB, AP-1, NFkappaB, Oct-1 and NF-1 bind to REs that are located close to HREs and synergise the effect of NRs (Bruggemeier *et al.* 1991; Pfahl 1993; McKay&Cidlowski 1999; Kassel&Herrlich 2007). These modulators of gene transcription can be distinguished by the nature of the target regulatory element. The different mechanisms of crosstalk are depicted in Figure 7 and can take place on a composite RE, an overlapping RE or a RE without sites for the modulating TFs (Kassel&Herrlich 2007).

Composite REs (Figure 7A) bind both the NR and the other TF. The resulting interaction can be either positive or negative. Examples of such modulation are the GR and Oct-1 synergistic action on the MMTV promoter (Bruggemeier *et al.* 1991) or the mutually influence of GR and CREB at each other's binding to their REs (Imai *et al.* 1993). In the positive crosstalk, the modulating transcription factor can strongly increase the transcription mediated by the nuclear receptor (Espinas *et al.* 1994) . Crosstalk on composite elements can also result in transcriptional repression. For example, GR

represses the C/EBP-mediated induction of the glutathione-S-transferase A2 gene via binding of response elements and recruitment of the corepressors silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Ki *et al.* 2005).

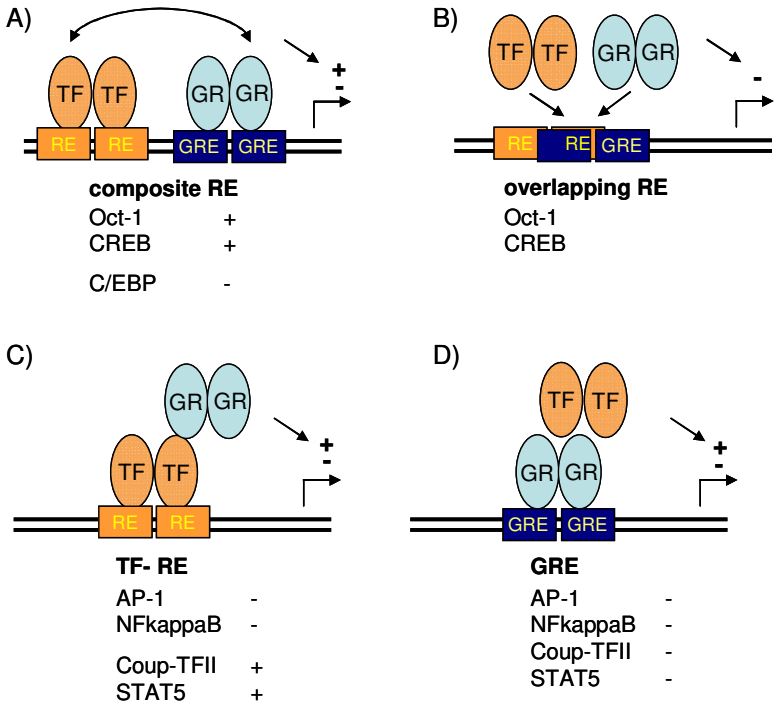

Figure 7: The different mechanisms of mutual crosstalk between GR and other transcription factors (TFs). GR and other TFs modulate each other's activity in a DNA binding-dependent (A and B) or DNA binding-independent manner (C and D).

Overlapping REs (Figure 7B) such as the GRE and TATA-box, Oct-1 or CRE results in a inhibition of transcription by GR binding to its GRE and negatively regulates osteocalcin (Stromstedt *et al.* 1991), prolactin (Sakai *et al.* 1988) or glycoprotein hormone α-subunit gene (Akerblom *et al.* 1988), respectively.

The last interaction mode is the crosstalk between NRs and other TFs at REs without binding sites for the modulating factor (Figure 7C-D). This interaction results, in the vast majority of cases, in gene repression and is termed as transrepression. GR can repress genes without GRE but containing TF relevant REs, and verse versa, TFs can repress genes without their regulatory element only containing a GRE. This finding suggests that the binding of only one partner to the DNA of the target genes is sufficient for transrepression (Kassel&Herrlich 2007). Important studies investigated these interactions for GR/AP-1 and GR/NFkappaB and could demonstrate that the DNA binding domain of GR is not important for the crosstalk but may be involved in the protein-protein-interaction with the

transcription factor (Yang-Yen *et al.* 1990; Heck *et al.* 1994; Heck *et al.* 1997). For the interaction between GR and STAT 5 it could be demonstrated that, depending on the levels of activated GR, a binding of GR to its HRE is not necessarily required. At low GR levels, DNA binding was necessary whereas at high GR levels GR DNA binding was dispensable (Doppler *et al.* 2001). The mechanism described for GR can be also applied to other NRs such as AR, ER, PR as well as the orphan receptor Coup-TF (Uht *et al.* 1997; McKay&Cidlowski 1999; Xing *et al.* 2002). Through these interactions NRs can influence transcription rates and hormone dependent responses can be restricted to cells and tissues expressing a appropriate set of TFs (Aranda&Pascual 2001).

Coactivators

If the crosstalk between NRs and TFs are necessary but not sufficient, coactivators act as bridging molecules that stabilise the interaction and mediate the transcriptional specificity, without binding to DNA themselves. The most abundant coactivators are proteins with a molecular mass of 140 and 160 kDa, known as the p140 and p160 family. The p160 family include important and well characterised factors such as steroids receptor coactivator 1 (SRC-1) and glucocorticoid receptor interacting protein 1(GRIP-1) (Aranda&Pascual 2001). Coactivators enhance ligand-dependent transcriptional responses and constitute common limiting factors recruited by the ligand bound receptor (Horwitz *et al.* 1996; Voegel *et al.* 1996). There are other potential coactivator functions as reviewed by Kassel and Herrlich with regard to the possible involvement of coactivators in transrepression (Kassel&Herrlich 2007). GRIP-1 but not SRC-1 was demonstrated to enhance GC mediated repression of AP-1 and NFkappaB dependent transcription after recruitment together with GR to the promoter (Rogatsky *et al.* 2002). Additional coactivators carry out subsequent downstream reactions in the transcription process, such as RNA processing, stabilisation and turnover of receptor-coactivator complex (Smith&O'Malley 2004).

Corepressors

Although there are far fewer corepressors, these proteins serve important roles in negative regulation of NR-dependent gene expression. NRs such as RAR, TR, Coup-TFs and dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1) repress basal transcription in the absence of their cognate ligands via binding to their HREs (Aranda&Pascual 2001). This repression is mediated by binding of SMRT and NR corepressor (NCoR) to the unligated NR. After ligand binding

conformational changes in the NR cause the dissociation of corepressors and recruitment of coactivators. This finding was supported by the analysis of the binding motifs implicated in the interaction with the NR. Corepressors and coactivators bind to similar or overlapping sites in the LBD of the receptor. However, the NH_2-terminal extension of the α-helix constitutes a critical distinction in the alternative ligand-dependent recruitment of coactivators or the ligand-independent binding of corepressors. Recent studies showed that coregulators together with NRs are not only important for direct gene regulation, but also for histone acetylation, deacetylation and chromatin remodelling (Aranda&Pascual 2001). The diversity of coativator and coreceptor functions provide additional knowledge in understanding the pharmacology of tissue specific actions of hormones and their receptors (Smith&O'Malley 2004).

Alternative splicing of nuclear receptor and different isoforms

Splicing events are used to generate a highly diverse proteome from a relative limited genome. Alternative splicing is frequent and it is estimated that more than 60% of eukaryotic gene transcripts are alternatively spliced (Thanaraj 1999; Kan *et al.* 2001; Clark&Thanaraj 2002). These events are specific to tissue type, developmental stage or physiological condition and up to 15% of human genetic diseases are caused by point mutations occurring at or near splice junctions (Clark&Thanaraj 2002). Four major alternative splicing modes can be defined, i) exon skipping, ii) 3'-alternative splicing, iii) 5'-alternative splicing and iv) intron retention (Figure 8). Exon skipping is the most commonly known alternative splicing mechanism where an alternative exon is either included or spliced out from the final transcript. In the 3'-alternative splicing and 5'-alternative splicing, different splice sites either at the 3' or the 5'end of an exon are used to create the alternative isoform. In this splicing mechanism, two or more alternative 5' splice sites compete for joining to two or more alternate 3' splice sites. Intron retention is the preservation of the entire intronic sequence within some final processed mRNA transcripts (Huang *et al.* 2005). In more complex pre-mRNA systems more than one alternative splice mode can be used to generate many different products from a single coding sequence.

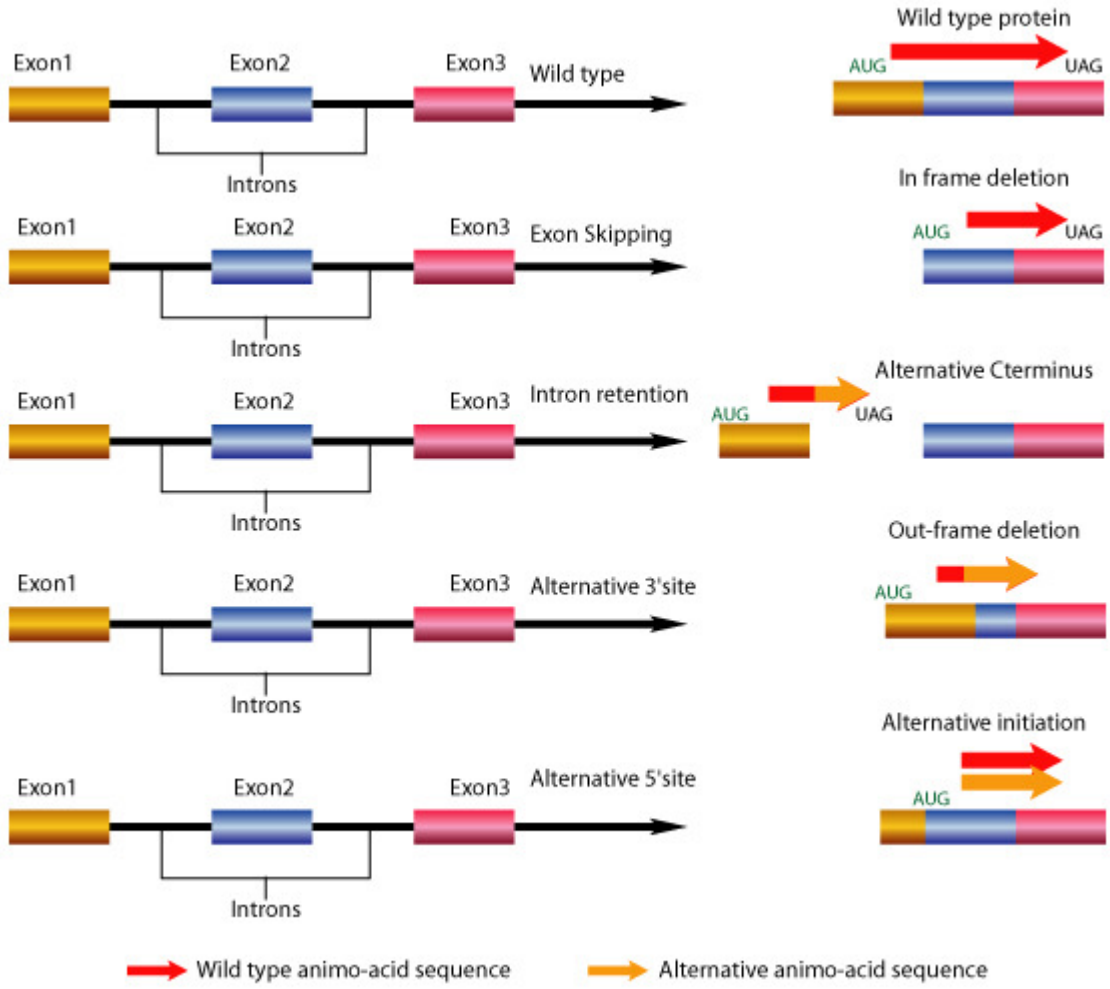

Figure 8: Alternative splicing mechanisms. Exon skipping: exon 1 is alternative spliced out leading to an in frame amino acid sequence deletion. Intron retention: In the final transcript the intronic sequence is retained and leads for example to an alternative C-terminus. In the 3' and 5'- alternative splicing different selection of the splicing sites causes alternative isoforms. Taken from: http://www.sciencesoftware.com/pages.php?pageid=603

Splicing mechanism and its regulation

Pre-mRNA splicing is a critical step in which intron sequences were removed and exons are jointed together to generate the mature protein-coding mRNA transcript. For intron removal the spliceosome recognise intron specific sequences. These splice donor and acceptor sites start mostly with GT and end with AG, also, there are exceptions GC-AG and AT-AC (Clark&Thanaraj 2002). Alternative splicing pattern use amongst other mechanisms protein factors that either stimulate the use of new or alternative splice sites or block the utilisation of default sites (House&Lynch 2008). During the splicing processes the use of multiple non-coding variable exons generates transcript diversity and coding exons create protein diversity in a tissue specific manner. Studies have shown that

alternative splicing regulation not only depends on the interaction of splicing factors with splicing enhancers and silencers in the pre-mRNA (House&Lynch 2008), but also on the coupling of transcription and splicing. This coupling is possible because splicing is often cotranscriptional and promoter identity and occupation may affect alternative splicing. Different mechanisms by which transcription regulates alternative splicing have been demonstrated i) the recruitment of splicing factors, ii) changes in the rate of transcriptional elongation that in turn affect the timing in which splice sites are presented to the splicing machinery, iii) changes in chromatin structure and iv) other factors effecting transcription elongation (House&Lynch 2008; Wang&Burge 2008). Steroid hormone receptors have been shown to induce formation of transcriptional complexes that stimulate transcription and control the nature of the splice variants produced by these genes. Ligand bound steroid hormone receptors and their coregulators recruited to a promoter may mediate the splice decisions and determine the alternative splice variants produced by the target gene. The stimulation of the production of specific variants is dependent on several parameter, such as nature and organisation of the gene, promoter context, specific set of available coregulators, modifications of coregulators and cellular context (Auboeuf *et al.* 2002; Auboeuf *et al.* 2004).

Detection of alternative splice events

Alternative splicing can be detected using expressed sequence tags (ESTs), the mRNA sequence and the protein sequence ideally from different species, because the mature mRNA embeds the alternative splicing information (Huang *et al.* 2005). Currently, the most efficient methods for large scale detection of splice variants include computational prediction methods, microarray analysis and sequencing. Microarray based splice variant detection is the most popular method currently in use. The highly parallel and sensitive nature of microarrays make them ideal for monitoring gene expression on a tissue-specific, genome-wide level (Nembaware *et al.* 2008). Microarray based methods provide a robust, scalable platform for high-throughput discovery of alternative gene splicing. A number of novel transcripts were detected using microarray based methods that were not detected by ESTs using computational methods (Purdom *et al.* 2008). Another commonly used method to discover novel isoforms is reverse transcription PCR followed by sequencing. This is a powerful approach and can be effectively used for analysing a small number of genes. However, it only provides a limited view of gene structure, is laboratory-intensive, and does not easily scale to thousands of genes or hundreds of tissues.

Among more than 15.000 human protein-coding genes more than 50%, explicitly 7674 genes were observed to consist of multiple putative promoter regions (PPRs). In the mouse, only 28% or 3816 genes of all protein-coding genes were found to contain PPRs (Kimura *et al.* 2006; Tsuritani *et al.* 2007). However, sequence comparisons showed that only 33% of the human PPRs are conserved in the mouse and that these conserved PPRs were enriched in CpG islands and poor in TATA-like elements (Tsuritani *et al.* 2007). Another recent study in human cell lines identified more than 15000 human protein coding and non-coding genes that are effected by alternative splicing (Johnson *et al.* 2003; Sultan *et al.* 2008). High throughput RNA sequencing of the human transcriptome identified over 90.000 splice junctions of which more than 4.000 were defined as novel. Many of the new junctions were associated with actively transcribed genes exhibiting more exons than average and pointing to rare splice events. Approximately 2000 genes had no splice junctions, which was correlated with the fact that those genes contain fewer exons and a lower activity than average. It was also shown that sequencing compared to microarray analysis was more sensitive, detecting 25% more genes and that approximately 6% of all splice junctions could be identified as alternative splicing events (Sultan *et al.* 2008).

Splicing and nuclear receptors

Members of the NR superfamily, such as GR (McCormick *et al.* 2000; Turner&Muller 2005; Turner *et al.* 2006; Presul *et al.* 2007), PPAR (Sabatino *et al.* 2005; Lundell *et al.* 2007), CAR (Savkur *et al.* 2003; Arnold *et al.* 2004), PXR (Fukuen *et al.* 2002) and VDR (Crofts *et al.* 1998) use alternative splice events to increase receptor functionality on gene regulation. One of the most intensively studied NRs, the GR, generates from over 150 kbp human genomic sequence i) five 3'variable mRNA transcripts (Zhou&Cidlowski 2005), ii) 13 different splice variants, based on 9 multiple first exon (Turner&Muller 2005; Presul *et al.* 2007) and iii) 16 protein isoforms using alternative translation initiations (Lu&Cidlowski 2006). Only few studies investigated until now the importance and function of GR alternative first exon transcript variants. In other NRs such as CAR alternative splicing generates 12 spliced transcripts. The resulting LBD isoforms of CAR demonstrate a loss of function phenotype, however, two variants retain distinct functional activities (Arnold *et al.* 2004). Interestingly, it was demonstrated that in mice interindividual variation in splice isoform selection is tightly controlled, shows tissue-specific and age-related changes to environmental variations and provides evidence for a robust, homeostatic control mechanism (Chisa&Burke 2007). NR isoforms are as well

associated with physiological variations and susceptibility to diseases. It has been demonstrated that splice variants of AR are implicated in androgen insensitive syndrome (Ris-Stalpers *et al.* 1994); of GR linked to asthma, childhood leukaemia and systemic lupus erythematosus (Hamid *et al.* 1999; Haarman *et al.* 2004; Piotrowski *et al.* 2007); and of ER changed in Alzheimer´s disease and breast cancer (Poola&Speirs 2001; Ishunina&Swaab 2008).

Crosstalk between neuroendocrine and immune system

The neuroendocrine systems comprise a tightly controlled network of specialised neurons and endocrine cells regulating metabolism, reproduction, blood pressure, mood, stress, sleep and homeostasis. The central nervous system (CNS) has direct endocrine activity or controls endocrine cells through the release of neurotransmitters and hormones. The CNS is also involved in the modulation of inflammatory and immune responses and excerpts its regulatory action at different levels: i) systematically through the hypothalamic-pituitary adrenal (HPA) axis, ii) regionally through the release of neurotransmitters in immune organs and iii) locally through nerve endings and neuroendocrine cells at the site of inflammation. In turn inflammatory mediators such as cytokines signal back to the CNS and enforce a bidirectional crosstalk (Figure 9). A wide variety of stressors activates the HPA axis which main components are the hypothalamus, the anterior pituitary gland and the adrenal gland. Corticotrophin-releasing hormone (CRH) is secreted from the hypothalamus and stimulates the expression of adrenocorticotropin hormone (ACTH) in the anterior pituitary gland. ACTH induces the expression and release of GCs in the adrenal glands. GCs negatively feed back to the HPA axis and control the HPA axis activity by maintaining basal and stress-related homeostasis (Eskandari&Sternberg 2002; Webster *et al.* 2002; Di Comite *et al.* 2007).

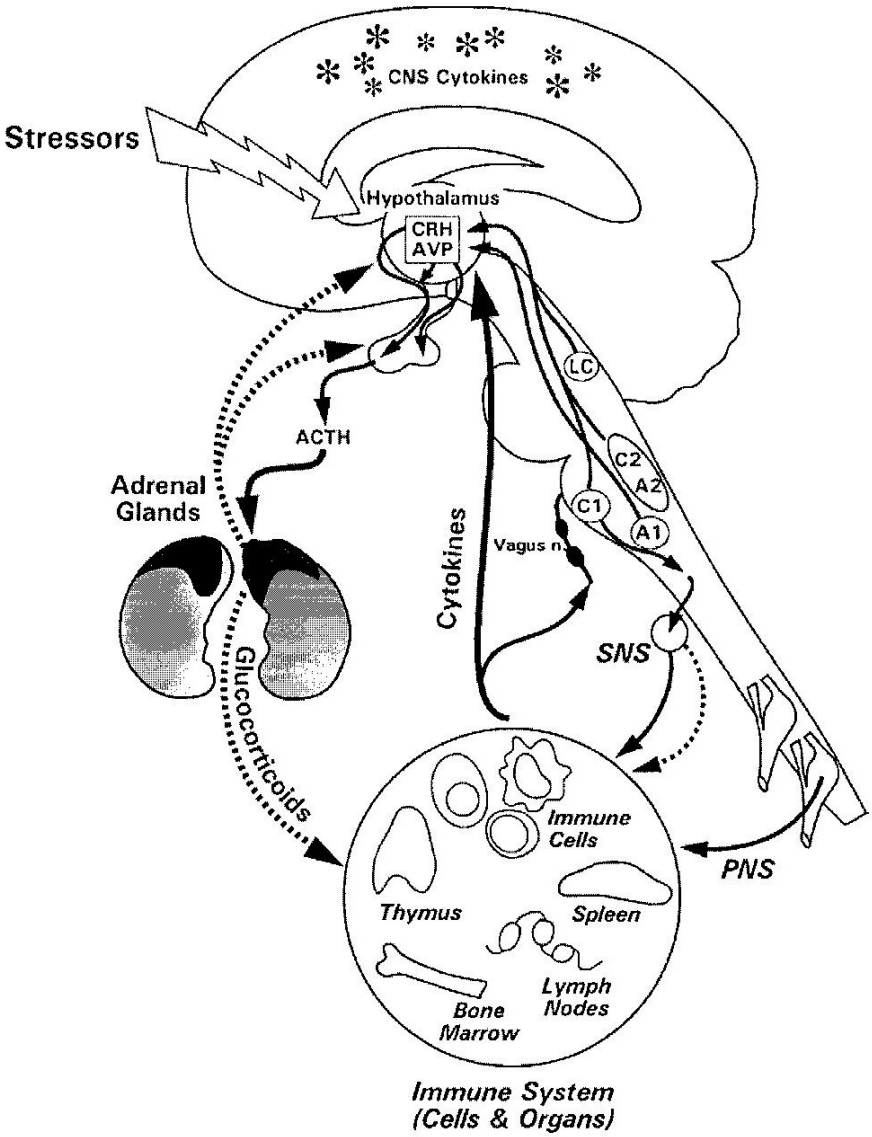

Figure 9: Crosstalk between brain and immune system, including HPA axis, sympathetic nervous system (SNS), peripheral nervous system (PNS) and cytokine feedback to the brain. (Webster *et al.* 2002)

Glucocorticoids and their receptors in the neuroimmune modulations

GCs regulate a variety of immune cell functions and expression of immune molecules with mainly immunosuppressive effects. This includes the regulation of immune related genes such as cytokines, cell adhesion molecules, chemoattractants and inflammatory mediators as well as a more general suppression of maturation, differentiation and proliferation of immune cells (Webster *et al.* 2002). GCs also influence physiologically the activity of neurons, memory functions, cardiovascular tone and sleep and have been associated with psychosomatic disorders such as depression, insomnia and anxiety (De Bosscher *et al.* 2008). The numerous actions of GCs are mediated by two closely related NRs, the GR and

the MR. While MR has a high affinity for naturally occurring GCs, such as cortisol, the GR binds avidly synthetic steroids such as dexamethasone but has a lower affinity for endogenous hormones (De Kloet *et al.* 1998). The GR shows a widespread tissue distribution, whilst the MR has a more restricted expression. However, both receptors are coexpressed in certain brain areas including the hippocampus where they control neurotransmission and plasticity in a GC mediated manner (De Bosscher *et al.* 2008). In general MR mediated effects are associated with an increased excitability of neuronal networks whereas GR activation sensitises the neurons for stimuli that decrease neuronal activity (de Kloet *et al.* 1999). When coexpressed, GR and MR may form heterodimers and as such add another level of complexity for regulation of learning processes, memory and mood. Disturbance of the GC actions at any level lead to an imbalance of the neuroendocrine and the immune system, and enhance the susceptibility to infection, inflammation and stress related disorders such as depression. Mutations of the GR have been associated with GC resistance, which is characterised by generalised or partial, target-tissue insensitivity to GCs. The differential diagnosis of GC resistance includes mild forms of Cushing's disease, pseudo-Cushing's states such as anxiety and melancholic depression as well as essential hypertension. However, it is difficult to distinguish mild forms of GC resistance from other mild forms of hypercortisolism such as Cushing's syndrome, anorexia nervosa, hypercortisolemic melancholic depression and intensive exercise (Charmandari *et al.* 2008). Other GC associated diseases are immune-related disorders including rheumatoid arthritis, asthma, inflammatory bowel syndrome; brain disorders such as Alzheimer's disease and Parkinson's disease; and cancer including acute T-cell leukemia and childhood acute lymphoblastic leukemia (ALL) (Tenbaum&Baniahmad 1997; Tissing *et al.* 2005; De Bosscher *et al.* 2008).

Impact of other nuclear receptors in the regulation of neuroimmune system

Other hormones such as estrogens were shown to have controversial and widely varying immunomodulating actions. On one side, estrogens inhibit bone resorption and suppress inflammation in several animal models of chronic inflammatory diseases. On the other hand, they play an immunosupportive role in trauma/sepsis, have proinflammatory effects in some chronic autoimmune diseases in humans and increase the risk of developing inflammatory diseases in women (Webster *et al.* 2002; Straub 2007). During early brain development especially for the determination of gender-specific differences in the HPA function estrogens play an important regulatory role (Patchev *et al.* 1995). They act as so

called “nature`s psychoprotectant” and have implications in female mood disorders and postmenopausal depression (Riecher-Rossler&de Geyter 2007). Beneficial effects of estrogens are reported in several mental and neurodegenerative diseases as well as multiple sclerosis and osteoporosis (Czlonkowska *et al.* 2006; Riecher-Rossler&de Geyter 2007). However, long use of estrogen hormone therapy is associated with increased risk of cancer including breast tumours. The actions of estrogens are mediated by the NRs; ERα and ERβ, which both are expressed in the brain and throughout the immune system, but exerting different effects. For example, ERβ plays a crucial role in the brain development and maintainance whereas ERα seems to be implicated in a variety of cellular events associated with neuroprotection (Marin *et al.* 2005).

As well as the steroid receptors, other members of the NR superfamily such as RAR ROR, PPAR, LXR and VDR play important role in the regulation of immune responses (Deluca&Cantorna 2001; Dzhagalov *et al.* 2004; Hammad *et al.* 2004; Mathieu *et al.* 2004; Kota *et al.* 2005; Wang&Wan 2008). PPARs are expressed throughout the cells of the immune system and are most effective in reducing chronic inflammatory processes such as inflammatory bowel disease, multiple sclerosis, Alzheimer’s disease and arthritis (Glass&Ogawa 2006; Wang&Wan 2008). LXR also shows anti-inflammatory effects via reciprocal repression of inflammatory genes upon bacterial, LPS or cytokine stimulation. The beneficial effects of anti-inflammatory LXR-activators could be also shown in neurodegenerative disease such as Alzheimer’s disease (Wang&Wan 2008). VDR is known as immunosupressants and its ligand, the 1,25-dihydroxyvitamin D3 was used successfully as treatment of autoimmune disorders such as rheumatoid arthritis and autoimmune encephalomyelitis in mice models (Deluca&Cantorna 2001). These studies showed that T cell mediated immunity can be regulated by exogenous administration of vitamin D and that the absence of that hormone can increase the incidence and severity of autoimmune diseases (Deluca&Cantorna 2001; Mathieu *et al.* 2004). Interestingly, non steroidal NRs are also involved in regulatory processes in the brain such as induction and early development as well as differentiation (Ruberte *et al.* 1993; Pereira *et al.* 2000; McGrath *et al.* 2004). For example, PPARs are expressed in the adult and developing brain, act as neuroprotective via direct influencing neuron cell viability and differentiation and may provide protection in neurodegenerative diseases (Heneka&Landreth 2007). RARs have been implicated in the early development of the nervous system, are expressed in the adult brain and show some neuroprotective implications in neurodegenerative diseases (Lopes da Silva&Burbach 1995; Chen&Napoli 2008; van Neerven *et al.* 2008).

Coup-TFs, as true orphan receptors, are important for the growth and differentiation of vertebrate brains. Coup-TFs are implicated in the neural-specific programming and loss-of-function mouse mutants died due to impaired embryonic development (Lu *et al.* 1994; Pereira *et al.* 2000). A deregulation of NR functions lead to disturbance of neuronal communications, signal transduction pathways and therewith to an increase of severe disease such as cancer, autoimmunity and neurological disorders (Tenbaum&Baniahmad 1997; McGrath *et al.* 2004; Matsuzaki *et al.* 2006; Riggs *et al.* 2006; Riecher-Rossler&de Geyter 2007; De Bosscher *et al.* 2008). Due to their widespread distribution, their enormous regulatory potential, the implications of agonistic and antagonistic ligands and the high numbers of interactions with other factors and pathways, NRs are in the spotlight of nowadays research.

Research objectives

As described in the previous paragraphs the molecular mechanisms of NR actions are highly complex and influenced by splicing events. These are used to generate a highly diverse proteome from a relative limited genome and are specific to tissue type, developmental stage or physiological condition. Up to 15% of human genetic diseases are caused by point mutations occurring at or near splice junctions and NR splice variants are associated with susceptibility to diseases such as autoimmune disorders, major depression and neurodegenerative diseases. In this thesis we aimed to analyse the role of NR splice variants relevant in stress responses. These are amongst others controlled by the immune system. Therefore, the first objective was to detect and correlate the expression of non-steroidal NRs in human immune cells. For their distribution and role throughout the immune system only few data are available in the literature, compared to the steroidal NRs such as GR. We expect a correlation of some receptors within the immune cells, influencing the regulation of stress responses by the formation of heterodimers. Receptor variants are known to positively or negatively regulate these interactions. We investigated possible isoforms of two NRs expressed in the immune system, the well characterised GR, in this thesis used as paradigm, and the Coup-TFII, which was coregulated with other NR in the first objective. Although alternative splicing for the GR was investigated by many groups, the 5´untranslated region was only poorly analysed. The second objective was to describe different GR alternative first exons, show their tissue distribution and extend this to other species providing further evidence for a conserved and tightly controlled function of these transcript variants in the immune regulation. These splice variants are not translated into protein but might influence protein functions including protein-protein interactions. One important region for these interactions is the N-terminal transactivation domain in exon 2 of the GR. The third objective was to elucidate GR protein variants with possible sequence modifications coded by exon 2. Tissue distributions will give evidence where normal GR activity can be impaired by new protein variants. In the last objective the non-steroidal and highly evolutionarily conserved orphan receptor Coup-TFII was investigated to show that splicing in the 5´untranslated region and sequence modifications in the protein can also occur in orphan NRs. The tissue distribution and intracellular location of the Coup-TFII variants will give insights of possible isoform functions that are expected to differ from the classical receptor. To investigate the relevance of Coup-TFII and its isoforms in stress responses, an immune cell line will be stimulated with stress

hormones. The major aim of this thesis is (i) to investigate NR variants in the immune system, (ii) to provide explanations on the possible increase of the enormous regulatory potential through interaction with other transcription factors and coregulators by NR variants, and (iii) to embed the variants in the network of the neuroendocrine and immune system.

Outline of the thesis:

In Chapter 2 the expression of non steroidal NRs was investigated in peripheral blood mononuclear cells (PBMCs) and isolated subgroups, such as CD4 and CD8 positive T cells, CD19 positive B lymphocytes and CD14 positive monocytes of healthy donors. PBMC specific and tissue specific expressions were determined using quantitative PCR. Protein expression was tested in different cell subsets as well as nuclear and cytoplasmatic fraction of PBMC. The expression pattern of each of the 24 investigated NRs was correlated to examine possible coregulations in the four analysed subgroups of immune cells. Chapter 3 provides a review of the current knowledge of alternative splicing at the 5' end of the GR. The 5' untranslated region in the CpG islands upstream of the GR exon 2 was analysed in five different species with respect to homogeneity and common TF binding sites. Finally, the implication of variable first exons in transcriptional control and disease were discussed. In Chapter 4 new transcript variants in the coding exon 2 of GR were investigated using PCR primers located at different positions within exon 2. Tissue specificity was analysed within different human biopsy samples and blood cells. A new GR protein isoform, generated due to an internal deletion, was identified in different cell lines with Western Blot techniques. The aim of Chapter 5 was to provide evidence that the 5' heterogeneity of the GR can be extended to the orphan receptor Coup-TFII. We analysed the human Coup-TFII genomic sequence concerning possible 5' transcript variants as well as protein isoforms. Tissue specific distribution was tested with PCR in different human tissue and brain biopsies as well as human PBMCs. Gene expression studies in stimulated THP-1 cells were used to investigate the implication of different Coup-TFII forms in immune regulation. To determine intracellular localisation of the identified protein forms, transfections of different cell lines were performed with green fluorescent protein tagged to both Coup-TFII forms. In Chapter 6 the major findings of this thesis are discussed in a broader context, summarised and future perspectives are given.

Chapter 2:

Nuclear receptors in human immune cells: Expression and Correlation

Andrea B Schote[1,2], Jonathan D Turner[1] , Jang Schiltz[3], and Claude P Muller[1,2,]

1. Institute of Immunology, Laboratoire National de Santé, 20A rue Auguste Lumière, L-1950 Luxembourg
2. Department of Immunology, Graduate School of Psychobiology, University of Trier, D-54290 Germany
3. Applied Mathematics Unit, University of Luxembourg, 162A, avenue de la Faïencerie, L-1950 Luxembourg

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Abstract

Nuclear receptors (NR) are key modulators of gene transcription. Their activity is ligand induced and modulates a large variety of tissue-specific cellular functions. However, for many NR little is known about their role in cells of the immune system. In this study, expression patterns and distribution of 24 NR were investigated in human peripheral blood mononuclear cells. We provide the first evidence of the expression of the 12 receptors CAR, CoupTFα, CoupTFβ, FXR, GCNF, HNF4α, PPARβ/δ, PXR, RevErbβ, TR2, TR4 and TLX in highly purified CD4, CD8, CD19, CD14 cells. The expression profile of RevErbα and LXRα previously observed in B cell and macrophages respectively has been extended to CD4, CD8 and CD14 cells. Except for RARβ, which was absence in any of the cells tested, our results suggest an almost ubiquitous expression of the NR in the different cell lineages of the immune system. The expression of CAR, CoupTFα, FXR was also confirmed at a protein level and despite conspicuous mRNA levels of HNF4α, only low levels of this receptor were detectable in the nuclear fraction of PBMCs. Expression of the latter receptors was mostly only a fraction (4-20%) of their expression in the thyroid gland, the adrenal gland, the lung or subcutaneous adipose tissue. The Spearman rank order correlation test was performed to examine the correlation in expression between individual nuclear receptor pairs in the four cell types for several donors. Distinct correlation patterns were observed between receptor pairs in the individual cell types. In CD4 T cells four NR, GCNF, PPARγ, PPARα7 and RevErbβ perfectly correlated with each other ($P \leq 0.0167$). In the other cell types correlations between NR pairs were more diverse, but also statistically highly significant. Interestingly, the relative expression level of a number of receptor pairs ranked identical or similar in at least 3 (CoupTFα and PPARβ/δ, CoupTFβ and HNF4α as well as RORβ and PXR) or 4 cell types (CoupTFα and CoupTFβ, PPARγ and RevErbβ). Despite the variability of NR expression in immune cells, these results suggest that some of the NR may be co-regulated in human immune cells.

Introduction

The nuclear receptors (NR) are a large superfamily of transcription factors with ligand-induced activity that modulates tissue specific functions as diverse as homeostasis, cell differentiation, reproduction, embryonic development and neurogenesis. According to their ligands and functions NR can be divided into three categories: (i) the endocrine or steroid hormone receptors (SHR), whose ligands were known before the receptors were identified; (ii) the adopted orphan receptors, whose ligands have been identified after the gene was cloned; and (iii) the true orphan receptors, whose ligands are still unknown (Barish *et al.* 2005).

SHR such as the glucocorticoid receptor (GR), mineralocorticoid receptor (MR) or estrogen receptor (ER), and adopted orphan receptors including the constitutive androstane receptor (CAR), pregnane X receptor (PXR) or liver X receptor (LXR) have been investigated to some extend in the immune system (Franchimont 2004). The adopted orphan receptors, whose ligands are mostly derived from dietary lipids are involved in the acute phase response during inflammation and infection (Beigneux *et al.* 2000; Kim *et al.* 2003). The physiological functions of orphan receptors such as chicken ovalbumin upstream promoter-transcription factor (CoupTF), germ cell nuclear factor (GCNF) or testicular receptors (TR2,4) are still largely unknown, but some evidence suggest their involvement in immune responses (Smirnov *et al.* 2001). For a wide range of tissues the distribution of NR is well established (Nishimura *et al.* 2004). However, the expression of many NR within cells of the immune system has not yet been determined.

In this study, we analysed the expression of 24 NR in PBMCs and subsets of lymphocytes and monocytes on the mRNA level using a quantitative real-time PCR normalised to a validated housekeeping gene. We provide the first evidence of the expression of CAR, CoupTFα, CoupTFβ, FXR, GCNF, HNF4α, PPARβ/δ, PXR, RevErbβ, TR2, TR4 and TLX in different subpopulations of immune cells. We also extended the expression profile of RevErbα and LXRα to these subsets and confirmed the expression of ROR (α, β, γ), RAR (α, β, γ), PPAR (α, γ), and VDR in immune cells. Although levels of expression greatly vary between donors and cell types NR analysis suggests that expression levels of some receptors may be co-regulated in immune cells.

Material and Methods

Preparation of cDNA from human blood cells and tissue samples

Human peripheral blood mononuclear cells (PBMC) were purified from 3 female and 2 male healthy volunteers and cellular subsets were purified by positive selection with monoclonal antibodies coupled to magnetic beads as previously reported (Turner&Muller 2005). A cell subset purity of 98-99.7 % was confirmed by flow cytometry (data not shown). Anonymous human biopsies were obtained from surgically removed organ tissues (thyroid gland, salivary gland, liver, prostate, skin, subcutaneous adipose tissue and lung) from the Clinic Sainte Therese, Luxembourg, following national and institutional ethical guidelines. Total RNA was purified using a High Pure RNA isolation kit (Roche, Mannheim, Germany) following the manufacturer's instructions. First-strand synthesis of total cDNA was carried out at 50 °C for 60 min using 200 U SuperScript III RT (Invitrogen, Paisley, UK) and 2.5 µM dT_{16} primer in a 40 µl reaction containing 250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol and 500 µM deoxynucleoside triphosphates (dNTPs).

Oligonucleotides and RT-PCR

Nuclear receptor- and housekeeping gene-specific primer sets were identified from the corresponding mRNA sequences using either the Vector NTi (Invitrogen) or the Primer Express software (Applied Biosystems, Nieuwerkerk, Netherlands). Primers and PCR conditions are shown in Table 2 and Table 3. All primers were synthesized by Eurogentec (Seraing, Belgium). Amplification of cDNA by PCR was performed in 25 µl reactions containing 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). Thermal cycling was performed either in an Opticon2 (MJ Research, Landgraf, Netherlands) or an ABI 7500 Sequence Detector System (Applied Biosystems). Opticon2 conditions were: 1 cycle 95 °C, 2 min; 40 cycles each at 95 °C, 20 sec; annealing, 20 sec; 72 °C, 30 sec, followed by 10 min, 72 °C. Using the ABI 7500 Sequence Detector System for amplification and detection the following profile was used: 1 cycle 95 °C, 10 min and 40 cycles each at 95 °C, 15 sec and 60 °C, 1 min. The PCR product was separated on a 3 % agarose gel and visualized with ethidium bromide under UV light. PCR products were cloned into pCR4-TOPO vector and confirmed by sequencing as previously reported (Turner and Muller, 2005).

Table 2: Primers and PCR conditions used for RT-PCR analysis on the Opticon2

	GenBank accession number	Primer Sequence	Amplified region	$[Mg^{2+}]$ (mM)	Tm [a] (°C)
Housekeeping genes					
Beta actin	NM_001101		208bp	2	60
Forward primer		5'-GGCCACGGCTGCTTC			
Reverse primer		5'-GTTGGCGTACAGGTCTTTGC			
GAPDH	BC_0023632		228bp	2	60
Forward primer		5'-GAAGGTGAAGGTCGGAGTC			
Reverse primer		5'-GAAGATGGTGATGGGATTTC			
PPIA	NM_021130		90bp	1	58
Forward primer		5'-TCCTGGCATCTTGTCCATG			
Reverse primer		5'-CCATCCAACCACTCAGTCTTG			
Nuclear receptors					
FXR	NM_005123		116 bp	3	60
Forward primer		5'-TGGGTCGCCTGACTGAATT			
Reverse primer		5'-ACTGCACGTCCCAGATTTCAC			
GCNF	HSU80802		200bp	3	60
Forward primer		5'-CGAACCTGTCTCATTTGTGG			
Reverse primer		5'-CCCATCTGGAGGCATTTG			
LXRα	NM_005693		107 bp	3	62
Forward primer		5'-AGACTTTGCCAAAGCAGGG			
Reverse primer		5'-ATGAGCAAGGCAAACTCGG			
PPARα2	NM_032644		225bp	3	63
Forward primer		5'-CTCTGGCCAAGAGAATCTACG			
Reverse primer		5'-CGCTGTCCTTTGCTTAACTGT			
PPARα7	BC071932		227bp	3	62
Forward primer		5'-TGATTTCCATTACGCTATTTCTGTG			
Reverse primer		5'-TGAGAAGCAAATGGTCAGTTGA			
PPARγ	NM_015062		239bp	2	63
Forward primer		5'-ATGTGAAGCCCATTGAAGACA			
Reverse primer		5'-GACTCTCTGCTAGTACAAGTCCTTG			
PXR	BC017304		221bp	5	62
Forward primer		5'-TGCGCATCCAGGACATACA			
Reverse primer		5'-GCCAGCTGTCATAGCAGGAA			
RevErbα	NM_021724		87 bp	2	62
Forward primer		5'-CTGCAAGGGCTTTTTCCGT			
Reverse primer		5'-ATGCGGACGATGGAGCAAT			
RevErbβ	BC045613		272bp	2	62
Forward primer		5'-TCTATGGGTGATAGAAACAGCTTTT			
Reverse primer		5'-CCCATTACCTACAGGAAGAATTTAT			
RORα	BC008831		221bp	2	62
Forward primer		5'-ATTGAGAACTATCAAAACAAGCAGC			
Reverse primer		5'-AAGTACACGGTGTTGTTCTGAGA			
TR2	NM_003297		222bp	2	60
Forward primer		5'-CATATCCAGATGACACCTACAGG			
Reverse primer		5'-GTTTACAGCACTGCAGTCACAG			
TR4	NM_003298		220bp	2	63
Forward primer		5'-AGACACCTACCGATTGGCC			
Reverse primer		5'-TTCTGTTGCTCCTTGGCAG			
VDR	J03258		228bp	2	60
Forward primer		5'-CACCTAGGCCACTCCTCCAA			
Reverse primer		5'-CTGTCTCTGGGAATCTTGTGG			

[a] Tm, annealing temperature

Table 3: Primers and PCR conditions used for RT-PCR analysis on the ABI Sequence Detection System

	GenBank accession number	Sequence	Amplified region	$[Mg^{2+}]$ (mM)	Tm [a] (°C)
Housekeeping genes					
Beta actin	NM_001101		208bp	2	60
Forward primer		5'-GGCCACGGCTGCTTC			
Reverse primer		5'-GTTGGCGTACAGGTCTTTGC			
GAPDH	BC_0023632		228bp	2	60
Forward primer		5'-GAAGGTGAAGGTCGGAGTC			
Reverse primer		5'-GAAGATGGTGATGGGATTTC			
PPIA	NM_021130		90bp	1	60
Forward primer		5'-TCCTGGCATCTTGTCCATG			
Reverse primer		5'-CCATCCAACCACTCAGTCTTG			
Nuclear receptors					
CAR	BC069626		270 bp	2	60
Forward primer		5'-CATGGGCACCATGTTTGAAC			
Reverse primer		5'-AGGGCTGGTGATGGATGAA			
CoupTFα	NM_005654		85 bp	2	60
Forward primer		5'-AGCACTACGGCCAATTCACCT			
Reverse primer		5'-GGCACGGCATGTGTAAGTTAA			
CoupTFβ	NM_021005		84 bp	2	60
Forward primer		5'-CACCGTCTCCTCCTCAGTCA			
Reverse primer		5'-CATATCCCGGATGAGGGTTTC			
HNF4α	NM_000457		70 bp	2	60
Forward primer		5'-AAGACAAGAGGAACCAGTGCC			
Reverse primer		5'-GGCTTCCTTCTTCATGCCA			
LXRβ	NM_007121		70 bp	2	60
Forward primer		5'-TGATGTCCCAGGCACTGATG			
Reverse primer		5'-CCTCTTCGGGATCTGGGAT			
PPARβ/δ	NM_006238		108 bp	2	60
Forward primer		5'-AGTACGAGAAGTGTGAGCGCA			
Reverse primer		5'-ATAGCGTTGTGTGACATGCCC			
RARα	NM_000964		97 bp	2	60
Forward primer		5'-CAGCTGGGCAAATACACTACG			
Reverse primer		5'-ATGCACTTGGTGGAGAGTTCA			
RARβ	NM_000965		89 bp	2	60
Forward primer		5'-CCTTTGGAAATGGATGACACA			
Reverse primer		5'-TTTTGTCGGTTCCTCAAGGTC			
RARγ	NM_000966		118 bp	2	60
Forward primer		5'-TGTCACCGCGACAAAAACTG			
Reverse primer		5'-TTCCGGTCATTTCGCACAG			
RORβ	NM_006914		92 bp	2	60
Forward primer		5'-CCTCACATCCGTACCCAACTT			
Reverse primer		5'-GCAATTCGGTCGATTTCAGTC			
RORγ	BC031554		77 bp	2	60
Forward primer		5'-TTCCGAGGATGAGATTGCC			
Reverse primer		5'-TTCCTTTTCTCTTGGAGCCCT			
TLX	NM_003269		69 bp	2	60
Forward primer		5'-GGAATAGCACAATGGGCCA			
Reverse primer		5'-TGTCACCGTTCATGCCAGAT			

[a] Tm, annealing temperature

Protein extraction and Western Blotting

Total protein was isolated from 10^8 PBMCs, CD4 T lymphocytes and CD14 monocytes using cell extraction buffer (Biosource, Nivelles, Belgium) supplemented with protease inhibitor cocktail and phenyl methyl sulphonyl chloride. Cells were lysed for 30 min (4 °C) in 1 ml of cell extraction buffer with vortexing after every 10 min. The extract was centrifuged at 13000 rpm for 10 min at 4 °C. Cytoplasmic and nuclear protein was isolated from 10^7 PBMCs with a nuclear extraction kit (Active Motive, Rixensart, Belgium). Total, cytoplasmic and nuclear protein extracts were aliquoted and stored at –80 °C until used. Proteins were resolved on a 4-16 % Bis-Tris Novex gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad, Herculis, CA). The membranes were subsequently developed with rabbit polyclonal hCAR, hCoupTFα, hFXR and hHNF4α antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-rabbit horseradish peroxidase labelled secondary antibodies (Pierce, Rockford, IL) using a standard protocol (Towbin *et al.* 1979).

Data analysis

The gene specific threshold cycle (Ct) is taken as the basis for semi-quantitative assessment. The relative expression of each target mRNA was calculated by the comparative $2^{-(\Delta\Delta Ct)}$ method using a stable internal housekeeping gene. For this normalisation three housekeeping genes, peptidylprolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were examined to find the most stable and reliable gene for calculations of $2^{-(\Delta\Delta Ct)}$ in immune cells. The amount of target mRNA relative to a donor specific reference sample (PBMC) or to a reference tissue was expressed as $2^{-(\Delta\Delta Ct)}$.

The Spearman rank order correlation was performed to investigate the relationship between the relative expression levels of individual receptors in the purified cell subsets between different donors. The probability to observe due to chance the number of correlated receptor pairs was calculated using the binomial law distribution function. The probability for a pair of receptors to correlate in three or four different cell types was computed using standard combinatorial reasoning.

Results

Housekeeping genes

The stability of three housekeeping genes β-actin, peptidylprolyl isomerase A (PPIA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured under the assumption that the threshold Ct ratio of at least two genes would be identical in a given cell subset for all donors. Figure 10 shows that in CD4, CD8 and CD19 lymphocytes the ratios between the Ct values of PPIA and GAPDH were relatively similar in the different donors. In contrast, β-actin seemed to be less stable in comparison to either PPIA or GAPDH. In CD14 monocytes the GAPDH/ β-actin ratios were relatively constant, suggesting that PPIA may be the variable gene. Based on these results, we selected GAPDH as the most stable housekeeping gene for normalisation of NR mRNA expression in the four immune cell subsets.

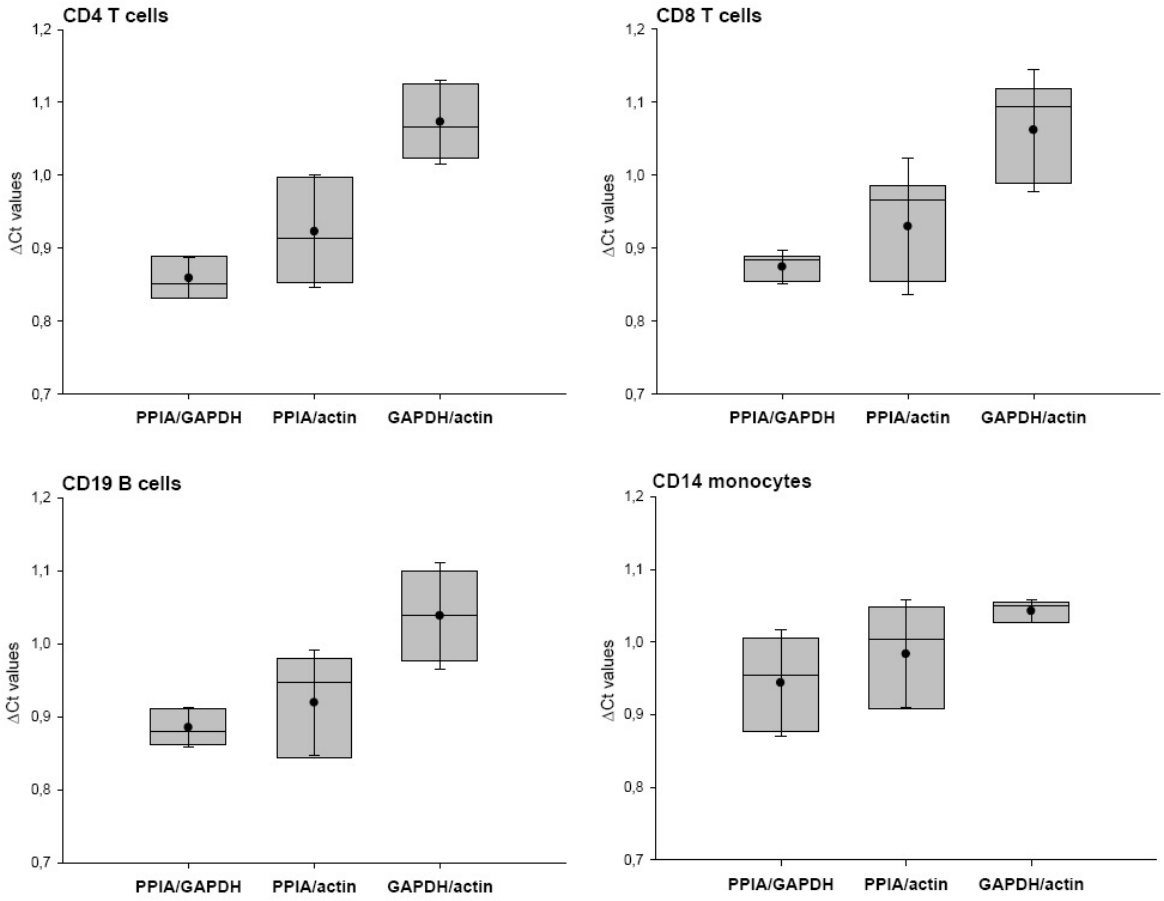
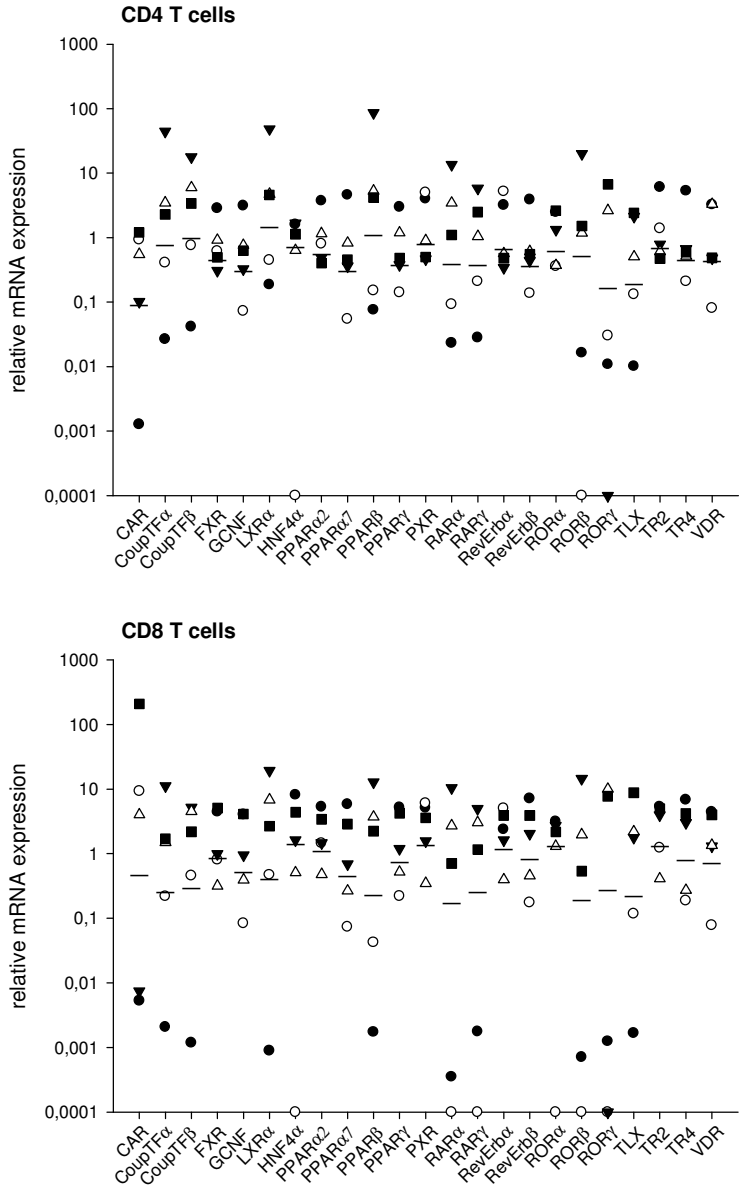


Figure 10: Housekeeping genes, GAPDH, PPIA and β-actin, plotted as threshold Ct ratios in four different cell types. The central line represents the median, box edges the 75 and 25 percentiles and error bars the standard deviation from the arithmetic mean (●)

Expression of nuclear receptors in different immune subsets

The mRNA expression levels of 24 different NR was quantified by real time-PCR and normalised to GAPDH. Figure 11 shows that the relative NR levels widely differed between receptors, cell types and donors. In all subsets of cells, 23 of the 24 NR were detectable although not necessarily in all donors. This is the first evidence that the 12 receptors CAR, CoupTFα, CoupTFβ, FXR, GCNF, HNF4α, PPARβ/δ, PXR, RevErbβ, TR2, TR4 and TLX are expressed in human CD4, CD8, CD19, and CD14 cells (and PBMC).



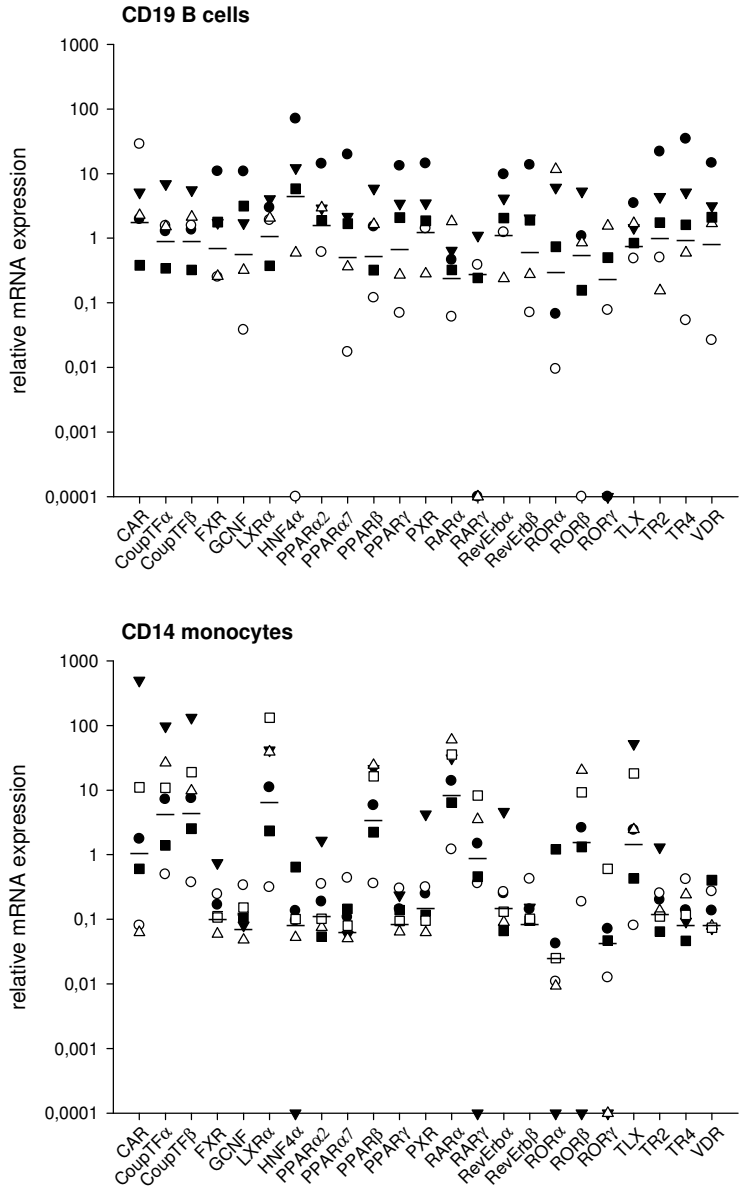


Figure 11: NR mRNA expression in lymphocytes and monocytes of five healthy donors (○ donor 1, ◆ donor 2, ■ donor 3, □ donor 4, ▲ donor 5). The values are given as $2^{-(\Delta\Delta Ct)}$ with the geometric means (–) normalised to GAPDH and related to PBMC values of the same donor. 0.0001 corresponds to the detection limit.

We have also extended the expression profiling of RevErbα, previously described in B cells (Hastings *et al.* 1997) and LXRα, previously detected in macrophages (Whitney *et al.* 2001) to the other cell types. RARβ was not detectable in any of the immune cells tested, while RARα and RARγ as well as the three ROR genes (α, β, γ) were detectable in all cells studied. However, RORα and RORβ were detectable in T cells and monocytes of only 4/5 donors and RORγ in only 3/5 donors suggesting a broader functional or constitutive variability of these receptors in different donors.

Tissue distribution of nuclear receptor mRNA

It was previously shown that CAR, FXR and HNF4α are relatively highly expressed in the liver and CoupTFα in the lung (Nishimura *et al.* 2004). Expression levels ($2^{(-\Delta\Delta Ct)}$) of CAR, CoupTFα, FXR and HNF4α in PBMC subsets were compared to the above tissues as well as a small panel of other tissues. Similarly to prostate and salivary gland expression of the latter NR is mostly much lower in immune cells than in adrenal and thyroid gland, the liver, the lung and subcutaneous fat tissue (Figure 11A-D). CAR expression in T lymphocytes and monocytes was similar to that in the liver. Thus CAR, a key player in xenobiotic and drug metabolism pathways, may have a role in immune modulation, especially since strong immune modulators such as glucocorticoids are inducers of CAR mRNA and protein (Pascussi *et al.* 2003).

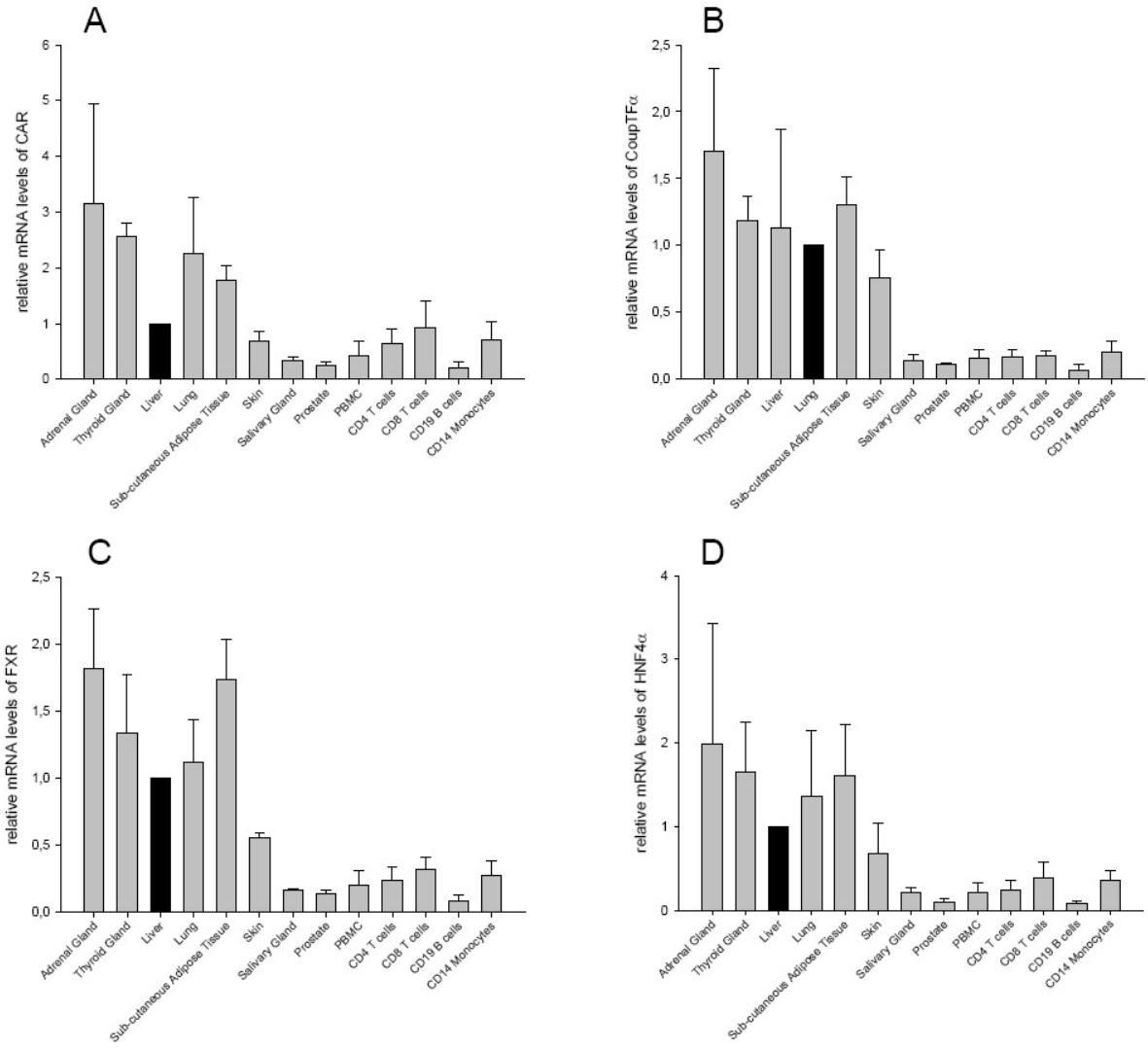


Figure 12: Relative mRNA expression of 4 different NR; A) CAR, B) Coup-TFα, C) FXR, D) HNF4α in immune cells and other tissues normalised to a reference tissue (full bar).

Detection of NR proteins in immune cells

For CAR, CoupTFα, FXR and HNF4α polyclonal antibodies were available for Western blotting. Figure 12 shows that these NR were detectable as proteins, albeit at different expression levels. For CAR, CoupTFα and FXR proteins were detected in PBMCs as well as in T lymphocytes and monocytes. In total and cytoplasmic protein extracts of PBMC two isoforms of these three receptors were detected. The CAR isoforms were also seen in CD4 cells. Smaller isoforms of CAR and CoupTFα (and FXR) were visible in the PBMC nuclear extract that may be explained by post activational modifications (Aranda&Pascual 2001; Dussault *et al.* 2002; Park *et al.* 2003). A weak expression of HNF4α protein was detected in the PBMC nuclear extract but not in the other extracts analysed.

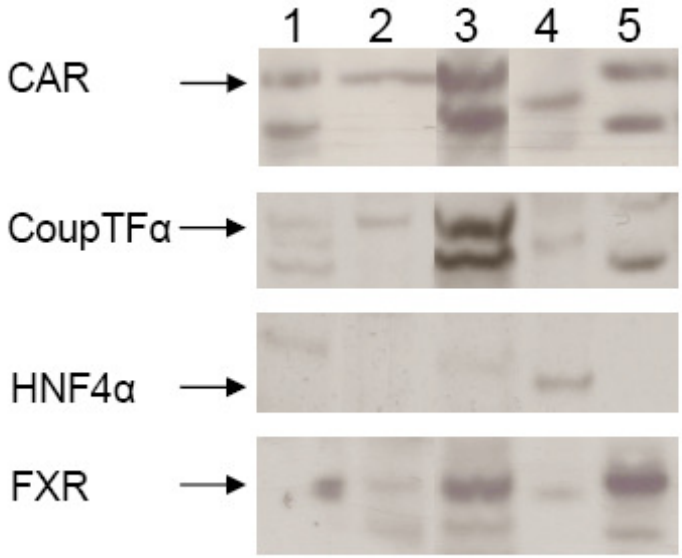


Figure 13: NR protein expression by Western analysis in CD4 (lane 1), CD14 (lane 2), PBMC total protein extract (lane 3), PBMC nuclear extract (lane 4) and PBMC cytoplasmic extract (lane 5).

Statistical interpretation

As levels of NR expression varied greatly between donors and cell types, we analysed the co-regulation of NR expression. Donor ranking (Spearman test) was compared within each cell type for each combinatorial pair of the 23 measurable NR (excluding RARβ), producing 253 (23x22/2) rank tests per cell type. Statistical significance was only found for perfect rank matches (PRM) of the rank orders ($P \leq 0.0167$) or for adjacent rank mismatches (ARM) ($P \leq 0.083$). In CD4 T cells 37 (14.6 %) pairs of NR displayed a statistically significant ($P \leq 0.083$) correlation. In CD8 T lymphocytes statistical

significance ($P \leq 0.083$) was found in 61 (24.1 %) pairs, in CD19 B lymphocytes in 59 (23.3 %) pairs and in CD14 monocytes in 30 (11.9 %) pairs of NR. PRM ($P \leq 0.0167$) were found in 7.5 %, 6.7 %, 7.9 % and 2.3 % for CD4, CD8, CD19 and CD14 cells respectively. The chance that a given receptor pair has ARM (correlation coefficient of 0.9 and -0.9) or a PRM (correlation coefficient of 1 or -1) is 6.67 % and 1.67 % respectively. Hence the number of receptor pairs among the 253 possible pairs with ARM follows a binomial law with parameters 253 and 0.067. The probability to find the above results by chance are 0.06 % in CD4 T cells, 3.24 % in CD14 monocytes and $<10^{-7}$ % in both CD8 T lymphocytes and CD19 B lymphocytes. Thus the percentages of correlations found are highly significant in the three lymphocyte subtypes and significant in the monocytes. The number of receptor pairs with PRM similarly follows a binomial law (parameters 253 and 0.0167). The probability to find the above results by chance are $2 \cdot 10^{-4}$ % in CD19 B lymphocytes, 24.98 % in CD14 monocytes and $<10^{-7}$ % in both CD4 T cells and CD8 T lymphocytes. Thus the percentages of correlations observed are highly significant in the three lymphocyte subtypes but not in the monocytes.

Table 4: Statistical analysis of the probabilities of a given receptor pair correlation in multiple cell types, the occurrence of such correlations within 23 NR studied and the probability for the number of observed receptor pairs to correlate significantly in the different observed situations.

Situation	Number of occurrences	Probability for a given receptor pair	Probability for the number of observed pairs
3 PRM in 3 subtypes	2	$5.79 \cdot 10^{-7}$	$< 10^{-9}$
1 PRM and 2 ARM in 3 subtypes	4	$2.32 \cdot 10^{-6}$	$< 10^{-9}$
2 PRM and 1 ARM in 3 subtypes	2	$2.12 \cdot 10^{-6}$	$< 10^{-9}$
3 PRM and 1 ARM in 4 subtypes	1	$1.93 \cdot 10^{-8}$	$5\ 10^{-6}$
1 PRM and 3 ARM in 4 subtypes	1	$1.93 \cdot 10^{-8}$	$5 \cdot 10^{-6}$

[a] PRM, perfect rank match
[b] ARM, adjacent rank mismatch

To calculate the statistical significance of receptor pairs correlating in 3 or 4 subsets, we considered the probability spaces $\Omega_3 = \{(i_1,i_2,i_3,j_1,j_2,j_3);\ 1 \leq i_k \leq 120,\ 1 \leq j_k \leq 120\}$ and $\Omega_4 = \{(i_1,i_2,i_3,i_4,j_1,j_2,j_3,j_4);\ 1 \leq i_k \leq 120,\ 1 \leq j_k \leq 120\}$, where i_k and j_k represent the 5 donors ranking for the first (i) and second (j) receptor respectively on the k-th cell type (using 5!=120 possibilities to rank 5 persons). Then $|\Omega_3| = 120^6$ and $|\Omega_4| = 120^8$.

CD4 T cells

	RORβ	RevErbβ	PXR	PPARγ	PPARβ	PPARα7	HNF4α	GCNF	CoupTFβ
CoupTFα					1,00**				1,00**
CoupTFβ							1,00**		
GCNF		1,00**		1,00**		1,00**			
HNF4α									
PPARα7		1,00**		1,00**					
PPARβ									
PPARγ		1,00**							
PXR	–1,00**								
RevErbβ									

CD8 T cells

	RORβ	RevErbβ	PXR	PPARγ	PPARβ	PPARα7	HNF4α	GCNF	CoupTFβ
CoupTFα					0,90*				0,90*
CoupTFβ							1,00**		
GCNF		0,90*		0,90*		0,90*			
HNF4α									
PPARα7		1,00**		1,00**					
PPARβ									
PPARγ		1,00*							
PXR	–0,90*								
RevErbβ									

CD14 monocytes

	RORβ	RevErbβ	PXR	PPARγ	PPARβ	PPARα7	HNF4α	GCNF	CoupTFβ
CoupTFα					0,90*				0,90*
CoupTFβ							0,90*		
GCNF									
HNF4α									
PPARα7									
PPARβ									
PPARγ		0,90*							
PXR	–1,00**								
RevErbβ									

CD19 B cells

	RORβ	RevErbβ	PXR	PPARγ	PPARβ	PPARα7	HNF4α	GCNF	CoupTFβ
CoupTFα									0,90*
CoupTFβ									
GCNF		0,90*		0,90*		0,90*			
HNF4α									
PPARα7		1,00**		1,00**					
PPARβ									
PPARγ		1,00**							
PXR									
RevErbβ									

Figure 14: Spearman rank order correlation test for 11 NR in four different subtypes of lymphocytes and monocytes. Values correspond to correlation coefficients. Black boxes highlight correlations that are found in the four cell lines, boxed values are shared by the three lymphocyte subtypes and light grey boxes show correlations found in CD4, CD8 and CD14 cells ** P≤ 0.0167, * P≤ 0.083.

Having 3 PRM and one ARM in the four cell types can thus be represented by $A=\{(i_1,i_2,i_3,i_4,i_1,i_2,i_3,j_4);\ 1\leq i_k\leq 120\}$, where j_4 represents an adjacent rank to i_4. Hence for every choice of i_4 there exist 4 different possibilities for j_4, since a ARM means that either the first and the second or the second and the third up to the fourth and the fifth ranked persons are inversed. Hence $|A|=4x120^4$ and the probability of finding by chance 3 PRM and one ARM in four subtypes for a given pair of receptors is $4x120^4/120^8=1.93\cdot10^{-8}$ %. The probabilities for all other events observed are computed the same way are all negligible (Table 4). Therefore our results represented in Figure 14 are all highly significant.

Discussion

In order to compare mRNA expression levels of NR between cell subsets in different donors, the yield of RNA extraction and the reverse transcription efficiency were normalised to genes with supposedly similar expression levels in the cells. β-actin, PPIA and GAPDH encode proteins of different functional classes, which reduces the chance that their expression may be co-regulated. We demonstrated that GAPDH was the appropriate housekeeping gene for our application due to its stability. Our findings confirm earlier studies with 3-15 housekeeping genes, showing that the stability is dependent upon cell type and may compromise their suitability as standards for normalisation between different cell types (Zhong&Simons 1999; Goidin *et al.* 2001; Vandesompele *et al.* 2002; Bas *et al.* 2004; Brunner *et al.* 2004; de Kok *et al.* 2005; Gabrielsson *et al.* 2005).

Here we show for the first time that CAR, CoupTFα, CoupTFβ, FXR, GCNF, HNF4α, PPARβ/δ, PXR, RevErbβ, TR2, TR4 and TLX are expressed in human CD4, CD8, CD19, and CD14 cells (and PBMC). While blood-derived human monocytes express all but one NR (RARβ) investigated here, Barish et al. (2005) found that a large number of orphan receptors were not expressed in murine bone marrow-derived macrophages (Barish *et al.* 2005). This may be due to the difficulty to detect some isoforms and splice variants (e.g. CAR with 12 possible splice variants (Arnold *et al.* 2004)) or may correspond to true biological, tissue or species dependent differences.

The receptors that we report here for the first time in immune cells can be divided into two functional categories. A first category includes CAR, FXR, LXR, PXR, HNF4α, PPARβ/δ, receptors for lipid, cholesterol and bile acid metabolism, which are known to be expressed in the liver, kidney and intestine (Jiang *et al.* 1995; Giguere 1999; Guo *et al.* 2003; Handschin&Meyer 2003; Nishimura *et al.* 2004; Handschin&Meyer 2005). Although the

role of these receptors in immune cells is not clear they seem to be under the control of cytokines at least in the liver. In the mouse, the expression of some of these metabolic NR (FXR, LXR, PXR, HNF4α) is inhibited by pro-inflammatory cytokines such as TNF-α and IL-1 released during the acute phase response (Kushner 1993; Kushner 1998; Miyake *et al.* 2000; Chiang 2002; Kim *et al.* 2003). During the development of a specific immune response the NR expression down-regulates cytochrome P450 activities e.g. CYP 7A1 (cholesterol 7α-hydroxylase) (Beigneux *et al.* 2000; Miyake *et al.* 2000; Beigneux *et al.* 2002; Kim *et al.* 2003). This in turn increases the pool of cholesterol, due to a reduced conversion of cholesterol to bile acids. Cholesterol a ligand for LXR, and bile acids, ligands for FXR have been shown to affect T cell mediated immune responses as well as T and B cell activation (Miyake *et al.* 2000; Chiang 2002; Han *et al.* 2003). The presence of these metabolic NR in lymphocytes and monocytes suggests that an autocrine loop involving cytokines and NR may be operational in these cells.

A second category includes CoupTFs, GCNF, TLX and TR2/TR4, receptors that are involved in cell differentiation, neurogenesis and reproduction and expressed in brain, testis, uterus and the prostate gland (Giguere 1999; Cooney *et al.* 2001; Lee *et al.* 2002; De Martino *et al.* 2004a; Nishimura *et al.* 2004; Zhang&Dufau 2004). CoupTF is one of the best characterized transcription factors (Tsai&Tsai 1997) but little is known about the function of most of the other orphan receptors of this group. CoupTFs can repress gene transcription by binding either as a homodimer (e.g. R2 site (Smirnov *et al.* 2000; Smirnov *et al.* 2001)) or as a heterodimer with RAR or the thyroid hormone receptor (Berrodin *et al.* 1992). CoupTF has been associated with down-regulation of MHC class I by adenovirus (Smirnov *et al.* 2000; Smirnov *et al.* 2001) in fibroblast cell lines but otherwise it is difficult to predict any function of orphan receptors in T and B cells or monocytes.

Interestingly, for a number of receptor pairs the ranking of the relative expression level was identical or similar in at least 3 or 4 cell types (Figure 14). PPARα7 and PPARγ as well as PPARα7 and RevErbβ show perfect correlations in all lymphocyte subtypes investigated. PPARγ and RevErbβ also correlate perfectly in these cell types and in monocytes with a single ARM ($P \leq 0.083$). If all statistically significant correlations ($P \leq 0.083$) are included CoupTFα and CoupTFβ also seem to be co-regulated in all four cell types; the same applies in the three lymphocyte subtypes for GCNF and PPARα7, GCNF and PPARγ as well as GCNF and RevErbβ; in T cells and monocytes but not in B cells for CoupTFα and PPARβ/δ, CoupTFβ and HNF4α as well as the negative correlation of RORβ and PXR (Figure 14). In CD4 T cells (Figure 15B) four NR, GCNF, PPARγ, PPARα7 and

RevErbβ match perfectly (P≤ 0.0167), suggesting a combined co-regulation. In the other lymphocyte subtypes these correlations were also found, but with a lower significance (P≤ 0.083). In Figure 15 we show single correlations in 4 or 3 subsets assuming a co-regulation of only two NR. The expression of each NR in all donors and cell types was measured simultaneously in one same assay and repeated twice. Thus rank orders of NR were obtained in independent experiments and the experimental design cannot explain the above correlations between NR rank orders.

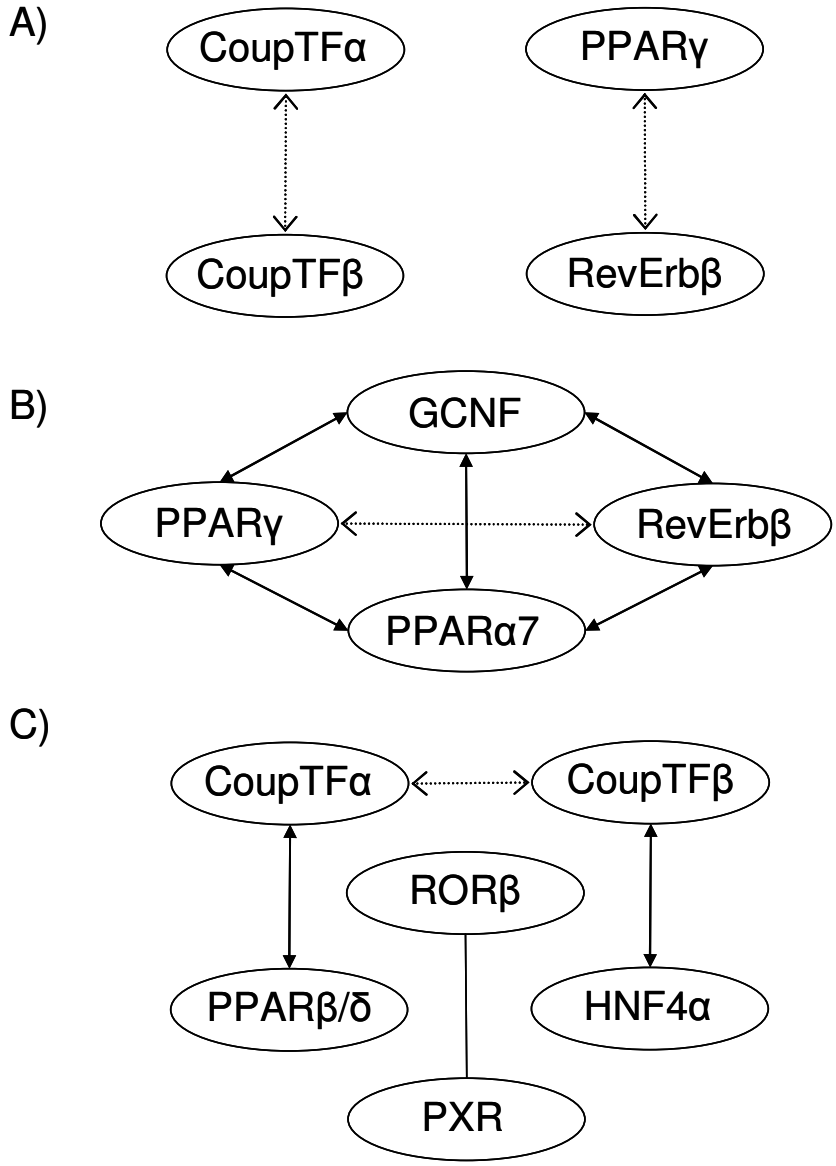


Figure 15: Scheme of putative co-regulation of NR in different lymphocytes and monocytes. (A) Correlations between 2 receptor pairs found in CD4, CD8, CD19 and CD14 cells; (B) correlation of 4 NR in the three lymphocyte subtypes; (C) positive correlation of CoupTFα with PPARβ/δ as well as CoupTFβ with HNF4α, and negative correlation between RORβ and PXR in CD4, CD8 and CD14 cells.

Among the NR newly observed in immune cells, CoupTFs and GCNF, showed interesting relationships with both known and newly reported receptors. The co-expression of CoupTFα and CoupTFβ in immune cells correlates with the ability of these receptors to form heterodimers and to repress hormonal induction of other NR at least in monkey kidney cells (Cooney *et al.* 1992; Cooney *et al.* 1993). The correlation of CoupTFα with PPARβ/δ suggests that the known role of both CoupTFs in PPAR-mediated signalling pathways in mammalian nephritic cells (Marcus *et al.* 1996) may operate also in immune cells. CoupTF regulates HNF4-mediated pathways in HepG2 cells (Yanai *et al.* 1999), which may provide a biological basis for a correlation between both receptors in immune cells. HNF4α and PXR modulate the gene expression induced by bile acids in the liver which links these NR to the induction of inflammatory cytokines (Chiang 2002). PPARα and PPARγ co-regulated with GCNF have been implicated in the inhibition of allergic inflammation (Woerly *et al.* 2003; Hammad *et al.* 2004), the regulation of B cell development (Yang&Gonzalez 2004) as well as the T cell activation (Jones *et al.* 2002).

In conclusion our study provides the first evidence that the expression of CAR, FXR, HNF4α, TLX as well as CoupTFα, CoupTFβ, GCNF, PPARβ/δ, PXR, RevErbβ, TR2 and TR4 are detectable in immune subpopulations and PBMC. Immune cells express all NR investigated except RARβ. This is in line with the findings of Nishimura et al. (2004) who analysed the mRNA expression profile of human NR in many other tissues and found that most NR (RAR (α,β,γ), PPAR (α,β,γ), ROR (α,β,γ), RevErb (α,β), LXRα, CoupTF (α,β), VDR, PXR, TR2 and TR4) were expressed in all 21 tissues analysed. CAR, FXR, HNF4α and TLX showed a slightly different expression profile and were not detectable in all tissues investigated (Nishimura *et al.* 2004). We showed that the expression at least of CAR, FXR, HNF4α, and CoupTFα was mostly lower in immune cells than in most other tissues, however the protein expression of these NR was confirmed in PBMCs. In mouse bone marrow-derived macrophages a more limited set of NR including VDR, RAR (α, γ), LXRα, PPAR (α,β), GCNF, RevErb (α,β), ROR (α,γ), TR2 and TR4 was detectable (Barish *et al.* 2005). Despite the vast variability of NR expression in immune cells, matching rank orders of a number of NR in different donors suggest that some of these may be co-regulated in human immune cells. Although this study provides interesting leads, further experiments are required to provide the physiological basis and the biological significance of such a co-regulation.

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Chapter 3:

Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage?

Jonathan D. Turner*[1], Andrea B. Schote*[1,2], Joana A. Macedo[1,2], Laetitia P.L. Pelascini[1], Claude P. Muller[1,2]

[1]Institute of Immunology, Laboratoire National de Santé, 20A rue Auguste Lumière, L-1950, Luxembourg

[2]Department of Immunology, Graduate School of Psychobiology, University of *Trier, D-54290, Germany*

* These two authors contributed equally to this work.

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Abstract

The CpG island upstream of the *GR* is highly structured and conserved at least in all the animal species that have been investigated. Sequence alignment of these CpG islands shows interspecies homology ranging from 64 to 99%. This 3.1 kb CpG rich region upstream of the *GR* exon 2 encodes 5' untranslated mRNA regions. These CpG rich regions are organised into multiple first exons and, as we and others have postulated, each with its own promoter region. Alternative mRNA transcript variants are obtained by the 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 unaffected.

Tissue specific differential usage of exon 1s has been observed in a range of human tissues, and to a lesser extent in the rat and mouse. The *GR* expression level is tightly controlled within each tissue or cell type at baseline and upon stimulation. We suggest that no single promoter region may be capable of containing all the necessary promoter elements and yet preserve the necessary proximity to the transcription initiation site to produce such a plethora of responses. Thus we further suggest that alternative first exons each under the control of specific transcription factors control both the tissue specific *GR* expression and are involved in the tissue specific *GR* transcriptional response to stimulation. Spreading the necessary promoter elements over multiple promoter regions, each with an associated alternative transcription initiation site would appear to vastly increase the capacity for transcriptional control of *GR*.

Introduction

Multicellular organisms with specialised tissues require mechanisms to control differential expression of proteins in a tissue-specific manner. Protein expression is either regulated at a transcriptional, post-transcriptional or a translational level. Chromatin condensation, initiation, and DNA methylation are important for the regulation of expression of many genes at the transcriptional level. Post-transcriptional mechanisms of gene regulation include e.g. alternative RNA splicing, and modulation of mRNA stability. Certain genes possess multiple promoter regions each associated with a specific alternative transcriptional start site in a specific, non-coding alternatively transcribed first exon. The alternative use of one of multiple exon 1s has only recently be recognized as another mechanism of transcriptional control of gene expression.

The use of alternative proximal promoters located immediately upstream of known transcription start sites results in RNA polymerase-generated primary transcripts of varying length. In this case, the location of the 5' 7-methyl-guanylate RNA cap structure may correspond to different positions within the genomic sequence depending upon the promoter used. Each primary RNA transcript is an exact copy of the genomic DNA and includes the first exon adjacent to the promoter region used, as well as all other downstream first exons that are excised only during further nuclear processing to produce mRNA.

Within the human genome more than 3000 genes contain multiple first exons (Zhang *et al.* 2004). In the mouse over 2000 genes with multiple first exons were identified. Zang et al (Zhang *et al.* 2004a) observed that, except for gene clusters encoding the immunoglobulins and T-cell receptors, only the variable 5' exons of the neural protocadherin (Pcdh), UDP glucuronosyltransferase 1 (UGT1), and I-branching β-1,6-N-acetylglucosaminyltransferase (IGnT) gene clusters displayed intra-gene sequence similarity and their first exons are translated giving a variety of closely related N-terminal protein variants. The variable 5' exons of all other genes investigated showed no such sequence similarity, nor were they thought to be translated. Although the majority of genes seem to have only a few alternative first exons, some genes, such as the glucocorticoid receptor have a remarkable number of first exons to choose from (McCormick *et al.* 2000; Turner&Muller 2005). This profusion of alternate first exons would be conducive to an important role in the transcriptional control of gene expression**.**

Glucocorticoids (GC) are involved in many physiological processes: they regulate the intermediary metabolism, control cellular differentiation, functions of the nervous and immune system and maintain homeostasis under stress. The activity of endogenous and exogenous GC is mediated by the type II glucocorticoid receptor (GR, *NR3C1* OMIM +138040). The *GR* is expressed in virtually all cell types. Levels of both *GR* mRNA and protein vary considerably between tissues. Also, within some tissues such as the hippocampus, *GR* expression is subjected to a significant level of plasticity (Herman *et al.* 1989). The specific level of *GR* expression is tightly controlled. An artificial reduction of *GR* levels by only 30-50% results in major neuroendocrine, metabolic, and immunological disorders (Pepin *et al.* 1992; King *et al.* 1995). It has been shown that post-transcriptional mechanisms such as the stability of different mRNA transcript variants do not play a significant role in controlling *GR* levels. In particular, the stability of mRNA transcripts was not influenced in vitro by the use of different alternatively spliced first and last *GR* exons (Pedersen *et al.* 2004).

The *GR* gene structure

The gene coding for the *GR* has been studied in some detail only in three species, humans, mice and rats. The human *GR* gene covers a region of more than 80 kb. Similarly, the rat and mouse *GR* genes span >80 and >110 kb respectively. The three species have a similar gene structure with 7 constant coding exons (exon 2 to 8), multiple alternative 5' -non-coding exon 1s, and two exon 9s encoding the alpha and beta protein isoforms (Hollenberg *et al.* 1985; Strahle *et al.* 1992). The alternative first exons were not designated sequentially but according to their order of discovery, which somewhat complicates the nomenclature. Most of the variable first exons of humans, rats, and mice are located in a CpG rich region of about 3.1 kb, starting some 4.6 kb and finishing 1.5 kb upstream of exon 2, although, a group of corresponding exons (human 1-A, rat 1_1 to 1_3 and mouse 1-A) is located 27-30 kb upstream.

Analysis of the 5' UTR of the human *GR* has revealed 11 splice variants derived from 7 exon 1s, (exons 1-A to 1-H (Turner&Muller 2005)). Similarly, the rat has 11 alternative exon 1s (1_1 to 1_{11} (McCormick *et al.* 2000)), and the mouse has 4 (1-A to 1-E (Chen *et al.* 1999)). With the exception of mouse exon 1-E there is a very good structural similarity and sequence homology between the alternative exons (Figure 16) as previously described for the rat and the human *GR*s (Turner&Muller 2005). All of the known exon 1 variants in the

human, mouse and rat have unique splice donor sites, splicing to a common exon 2 splice acceptor site. All mRNA splice variants have an in-frame stop codon (mouse and rat TAA; human TGA) before the ATG translation start site in exon 2 that is common to all mRNA variants. Since the translation start codon lies within the exon 2 the 5'-heterogeneity is untranslated and does not affect the sequence of the receptor.

GR tissue specific expression

Tissue specific differential usage of exon 1s has been examined systematically in a range of human tissue. Our earlier data suggest that no first exon is expressed in all tissues (Turner&Muller 2005). Conversely, at least the human hippocampus expressed all of the known exon 1 transcript variants, with exon 1 D being unique to this brain region. In addition to their presence in the hippocampus, exons 1-E and 1-F, seem to be biased towards the immune system. Exon 1-E was observed in both CD8+ T cells and CD14+ monocytes, while exon 1-F was best expressed in CD19+ B lymphocytes and BCDA2+ peripheral blood dendritic cells. Exon 1-B and 1-C appeared to be broadly expressed in many tissues, although they were absent from both subcutaneous adipose tissue and heart muscle and from the liver respectively.

Exon 1_{10} and 1_6 the rat homologues of the latter human exons also showed a broad expression pattern (McCormick *et al.* 2000). In the rat, 1_{10} represents at least 50% of the total *GR* transcripts, while exon 1_6 accounts for far less. As in humans, the other rat exons were expressed in a tissue-specific manner. Exon 1_1 was observed in the thymus, but not in the hippocampus and the liver. As in humans, most other exons 1s were expressed in the hippocampus, including significant levels of transcripts containing the minor exon 1_5, 1_7, and 1_{11}. As its human homologue 1-D, exon 1_7 is hippocampus-specific. Exon 1_5-, 1_7-, and 1_{11}-containing transcripts were either low or undetectable in the liver and thymus (McCormick *et al.* 2000).

Much less is known about the promoter usage and first exon expression in the mouse. Strähle et al (Strahle *et al.* 1992) showed that mouse exon 1-A was found in two T-lymphoma cell lines (S49 and WEHI-7 cells) but not in fibroblasts, the liver, or brain. In contrast, transcripts containing exons 1-B and 1-C were found in the latter tissues and at lower levels in T lymphocytes. Exons 1-D and 1-E have only been observed in mouse S49 T lymphoma cells, and AtT-20 pituitary tumour cell lines (Chen *et al.* 1999), but otherwise have not been investigated.

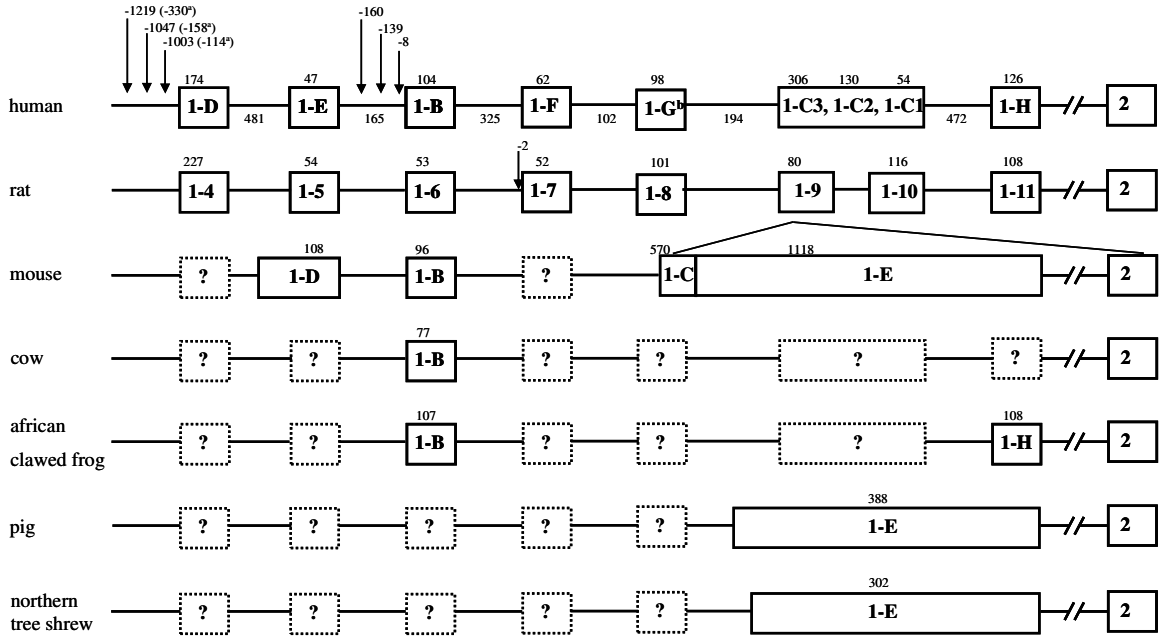

Figure 16: Map of the known alternative exon 1s of *GR* genes from all species for which the data is available. Experimentally identified exons (line boxes) and those predicted by genomic homology (dotted boxes) are shown. Incomplete sequencing data is indicated by dashed lines. Drawing not to scale, exon and intron sizes given in basepairs.

The *GR* CpG island is common to many species.

The human alternative 5'UTRs in the CpG islands upstream of *GR* exon 2 have been compared to those of the rat based on their high homology (Turner&Muller 2005).The alignment of the corresponding CpG islands demonstrates that this region is highly conserved among species as shown in Figure 16 for exon 1 coding stretches. Over the entire CpG island (3.1 kb) the total inter-species homology ranges from 65.5 % (mouse vs cow) to 88.1 % (mouse vs rat) and even 98.9 % (human vs chimpanzee). The cow seems to share a greater homology with the human and the chimpanzee than with either the rat or the mouse (77.3 and 77.0 % vs 66.2 and 65.2 % respectively) (Table 5).

The *GR* mRNA sequence is conserved between species.

Since the human *GR* was first cloned and sequenced in 1985 (Hollenberg *et al.* 1985) full length *GR* sequences have been obtained from more than a dozen species, and partial sequences from a dozen other species. As expected, the phylogenetic tree of the *GR* alpha mRNA (Figure 18), shows that the sequence homology between mammals is high, while length and sequence are less well conserved in other species (Ducouret *et al.* 1995; Takeo *et al.* 1996). The DNA binding and ligand binding domains show the greatest sequence

homology between species (Yudt&Cidlowski 2002). In our hands, the rat sequence showed a higher homology with the mouse *GR* sequence than with the human, although, a higher homology between rat and human was previously reported (Zhang *et al.* 2004b).

Table 5: Sequence homology exists between species.

		Cow	Chimp	Human	Rat
500 bp intron [a]	Mouse	61.9[b]	65.8	65.7	82.5
	Rat	61.9	65.3	66.0	
	Human	78.0	100.0		
	Chimp	76.0			
CpG Island [c]	Mouse	65.2	66.1	66.4	88.1
	Rat	66.2	67.5	67.6	
	Human	77.3	98.9		
	Chimp	77.0			
CDS [d]	Mouse	85.3	87.8	88.0	92.6
	Rat	81.9	86.2	81.5	
	Human	90.0	99.7		
	Chimp	90.2			

[a] 500bp of intron sequence between the CpG island and exon 2
[b] Data are expressed as the percentage of identical base pairs
[c] 3.1 kb genomic sequence used
[d] Alignment of *GR* alpha isoform RefSeq mRNA from the start of exon 2 to the end of the CDS

Alternative *GR* 5'UTRs can be identified in many species.

Alignment of the known human first exons with the genomic sequence of the cow CpG island, shows that the human exons 1-B, -D, -E and -F have homologies of 82.1, 80.6, 84.0, and 64.2% respectively with the cow sequence. While the homology with exon 1-H was still significant although including of a small segment (25bp) of unknown genomic sequence, there was none with 1-G.

The known 5'UTRs of *GR* of the species for which they are known align with one of the known rat or human variable exon 1s. The published mRNA sequences suggest that only the African clawed frog (Xenopus laevis) has alternative first exons and these correspond to the human exons 1-H and 1-B, only the latter exon being shared also with the cow. The sequences of the pig (*Sus scrofa*) and northern tree shrew (*Tupaia belangeri*) seem homologous to the mouse 1-E, but are significantly different from the corresponding rat and human region. The corresponding human region encodes the multiple intra-exon spliced 1-C as well as 1-H, while in the rat three independent exons, $1_{9,}$ 1_{10} and 1_{11} are found here. The mouse exon 1-C, included in the 5' end of exon 1-E, is spliced to exon 2 similarly to the intra-exon splicing of the human 1-C. This mouse exon 1-C overlaps with the ephimeric human 1-G and the rat exon1_8.

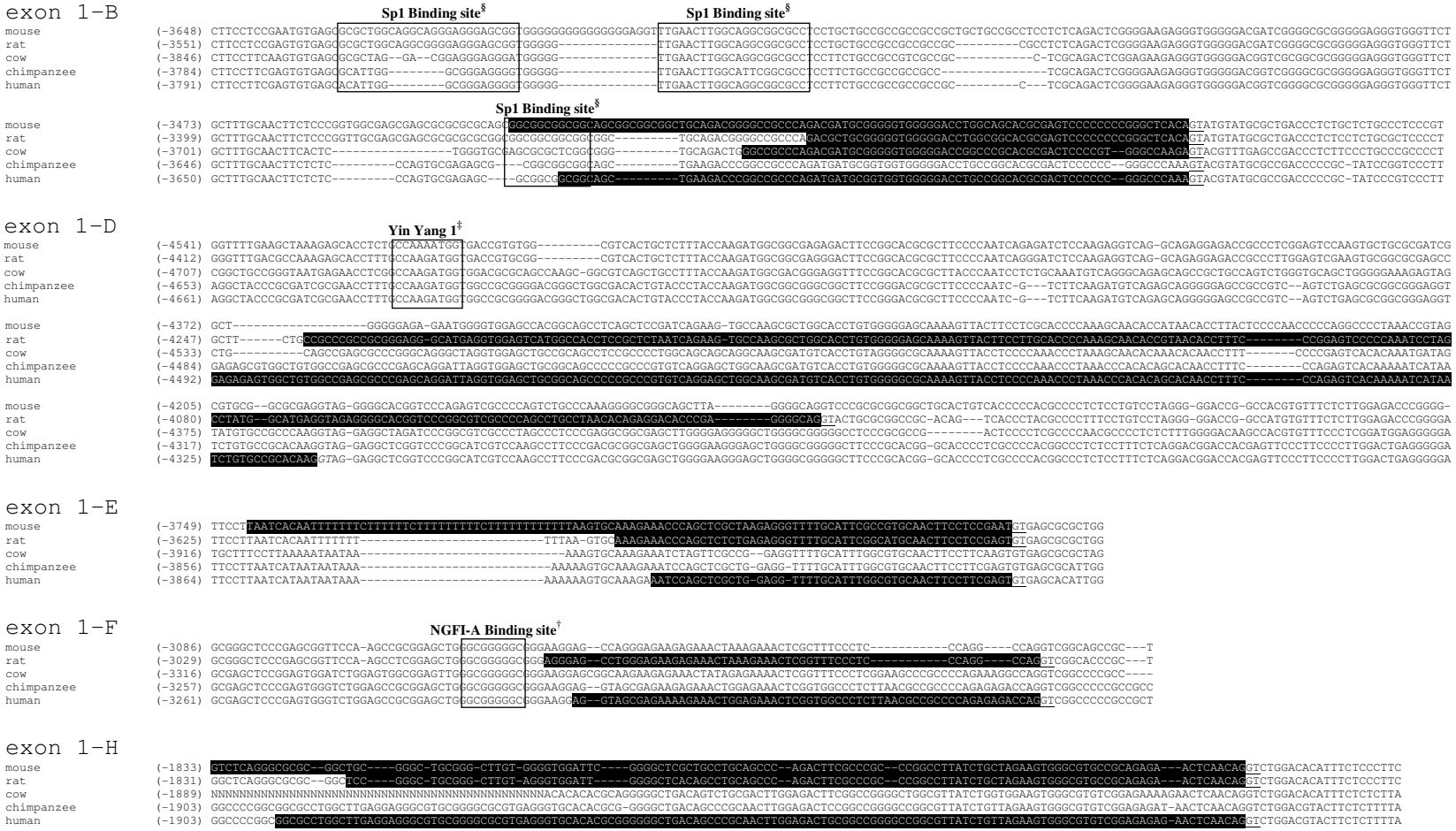

Figure 17: Alignment of genomic sequences coding transcribed first exons 1-B, 1-D, 1-E, 1-F and 1-H. Numbering is with respect to the translation start site within exon 2 for each species. The start of the human exon 2 is at –13 bp. Shaded sequences represent previously identified alternative exon 1s. Previously identified transcription factor binding sites are boxed. ‡ from (Breslin&Vedeckis 1998); † from (Weaver *et al.* 2004); § from (Nunez&Vedeckis 2002). Alignments of the genomic DNA of the human chromosome 5 (July 2004, UCSC), the rat (AJ271870, NCBI), mouse (NCBIM35:18: 39863309: 39869358:1, Ensembl) chimpanzee (CHIMP1A:5: 149403812:149409861:1, Ensembl) and cow (ChrUn.313: 334239:339538:1, Ensembl) upstream of the *GR* exon 2 was performed using Vector NTi (Invitrogen, Paisley, UK).

Figure 17 shows that exons 1-B, -E, -F, and -H share identical 3' splice donor sites. As previously noted (Turner&Muller 2005) exon 1-D is slightly longer in the rat than in the human. From these homologies we can predict with reasonable accuracy the 3' end of exons 1-B, D, E, F and H in the cow, all 5 alternative first exons in the chimpanzee, and exons 1-D and F of the mouse. Although, it is much more difficult to predict the 5' end of these potential exons, the predicted genomic region covered by these exons can be narrowed down. These comparisons suggest that the 3' ends of alternative first exons of *GR* will be predictable in new genomic sequences as they become available from other species. As the repertoire of alternative first exons grows, it would however seem logical that a unified system of nomenclature be developed. Such a system should allow for the comparison between species and also prevent the situation arising, as currently exists, with homologous exons having different names between species.

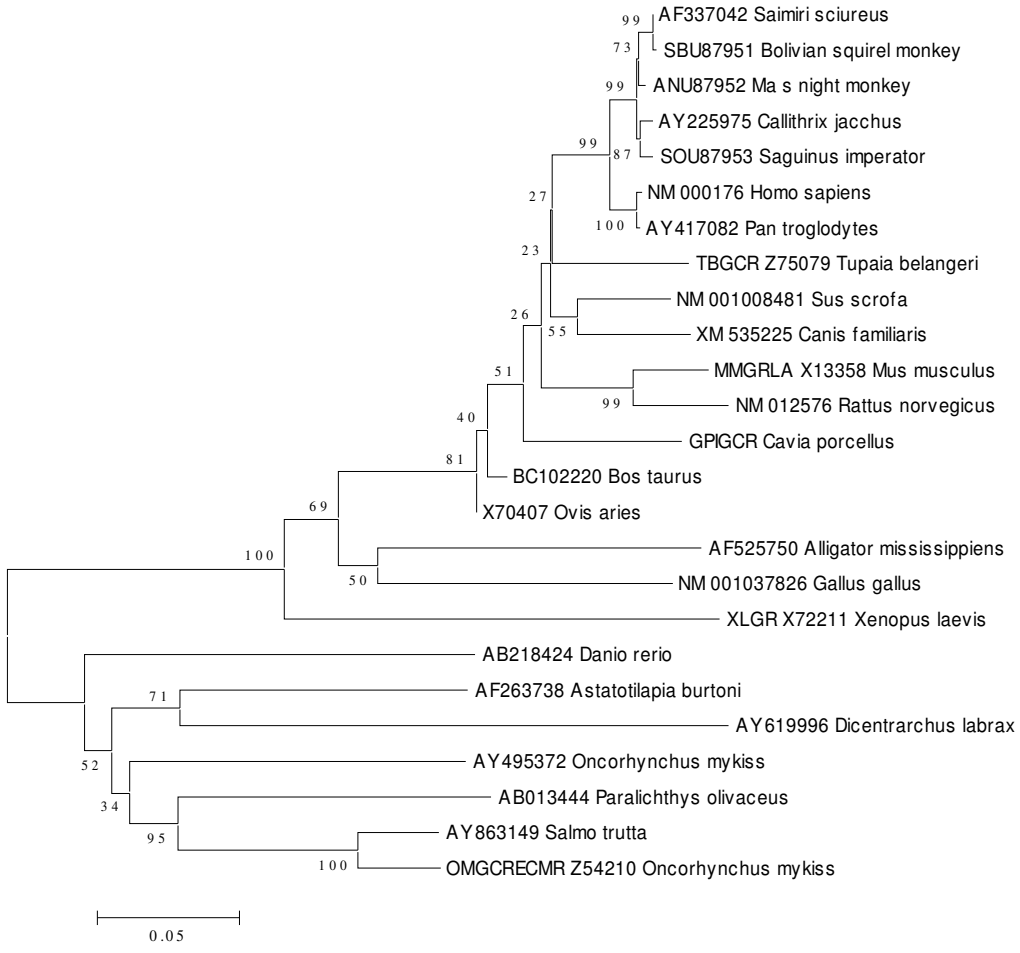

Figure 18: Phylogenic analysis of 25 complete NR3C1 coding sequences currently available on GenBank. Numbers at nodes correspond to bootstrap values. Alignment of the mRNA sequences was performed using ClustalX. . Phylogenetic analysis was performed using neighbour-joining Kimura 2 parameter method and the tree was constructed using Mega 3.1 (Kumar *et al.* 2004b)

Alternative 5'UTRs may have their own promoters and transcription factor binding sites

Assuming that each variable first exon has its own proximal promoter, the high degree of inter-species genomic homology suggests that transcriptional control mechanisms may be similar among different species. The known transcription factor binding sites within the alternate promoter elements are summarised in Table 6 and their location shown in Figure 16.

Table 6: Regulatory elements of the *GR* proximal promoter regions

Associated exon	Transcription factor	Sites	Technique[a]	Location[b]	Comments	Ref.
1A	Ets-1/2[c]	1	RG	-34412 (95)	Steroid induced recruitment confirmed by ChIP	(Geng&Vedeckis 2005)
	PU.1[c]	1				
	Spi-B[c]	1				
	c-Myb	1	RG	-34416 (91)		
	GR[d]	1	RG,	-34432 (75)		(Breslin *et al.* 2001)
	GR[e]	1	RG, FP, E	-34546 (175)		(Nunez *et al.* 2005)
	IRF-E	1	RG, E			
1B	SP1	3	RG, FP, E	-3774 (-160) -3753 (-139) -3622 (-8)		(Nunez&Vedeckis 2002)
	YY-1	3	FP, D, E	-4808 (-1219) -4636 (-1047) -4592 (-1003)	-330 relative to 1D -158 relative to 1D -114 relative to 1D	(Breslin&Vedeckis 1998)
1C	SP1	numerous	FP, E		11 footprints observed in the region covering 1C and 1D promoters	(Breslin&Vedeckis 1998)
	AP2	1	FP,E			
1D	n/d[f]					
1E	n/d					
1-7 (1F)	NGFI-A	1	ChIP	-3226 (-2)	Identical binding site at -7bp from human homologue 1F	(Weaver *et al.* 2004)
1G	n/d					
1H	n/d					

[a] RG, Reporter gene; FP, DNAse protection / DNA footprinting; ChIP, chromatin precipitation; D, deletion analysis; EMSA, electrophoretic mobility shift assay.
[b] Locations - with respect to ATG start codon and to associated first exon in parentheses
[c] Ets-1/2, PU.1 and Spi-B are members of the Ets family, binding to the same site. Overexpression of all three factors increases exon 1A expression(Geng&Vedeckis 2005)
[d] FP11 from (Geng&Vedeckis 2005) one non-consensus GR half site (GTAAAATGCGC) is present and this may not be enough for complete steroid responsiveness in and of itself.
[e] FP6 from (Breslin *et al.* 2001) two GR half sites AGAAAA and TCTTCT
[f] n/d not determined

The human 1-B promoter contains previously identified binding sites for SP1 and Yin Yang 1 (YY1) (Nobukuni *et al.* 1995; Breslin&Vedeckis 1998; Nunez&Vedeckis 2002). Detailed analysis showed that SP1 sites are found in all species some 127 and 158 bp upsteam of the exon 1-B transcription start site. The YY1 sites were identified 1219, 1047, and 1003 bp upstream of the transcription initiation site of exon 1-B. However, later only 330, 158, and 114 bp downstream of these YY1 sites the initiation site of exon 1-D was discovered (Figure 2). Therefore, these YY1 binding sites may be part of the proximal promoter region of exon 1-D rather than 1-B. From the latter work (Nunez&Vedeckis 2002) it was not possible to establish exactly which elements control the transcription starting at exon 1B, 1C, or 1D. In the rat, analysis of the proximal promoter region of exon 17 showed that the transcription factor NGFI-A binds only 2 bp upstream of the transcription initiation site of this exon. Figure 17 shows the known SP1 and NGFI-A binding sites upstream of the human exon 1-B and the rat exon 17 respectively. Sequence homology in these regions suggests that these transcription factors are also operational in other species such as the chimpanzee, cow and mouse. The proximal promoter of human exon 1-A has received more attention, because of its role in *GR* auto-regulation. This promoter is reportedly activated selectively in hematopoietic cells (Nunez&Vedeckis 2002; Adamson *et al.* 2004) and highly sensitive to dexamethasone stimulation. Its sensitivity to dexamethasone may be partially explained by the presence of two distinct GRE-like sequences found in DNA footprinting experiments including a half-site pair (Breslin *et al.* 2001), and an isolated half-site (Geng&Vedeckis 2005), although neither footprint corresponded to a consensus GRE. In super-shift assays only *GR*beta bound to the half-site pair footprint, but technical reasons may have prevented the detection of *GR*alpha binding (Breslin *et al.* 2001). The second single GRE-like half-site appears to be paired with a second footprint that binds c-Myb and members of the ETS family. The T and B cell lines differentially express members of these families, explaining the different results upon dexamethasone stimulation. It is difficult to attribute transcription factor binding sites to specific alternate first exons. Experimentally, it has been shown that the footprints observed display promoter activity using techniques such as luciferase reporter genes. However, examination of promoter elements upstream of exons 1-B and 1-C was performed on approximately 1kb segments (Breslin&Vedeckis 1998; Nunez&Vedeckis 2002). Promoter constructs for exon 1-B included exons 1-D and 1-E, whilst for 1-C both 1-F and 1-G were included, in the test constructs. Therefore these experiments were unable to unequivocally assign the promoter activity observed to the alternative exons.

Identification and localisation of alternative 5' UTRs.

Whilst coding sequences can be found in databases such as the NCBI RefSeq database (Pruitt *et al.* 2005) with relative ease, multiple alternative 5' UTR are more difficult to retrieve. Also experimentally, their identification is complicated. Expressed Sequence Tag (EST) represents single pass, partial sequences generated from either the 5' or the 3'-end of a cDNA clone prepared from various tissues or cell lines. EST databases provide a view of the mRNA species within given cell types and conditions. However, 5' sequences are under-represented in EST databases. This, together with sequencing errors, frequent insertions and deletions, contaminations by vector and linker sequences, and the non-random distribution of sequence start sites in oligo(dT)-primed libraries reduces their overall usefulness. In addition, the patterns of overlapping sequences of the same gene caused by alternative transcripts are different from those obtained from genomic sequencing (Liang *et al.* 2000). For the identification of alternative exon 1 splice variants the EST database is only partially useful because while only transcribed regions of the genome are represented, the number of tissues included is limited, and expression of the different first exons of the *GR* is tissue specific. BLAST searches for the reported human alternative first exons provided no matches with any EST clones. Similarly, experimental determination of alternative 5'UTR sequences is tedious as it relies upon techniques such as 5'RACE PCR applied to each individual tissue.

GR variable exons and disease.

Although differential expression of the *GR* variable first exons has received little attention in various disorders, modulation of their expression has been observed in certain psychobiological conditions. The hippocampus is well known to be a site of inhibitory feedback of glucocorticoids on the hypothalamic-pituitary-adrenal (HPA) axis. Perinatal manipulations (e.g. postnatal handling) significantly increased *GR* mRNA level in the rat hippocampus and attenuated the stress response of the HPA axis. This increase in hippocampal *GR* mRNA levels was due to the selective upregulation of the rat hippocampus specific exon 1_7. As a result, adult rats that were handled after birth were more sensitive to negative glucocorticoid feedback and had a decreased HPA reactivity to stress throughout their adult life (Francis *et al.* 1996; Liu *et al.* 1997).

In conditions such as major depression where the HPA axis is hyperactive, because of lower hippocampal *GR* (mRNA) levels resulting in an impaired glucocorticoid feedback,

the selective upregulation of exon 1_7 has been considered to play a role in the therapeutic mechanism of certain antidepressant agents. In rats, a four-week antidepressant treatment with fluoxetine increased hippocampal exon 1_7 expression (Yau *et al.* 2002) associated with increased total *GR* expression. *MR* and *GR* expression was also increased in the rat hippocampus after treatment with other antidepressant drugs, but the role of alternative first exon usage was not investigated (Mitchell *et al.* 1992; Yau&Seckl 2000; Lai *et al.* 2003). Similarly, it was shown that prenatal exposure of rats to synthetic glucocorticoids such as dexamethasone increased hepatic *GR* mRNA levels. As a result, the relative expression of the predominant hepatic first exon, 1_{10}, was reduced suggesting an increase of any of the other variable exons, althought the responsible exons have not been identified.

In mouse lymphocytes exon 1-A expression was upregulated by GC exposure in a similar manner to the rat, although this effect was dependent on the organ source and the phenotype of lymphocytes (Purton *et al.* 2004). In humans *GR* regulation in acute lymphoblastic leukemia (ALL) has been investigated in an effort to understand GC resistance, which is associated with a poor prognosis. Chemotherapy of childhood ALL includes GCs, which in GC-sensitive ALL cell lines upregulates *GR* mRNA and protein levels, and induces lymphoblast apoptosis (Eisen *et al.* 1988; Obexer *et al.* 2001; Tonko *et al.* 2001). The *GR* promoter 1-A has a weak glucocorticoid response element (GRE), whereas promoters 1-B to 1-H had no obvious predictable GRE. It was hypothesized and later confirmed in the human ALL cell line CEM-C7 (Breslin *et al.* 2001; Pedersen&Vedeckis 2003) that the upon GC exposure alternative *GR* promoters are regulated independently and in different ways (Geng&Vedeckis 2004). In ALL patients baseline promoter usage and transcript variant expression levels for the 3 exons investigated (1-A, -B, and -C) showed no difference between either GC-sensitive or insensitive ALL patients and with healthy controls. Similarly, after GC stimulation GR expression was induced in both GC-sensitive and insensitive ALL cells in the same manner as in healthy controls. Therefore, GC resistance in primary ALL cells can not be attributed to the inability of resistant cells to upregulate the expression of the *GR* upon GC exposure, nor to differences in *GR* promoter usage upon GC treatment (Tissing *et al.* 2006). Thus, in humans in vitro and in vivo studies seem to give conflicting results, or *GR* expression is simply regulated differently in both cell types. Although alternative mechanisms may explain the above results, differences in mRNA half-life of the transcript variants have so far not been reported (Pedersen *et al.* 2004).

Conclusion

The CpG island upstream of the *GR* is highly structured and conserved at least in all the animal species that have been investigated. Sequence alignment of these CpG islands shows interspecies homology ranging from 64 to 99%. The 3.1 kb CpG rich region upstream of the *GR* exon 2 encodes 5' untranslated mRNA regions, organised into multiple first exons and, as we and others have postulated, each with its own promoter region. Alternative mRNA transcript variants are obtained by the 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 unaffected.

Tissue specific differential usage of exon 1s has been observed in a range of human tissues, and to a lesser extent in the rat and mouse. Baseline and stimulated levels of *GR* expression are tightly controlled within each tissue or cell type. We suggest that no single promoter region may be capable of containing all the necessary promoter elements and yet preserve the necessary proximity to the transcription initiation site to produce such a plethora of responses. Thus we further suggest that alternative first exons each under the control of specific transcription factors control the tissue specific *GR* expression and are involved in the tissue specific *GR* transcriptional response to stimulation. Spreading the necessary promoter elements over multiple promoter regions, each with an associated alternative transcription initiation site would appear to vastly increase the capacity for transcriptional control of *GR*.

Transcriptional control via multiple alternate first exons provides new possibilities for control also at the translational level. Many mRNA features control its translation but most translational control elements are located within the UTRs, especially the 5'UTR (reviewed in (Wilkie *et al.* 2003; Pickering&Willis 2005)). In eukaryotes, translation is mostly initiated by a cap scanning mechanism and to a lesser extent through internal ribosome entry. The key features of the 5' UTR involved in translational control include their length initiation consensus sequences, together with characteristic secondary structural elements, upstream start codons and micro RNAs, and internal ribosome entry sites (IRES) (Gray&Wickens 1998; Mignone *et al.* 2002). In addition, 5' UTRs can contain sequences that function as binding sites for regulatory proteins. So far, the effect of multiple alternate 5'UTRs on translation of the *GR* mRNA has received little attention,

although, the conspicuous and evolutionary conserved diversity in 5'UTR length and sequence, suggest an important role for subsequent translational mechanism. For instance it has recently been shown that secondary structure elements within one of the alterative sequences of the early onset breast cancer gene (BRCA1), on down-regulate protein production (Sobczak&Krzyzosiak 2002). This gene has a transcriptional control mechanism similar to the *GR*, although using only two alternative promoters and associated first exons. Although, the UTR of the *GR* is much more complex GR translation may be controlled in a similar fashion. It is interesting to hypothesise that different first introns in these alternative primary RNA transcripts may also play a role in controlling the subsequent processing of the RNA to mRNA.

In summary, tissue, cell and stimuli specific transcriptional control of the *GR* appears to rely on alternative promoters and the alternative transcription start sites associated with them and this mechanism appears to be conserved across many species. Such a transcriptional control mechanism has significant, although currently unexplored implications for the translation of the *GR* mRNA

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Chapter 4:

A new transcript splice variant of the human glucocorticoid receptor: identification and tissue distribution of hGRDelta313-338, an alternative exon 2 transactivation domain isoform.

Jonathan D. Turner[1], Andrea B. Schote[1,2], Marc Keipes[3] and Claude P. Muller[1,2]

[1]Institute of Immunology, Laboratoire National de Santé, 20A rue Auguste Lumière, L-1011, Grand Duchy of Luxembourg

[2]Department of Immunology, Graduate School of Psychobiology, University of Trier, D-54290, Germany

[3] Clinique St Thérése, 36 rue Ste-Zithe, 2763 Luxembourg, Grand Duchy of Luxembourg

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Abstract

All human glucocorticoid receptor isoforms (hGR) are encoded by the *NR3C1* gene consisting of 7 core exons (exons 2-8) common to all protein isoforms. The gene has two major exon 8-9 splice variants and a 5'-UTR consisting of 11 alternative splice variants. The N-terminal region of the hGR includes a tau-1 transactivation domain that interacts with proteins in the basal transcriptional apparatus, including the TATA box-binding protein. Here, we report the existence and the tissue distribution of a novel splice variant, hGRΔ313-338, with a 26 residue (78 bp) deletion in this N-terminal region encoded by exon 2, between amino acids 313 and 338. The hGRΔ313-338 observed at the mRNA level represents a transcript variant encoding a predicted protein isoform with a deletion between the tau-1 domain and the DNA binding domain (DBD) encoded by exons 3 and 4. Previous studies in transgenic mice showed that the removal of the entire exon 2 covering both the tau-1 transactivation domain and our deleted region produced a functional receptor albeit with an altered glucocorticoid-induced gene transcription pattern. Interestingly, the deleted residues show a number of potential phosphorylation sites including serine 317, known to be phosphorylated. It is thought that phosphorylation plays an important role in transactivation action of hGR. Thus, we hypothesise that hGRΔ313-338 represents a hGR isoform with an altered glucocorticoid induced transactivation profile.

Introduction

The activities of endogenous and exogenous glucocorticoids are mediated by the type II glucocorticoid receptor (GR, OMIM +138040). The GR is a transcription factor and a member of the nuclear receptor family (NR3C1, subfamily 3, group C, receptor 1). Upon glucocorticoid binding, the GR dimerises and translocates to the nucleus, where it exerts its transcriptional activity by binding to its cognate genomic DNA sequences such as the glucocorticoid response element (GRE). The GR consists of three domains: an N-terminal domain, a DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). *hGR* transcripts are encoded by nine exons in the *NR3C1* gene. The variable exon 1 (1A-1H) encodes a 5' untranslated region, with at least 11 potential sequences, the expression of which is thought to be controlled by alternative promoter usage (Breslin *et al.* 2001; Turner&Muller 2005). Exons 2 to 9 encode the GR with an ATG translation start site located in exon 2 and alternative splicing of the two different exon 9 isoforms (exons 9α- and 9β) generates either a GRα- or GRβ- encoding transcript (hGRα, 777 amino acids, and hGRβ, 746 amino acids). Within exon 2 there are two in-frame alternative ATG start codons, 27 bp apart. The use of these two sites produces two protein isoforms (A and B) of both the GRα and GRβ. The N- terminus of the GR-A isoform of both GRα and GRβ is 9 amino acids longer than the GR-B isoform.

Here we describe for the first time of a new mRNA spice variant with a deletion (hGRΔ313-338) in the exon 2, its sequence and its expression in a range of human tissues. The location of the deletion suggests that the splice variants encode a new hGR isoform with an altered glucocorticoid induced transactivation profile.

Materials and Methods

PCR detection, cloning and sequencing

Amplification of cDNA by PCR was performed using 20 mM Tris HCl (pH 8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates (dNTPs), and 2.5 U Platinum *Taq*DNA polymerase (Invitrogen, Paisley, UK), (35 cycles: 96 °C, 20 sec; annealing, 20 sec; 72 °C, 20 secs), followed by 10 min, 72 °C. PCR primers used to amplify exon 2 of the hGR, annealing temperatures, and $MgCl_2$ concentrations are shown in Table 7. Oligo-dT primed universal human cDNA from a variety of human tissues (BD Clontech, Belgium) was used as a template. PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and

used to transform Top10 electrocompetent *E. coli* (Invitrogen). Colonies were screened using M13 primers. M13 PCR products of the correct size were purified with spin columns (Life Technologies, UK), and sequenced using 100 nM M13 primers (Table 7) and the BigDye 3.1 terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, NL). After 35 cycles (96 °C, 20 sec; 50 °C, 20 sec; 72 °C, 2 minutes) the reaction was completed by incubating for 10 minutes at 72 °C. The products were precipitated with ethanol and sodium acetate (0.3 M). Sequencing was performed on an ABI 3130 sequencer (Applied Biosystems).

Table 7: Primers and PCR conditions

Primer pairs	Sequence	Nt position1	$[Mg^{2+}]$ (mM)	[Primer] (μM)	Tm[a] (°C)
Detection primers					
$PCR1_{fwd}$ $PCR1_{rev}$	5'-TTTGCTTTCTCCTCTGGC 5'-GCAATCATTCCTTCCAGC	626 - - 1324	2	1	49.5
$PCR2_{fwd}$ $PCR2_{rev}$	5'-GACCAAAGCACCTTTGAC 5'-ATAGCGGCATGCTGGGCA	618 - - 1369	2	1	53
$\Delta 313\text{-}338_{fwd}$ $\Delta 313\text{-}338_{rev}$	5'-TCCTGGAGCAAATATAATTGCAT 5'-CGGGAATTGGTGGAATGA	931 - - 1010	3	1	52
2_{Fwd} 2_{Rev}	5'-GTTGATTTTCCAAAAGG 5'-CAATGCTTTCTTCCAA	200 - - 392	3	1	48
Housekeeping gene					
$Actin_{fwd}$ $Actin_{rev}$	5'-GGCCACGGCTGCTTC 5'-GTTGGCGTACAGGTCTTTGC	754 - - 961	2	1	60
Sequencing Primers					
$M13_{Fwd}$ $M13_{Rev}$	5'-GTAAAACGACGGCCAG 5'-CAGGAAACAGCTATGAC		2	0.1	55

[a] Annealing Temperature

Western blot

Total proteins were isolated from either 1 x 10^8 HeLa or THP-1 cells lysed for 30 min (4°C) in 1ml of cell extraction buffer (Biosource, Nivelles, Belgium) with vortexing after every 10 min. The extract was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was aliquoted and stored at –80°C until used. Proteins were resolved on a 4-16% Bis-Tris Novex gel (Invitrogen) and transferred to a nitrocellulose membrane (BioRad, Richmond CA). The membranes were blocked and blotted using a standard

protocol (Towbin *et al.* 1979). Rabbit polyclonal anti-hGR antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA), and anti-rabbit horseradish peroxidase labelled secondary antibodies (Pierce, Rockford, IL) were used for detection.

Tissue distribution of hGRΔ313-338

Anonymysed human biopsies were obtained from surgically removed organ tissues (thyroid gland, salivary gland, liver, prostate, skin, sub-cutaneous adipose tissue, lung and heart muscle) from the Clinic Sainte Therese, Luxembourg, following national and institutional ethical guidelines. Purified immune cells included CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells and CD11c- CD123+ (BDCA-4+) plasmacytoid dendritic cells. Hippocampal cDNA (dT_{16} primed) was obtained from Biochain (Gentaur, Brussels, Belgium). Briefly, 10^6 purified cells or 10 mg of tissue were lysed in lysis buffer (Miltenyi Biotech Gmbh, Germany); after removal of debris, samples were incubated with oligo-dT labelled magnetic beads (Miltenyi). mRNA was subsequently isolated using μMacs magnetic columns. First-strand synthesis of total cDNA was carried out at 50°C for 60 min using 200 U SuperScript III reverse transcriptase and 2.5 μM dT_{16} primer in a 40 μl reaction containing 250 mM Tris-HCl (pH 8.3, at room temperature), 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol and 500 μM dNTPs. Tissue distribution of the exon 2 deletion variants was determined by classical PCR (Table 7). Forward primers covering the wild-type and mutant forms were paired with a common reverse primer. To ensure that the hGR was detectable in each sample a PCR of the coding region of exon 2 (2_{Fwd} and 2_{Rev}) was included in each sample.

Results

Detection of hGRΔ313-338

Amplification of cDNA from mixed human tissues using the PCR-1 primers (Table 7) generated several amplicons smaller than the expected exon 2 transcript (Figure 19B). Similarly, amplification of exon 2 with a primer set external to PCR-1 (PCR-2, Table 7) revealed a PCR product with a similar mass difference (Figure 19B). Cloning and sequencing showed that these smaller amplicons were identical to the normal hGR transcript cDNA sequence except for a deletion in exon 2. The 78 bp deletion corresponds to 26 residues between amino acids 313 and 338 in the N-terminal domain of the GR. The

mRNA sequence of this new splice variant has been submitted to GENbank (accession number AM183262).

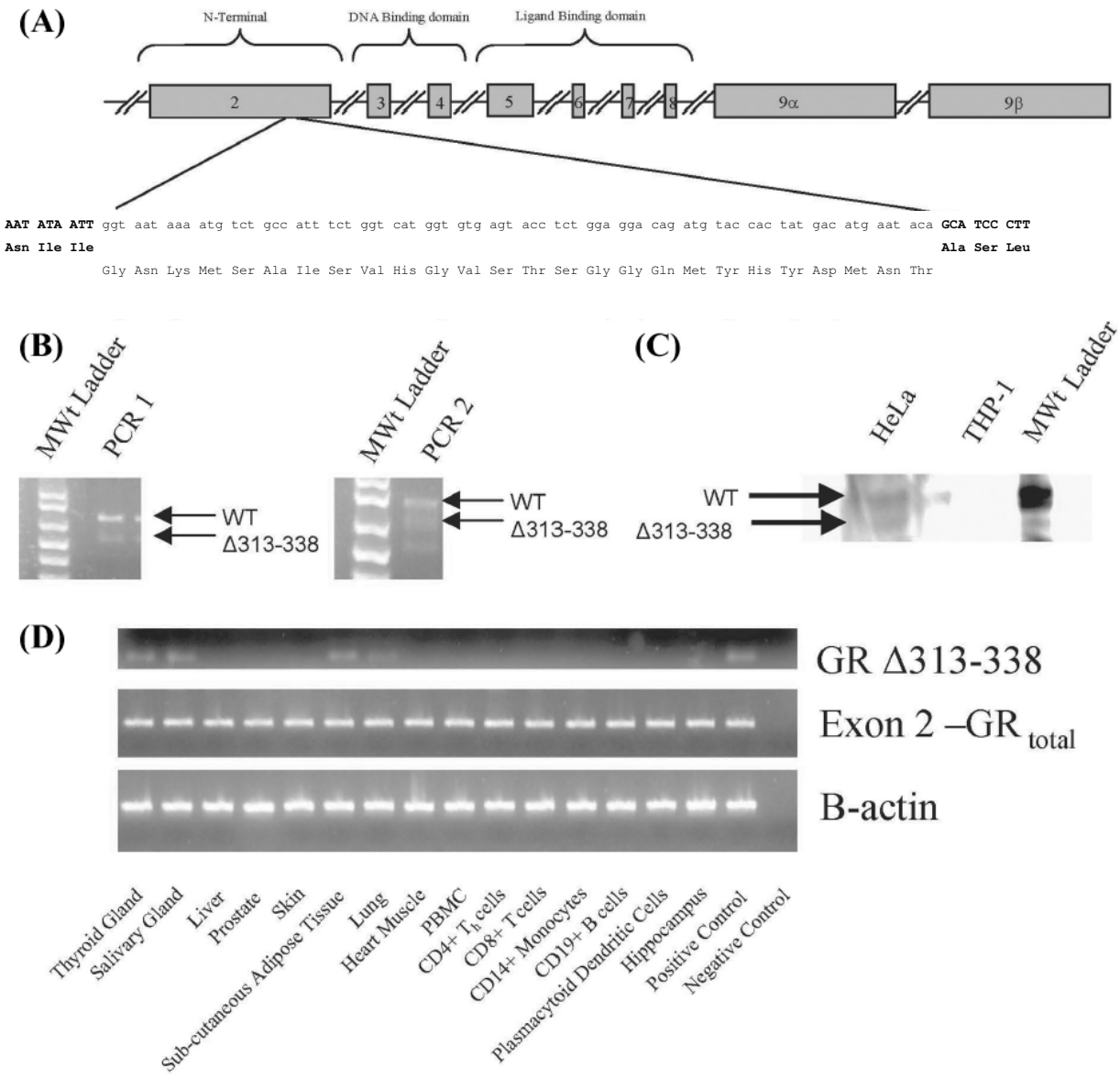

Figure 19: Identification and tissue distribution of the hGR Δ313-338 isoform. (A) The 26-residue (78 bp) deletion within exon 2 of the hGR. Exon 2 codes for the N-terminal transactivation domain. (B) The PCR product containing the 78 bp deletion is smaller than the wild-type (WT) in two independent PCR reactions using different primer pairs, (PCR-1, PCR-2; Table 1). (C) Western blot of protein product of Δ313-338 and the larger band of the wild-type hGR in HeLa cells; wild-type hGRbut not the Δ313-338 variant in THP-1 cells.

A PCR primer spanning the splice donor and acceptor site was developed to specifically amplify the hGRΔ313-338 isoform. Specificity resulted from the incorporation of three isoform-specific nucleotides at the 3'-terminus of the forward primer. Under appropriate experimental conditions, efficient primer elongation is dependent on matching nucleotides at the 3' end (Newton *et al.* 1989). Amplification with the hGRΔ313-338 specific primers produces a single band. Figure 19D shows that the deleted isoform can be detected in a tissue specific manner, confirming that the observation of hGRΔ313-338 is not a PCR artefact.

Detection by Western blot

Western blot analysis of HeLa cell total protein extract revealed the dominant GRα wild-type with the expected molecular weight and a minor second band approximately 3 kDa smaller (Figure 19C). This latter band was absent from the THP-1 cells. GRβ can be excluded as an explanation for this band as this lower molecular weight splice variant was not detectable by exon 8-9β specific PCR.

Tissue distribution of hGRΔ313-338

The reverse transcription PCR specific for hGRΔ313-338 mRNA was also used to investigate expression patterns of hGRΔ313-338 in blood cells isolated from normal volunteers and organ tissue biopsies. While the wild-type GRα was abundant in all tissues, Figure 19D shows that the deletion splice variant was differentially expressed in tissues. hGRΔ313-338 was detected only in the lung, subcutaneous adipose tissue, thyroid and salivary gland but not in liver, prostate, skin, heart muscle, hippocampus or any immune cell studied.

Discussion

We have observed at the messenger RNA level a new exon 2-deleted isoform of the hGR. The 78bp deletion observed within the hGR mRNA was not only confirmed by 2 different PCR reactions amplifying different fragments including the region of interest, but also by an isoform specific PCR reaction with primers bridging the deletion. Western blot analysis revealed a protein band of the proper size but too weak to be confirmed by sequencing, or peptide mass fingerprinting by MALDI-TOF. Recently there has been significant interest in GR splice variants also because they may play a role in cancer pathology. Vedeckis (Geng *et al.* 2005; Geng&Vedeckis 2005; Nunez *et al.* 2005) investigated the role of exon

1As (1A1 to 1A3), and their link to exon 2 splice variants. Exon 2 splice variants are of significant interest due to the prominent role of exon 2 in the transcriptional activity of the GR. The tissue distribution that was observed may be biased because the solid biopsies were obtained from tumour-infiltrated organs. Interestingly, the deleted residues show a number of potential phosphorylation sites including serine 317, known to be phosphorylated. It is thought that phosphorylation plays an important role in transactivation action of hGR.

The GR possesses two intrinsically transcriptionally active regions, designated tau 1 and tau 2 (amino acids 85-269 and 533-562 (Mittelstadt&Ashwell 2003)). The tau 1 region containing AF-1 is located in the N-terminal transactivation domain and its activity is thought to be glucocorticoid-independent. AF-2 is located in the carboxy-terminal tau 2 domain and is glucocorticoid-dependent (Hollenberg&Evans 1988). It is thought that the GR initiates transcription by recruiting chromatin remodelling factors and transcription factors to its transcriptionally active domains (McKenna *et al.* 1999a; McKenna *et al.* 1999b; McKenna&O'Malley 2002) together with proteins of the basal transcriptional apparatus such as the TATA box-binding protein. In addition nuclear receptor coactivators from several families have been described, including the steroid receptor coactivator 1 (a p160 coactivator), the GR-interacting protein 1 (GRIP1) and the p300/cAMP response element-binding protein (CREB)-binding protein (CBP). The p160 coactivators interact directly with both the AF-1 of the GR through their carboxy-terminal domain and the AF-2 through multiple amphipathic LXXLL signature motifs located in their nuclear receptor-binding (NRB) domain. These coactivators play an important role in transcription initiation mediated by their histone acetyltransferase activity, which is believed to prepare target gene promoters for transactivation by decondensation of the target chromatin (McKenna *et al.* 1999a; McKenna *et al.* 1999b; McKenna&O'Malley 2002). Interactions of the N-terminal domain of the GR with the above regulatory proteins have been described, nevertheless, removal of the entire exon-2 and hence the entire N-terminal domain does not inactivate the transcriptional activity of the receptor. The relative roles of the N- and C-terminal portions of the GR in transactivation has been a matter of debate (Mittelstadt&Ashwell 2003). Even in cells expressing the GR2KO gene product lacking the N-terminal tau 1 domain many genes were still glucocorticoid responsive, although to a lesser extent than in wild-type cells. In such cells, some genes showed a normal response (IL- 7Ra and glutamine synthetase), while others showed a reduced (GILZ) or no response (FKBP51) to glucocorticoids. The tau2 domain in the C-terminal half of the GR harbours a

considerable ligand-regulated transcriptional activity, although with a different glucocorticoid induced transactivation profile. On the basis of these observations, it seems reasonable to hypothesise that changes in the N-terminal sequence would modulate transcriptional activity of the tau 2 domain.

In summary, we report a mRNA splice variant encoding a deletion in exon 2 (hGRΔ313-338), together with a protein band of the appropriate size, and its differential expression in a range of human tissues. We hypothesise that this deletion represents a hGR isoform with an altered glucocorticoid- dependent transactivation profile.

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Chapter 5:

Identification and characterisation of a new human chicken ovalbumin upstream promoter-transcription factor II isoform

Andrea B Schote[1,2], Jonathan D Turner[1,2], Patrick Pirrotte[2] and Claude P Muller[1,2]

1. Institute of Immunology, National Public Health Laboratory Luxembourg,

   20A rue Auguste Lumière, L-1950 Luxembourg, Grand Duchy of Luxembourg
2. Institute of Psychobiology, University of Trier,

   Johanniterufer 15, D-54290 Trier, Germany

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Abstract

Chicken ovalbumin upstream promoter transcription factors (Coup-TFII) are well characterised orphan nuclear receptors involved via gene regulation in differentiation, development, homeostasis and diseases. We have investigated the existence of alternative first exons in Coup-TFII. Species alignments of known Coup-TFII genomic and transcript sequences (Ensemble and NCBI database) were used to identify an alternative first exon in the human Coup-TFII receptor. The predicted transcript showed similarity to alternative transcripts of mouse and macaque. To determinate the 5' location of this new exon, exon overlapping PCR, 5'RACE PCR and subsequent sequencing were performed. The results of this analysis demonstrated that the new alternative exon splices directly to exon 3 with deletion of exon 1 and exon 2 of the classical Coup-TFII. Upon examination of the nucleotide sequence, an ATG start codon, in frame with the coding sequence in the common exon 3, was found. This leads to a protein without a DNA binding domain, but with an intact ligand binding domain containing complete nuclear translocation site, dimerisation domain and AF-2 core sequence. Further investigations with GFP fused Coup-TFII and Coup-TFIIiso proteins demonstrated a different intracellular localisation of the classical receptor and its isoform. Coup-TFII was, independently of the cell type, located in the nucleus, whereas our new isoform was always in both, cytoplasm and nucleus. Based on the localisation profile we hypothesize that the Coup-TFII isoform is not constitutively active on the DNA but rather function competitively, due to protein-protein-interaction with other nuclear receptors or cofactors in the cytosol. To understand the implication of Coup-TFII and Coup-TFIIiso in the immune systemTHP-1 monocytes and THP-1 macrophages were analysed after stimulation with cortisol and LPS. Interestingly we determine a difference in the expression profile of both receptor forms. In undifferentiated THP-1 monocytes Coup-TFIIiso is not at all, in differentiated THP-1 macrophages mainly expressed after cortisol treatment. In addition we observe after LPS treatment in THP-1 macrophages a decrease, in THP-1 monocytes an increase of Coup-TFII expression. We suggest that both Coup-TFII forms have some implications in immune regulation in disease rather than health and that the isoform could act as an ubiquitous repressor of other nuclear receptors such as the GR.

Introduction

Chicken ovalbumin upstream promoter transcription factors (Coup-TFs) are members of the nuclear receptor superfamily with two distinct types in humans; Coup-TFI/Ear-3 (Coup-TFI) and Coup-TFII/ ARP-1 (Coup-TFII) (Wang *et al.* 1989; Ritchie *et al.* 1990; Ladias&Karathanasis 1991; Tsai&Tsai 1997). As no specific ligand has yet been identified, the Coup-TFs are classified as orphan nuclear receptors. They are highly conserved across different species suggesting important biological functions (Tsai&Tsai 1997). Coup-TFs play a critical role in neurogenesis, angiogenesis and heart development and leads in Coup-TF deficient mice to early embryo lethality (Pereira *et al.* 2000; Cooney *et al.* 2001). Coup-TFs act predominantly as transcription repressors, however, they also exert positive gene regulation (Cooney *et al.* 1993; Leng *et al.* 1996; Zhang&Dufau 2004). Recent findings suggest an important role of Coup-TFII in normal female reproduction, in particular uterine endometrial functions based on Coup-TFII mediated control of estrogen receptor (ER) activity (Takamoto *et al.* 2005; Lee *et al.* 2006; Kurihara *et al.* 2007; Petit *et al.* 2007). Association of Coup-TFs with other nuclear receptors and cofactors are crucial for their gene silencing function (Cooney *et al.* 1993; Klinge *et al.* 1997; Shibata *et al.* 1997; De Martino *et al.* 2004b). Protein-protein interactions of Coup-TFII with the glucocorticoid receptor (GR) showed a reciprocal effect on each other's target gene expression; Coup-TFII suppressed GR induced gene transactivation whereas GR enhanced Coup-TFII stimulated gene expression (De Martino *et al.* 2004b). Formation of inactive heterodimers, for example with retinoid X receptor (RXR), titrates out RXR for binding with other nuclear receptors and thereby diminishes hormone induced gene expression (Zhang&Dufau 2004). The dimerisation domain important for this interaction is located in the ligand binding domain (LBD) of the receptor, that together with the DNA binding domain (DBD) and the hinge regions build the typical structure of nuclear receptors (Achatz *et al.* 1997; Bain *et al.* 2006). Our own studies on the 5' untranslated region (UTR) of the GR showed that alternative splicing affect tissue and cell specific transcriptional control mechanism and cause diseases (Turner *et al.* 2006). Analysing the 5' UTR of Coup-TFII we identified a new alternative first exon of Coup-TFII containing an alternative translational start site leading to a protein isoform of Coup-TFII. This isoform of the receptor is lacking the complete DBD but containing an intact LBD. To further characterise Coup-TFIIiso we determined its tissue distribution, intracellular

translocation and implication in immune regulation in order to detect differences compared to the classical form of the receptor.

Material and Methods

Identification of the new first exon of Coup-TFII by exon overlapping PCR

Sequence alignments of the nuclear receptor Coup-TFII genomic DNA of nine species, including Homo sapiens, Rattus norvegicus, Mus musculus, Pan troglodytes, Macaca mulatta, Bos Taurus, Gallus gallus, Xenopus tropicalis and Danio rerio was perfomed using Vector NTi (Invitrogen, Paisley, UK). The known exons of each species were localised by the transcript and/or exon published in the Ensembl genome database (release 49). An overview of these transcripts with highlighted coding regions is given in Figure 20. Human forward primer was designed on the basis of the homology to the known exon 1 of the mouse transcript NM_183261 and combined with a reverse primer located in the human exon 3. Amplification of cDNA was performed in 25µl reactions containing 20mM Tris–HCl (pH 8.4), 50mM KCl, 200mM deoxynucleoside triphosphates (dNTP) and 2.5U Platinum TaqDNA polymerase (Invitrogen). Oligo-dT-primed universal human cDNA (BD Clontech, Belgium) was used as template providing cDNA from a variety of human tissues.

5' –rapid amplification of cDNA ends (RACE) PCR

The first strand of Coup-TFII specific cDNA was reverse transcribed for 50min at 42°C using 200U SuperScript II RT(Invitrogen, Paisley, UK) and 100nM gene specific reverse primer (GSP, Table 8) in a 25µl reaction containing 50mM Tris-HCL (pH 8.3), 75mM KCl, 3mM $MgCl_2$, 10mM dithiothreitol and 500µM dNTPs. After RNAse H (Invitrogen) treatment for 10min at 37°C and potassium-acetate precipitation, a poly-C tail was added to the 3' end of the cDNA. Poly-C-tailed DNA was subsequently amplified using a nested PCR reaction. The first-round PCR was performed with 400nM abridged anchor primer (AAP, Table 8, Invitrogen), 400nM exon specific reverse primer (R2, Table 8), 20mM Tris-HCl (pH 8.4), 50mM KCl, 150nM $MgCl_2$, 200mM dNTPs and 2.5U Platinum *Taq* DNA Polymerase (35cycles: denaturation, 95°C, 20sec; annealing 50°C, 20sec; elongation 72°C, 1min) with a final elongation for 10min at 72°C. Nested exon specific 5'RACE products were subsequently generated using the protocol above and the nested reverse primers (R1; AUAP, Invitrogen) are shown in Table 8.

Table 8: PCR primers and associated conditions

	Primer Sequence	$[Mg^{2+}]$ (mM)	Tm [a] (°C)	comment
Detection primer				
Coup-TFII	Fwd[b] 5'-GGCGCCTATAAATCGCATTCC Rev[c] 5'-TAGAAGCACATCCTTCGTGCGCAG	2.5	62	exon 1
Coup-TFIIiso	Fwd 5'-AAAGTTAGTGTGCAGGGTTTTCC Rev 5'-GTTGTTGGGCTGCATGCATT	3	60	exon 1A
β-actin	Fwd 5'-GGCCACGGCTGCTTC Rev 5'-GTTGGCGTACAGGTCTTTGC	2	60	
5'RACE primer				
GSP[d]	Rev 5'-CATATCCCGGATGAGGGTTTC			
AAP	Fwd 5'-GGCCACGCGTCGACT-AGTACGGGIIGGGIIGGGIIG	1.5	53	first round RACE
R2	Rev 5'-ACCTCAAGGAAGTGCTTCCCCA	1.5	53	
AUAP	Fwd 5'-GGCCACGCGTCGACTAGTAC	1.5	50	nested
R1	Rev 5'-AGCCTTGGAAAACCCTGCACAC	1.5	50	RACE
Cloning primer				
Coup-TFIIiso_ORF	Fwd 5'-ATGCAAGCGGTTTGGGACCT Rev 5'-TTATTGAATTGCCATATACGGCCAG	2	58	
Coup-TFIIiso_adap	Fwd 5'-GAAATACTTAACCATG-GTCATGCAAGCGGTTTGGGAC Rev 5'- ATATCTCGAGTGCGGCC-GCTATTATTGAATTGCCATATA	3	63	NcoI site attached NotI site attached
Sequencing primer				
M13	Fwd 5'-GTAAAACGACGGCCAG Rev 5'-CAGGAAACAGCTATGAC	2	55	

[a] annealing temperature, [b] forward primer, [c] reverse primer, [d] gene specific primer for reverse transcription

Fresh PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and colonies screened using M13 primers (Tab.1). M13 PCR products of the correct size were purified using the Jet Quick-PCR Purification Kit (Genomed, Löhne, Germany) and sequenced with 100nM M13 primers and the BigDye 3.1 terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, NL). After 35 cycles (96°C, 20sec; 50°C, 20sec; 72°C, 2min) the reaction was completed by incubation at 72°C for 10min. The products were precipitated with ethanol and sodium acetate (0.3M). Sequencing was performed on an ABI 3130 sequencer (Applied Biosystems).

Preparation of cDNA from human blood and biopsy samples

Human peripheral blood mononuclear cells (PBMCs) were purified from healthy volunteers and cellular subsets were purified by positive selection with monoclonal antibodies coupled to magnetic beads (Miltenyi Biotec, Amsterdam, NL) as previously reported (Turner&Muller 2005). Anonymous human biopsies were obtained from

surgically removed organ tissues (adrenal gland, thyroid gland, salivary gland, liver, skin, lung and heart muscle) from the Clinic Sainte Therese, Luxembourg, following national and institutional ethical guidelines. Human post-mortem brain samples from non-demented subjects were obtained from the Netherlands Brain Bank (Netherland Institute of Neuroscience, Amserdam, NL) following international and institutional ethical guidelines. RNA isolation was performed for blood and tissue samples with a mRNA isolation kit (Miltenyi Biotec), for the brain samples with a RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, NL) according to the manufacturer's instruction. First-strand synthesis of total cDNA was carried out at 50°C for 60min using 200U SuperScript III RT (Invitrogen) and 2.5µM dT16 primer in a 40µl reaction containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 5mM $MgCl_2$, 10mM dithiothreitol and 500µM dNTPs, for brain samples using the iScript cDNA Synthesis kit (BioRad, Richmond, CA).

Tissue distribution of Coup-TFII and Coup-TFIIiso

Tissue distribution was determined by classical PCR using the reaction conditions and detection primer sequences listed in table 1. To ensure cDNA quality, β-actin was included as housekeeping gene and internal control.

Quantitative Real Time PCR

Total RNA was purified from THP-1 cells using the High Pure RNA isolation kit (Roche, Mannheim, Germany). First-strand synthesis was carried out at 50 °C for 60 min using 200 U SuperScript III reverse transcriptase and 2.5 µM dT16 primer in a 40 µl reaction containing 250 mM Tris- HCl (pH 8.3, at room temperature), 375 mM KCl, 15 mM MgCl2, 10 mM dithiothreitol and 500 µM dNTPs. Relative PCR quantification was performed in 25 µl reactions containing 20mM Tris–HCl (pH 8.4), 50 mM KCl, 200 mM dNTP, 1x concentrated SYBR Green (Cambrex, Verviers, Belgium) and 2.5 U Platinum Taq DNA polymerase (Invitrogen). Thermal cycling was performed using an Opticon 2 (Bio-Rad). Cycling conditions were: 1 cycle 95 °C, 10 min and 45 cycles each at 95 °C for 20 s and 60 °C (for Coup-TFII 62 °C), 20 s. Primer sets used are listed in Table 8 and relative expression calculated using a standard method (Livak&Schmittgen 2001)

Cloning and expression vector construction

The full length Coup-TFII isoform was amplified by classical PCR using primers (Table 8) covering the complete open reading frame. Subsequent amplification was performed using adaptor primers with NcoI and NotI restriction sites (Table 8). The Coup-TFIIiso entry

clone was generated using restriction cloning of the gene of interest into a pENTR11 vector compatible with the Gateway System (Invitrogen). The classical Coup-TFII receptor entry clone was obtained from the Ultimate ORF collection from Invitrogen. Entry clones were combined with the pDEST53 destination clone by LR reaction following the manufacturer's instruction to generate a mammalian expression vector coding for GFP-fusion Coup-TFII or Coup-TFIIiso.

Cell culture and transfection

Unless otherwise stated, all cell culture material was obtained from Lonza (Verviers, Belgium) and plasticware from Greiner (Wemmel, Belgium). All cultures were maintained at 37°C in a humidified atmosphere with 5% CO2. The human monocytic cell line THP-1 was cultured in RPMI 1640 supplemented with 5% heat-inactivated FBS, 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100µg/ml). Cells were seeded at a density of 10^6 cells per ml into a 24 well plate. Before stimulation cells were either differentiated using PMA or not and finally incubated with or without 10^{-6} M cortisol (Sigma-Aldrich, Bornem, Belgium) and with or without LPS (1µg/ml) (Sigma-Aldrich) for 6h. Human breast adenocarcinoma cell line MCF-7 was cultured in RPMI medium supplemented with 10% FBS, 1% NEAA and 1% sodium pyruvate. Human embryonic kidney cell line HEK 293T was cultured in DMEM medium containing 2mM L-glutamine and 10% FBS. Chinese hamster ovary cell line CHO K-1 was cultured in G-MEM (Invitrogen) supplemented with 10% FBS. 24h before transfection cells, were seeded onto 4-well BD Falcon culture slides (BD Biosciences, Erembodegem, Belgium) at $4x10^4$ cells per well. 24h post seeding, cells were washed once with 1xPBS, 500µl fresh medium was added and 1µg of DNA was transfected by adding 100µl of DNA-lipofectamine LTX (2.25 µl, Invitrogen) complexes in Opti-MEM (Invitrogen) media to the culture.

Microscopy

24h post transfection cells were washed three times with 1xPBS, fixed for 20 minutes in 4% formaldehyde, washed twice in Opti-MEM (Invitrogen) and mounted in anti-fade medium (0.5mM ascorbic acid, 50% glycerol in 1xPBS). Intracellular localisation of GFP-fusion proteins was performed on an Axio Observer Z1 inverted fluorescence microscope fitted with an apotome (Zeiss, Jena, Germany), using a plan-apochromat 63x oil immersion objective lens (N.A. 1.4). Differential interference contrast (DIC) images of whole cells and fluorescence images of GFP distribution were acquired using the Zeiss Axiovision software version 4.6.3.

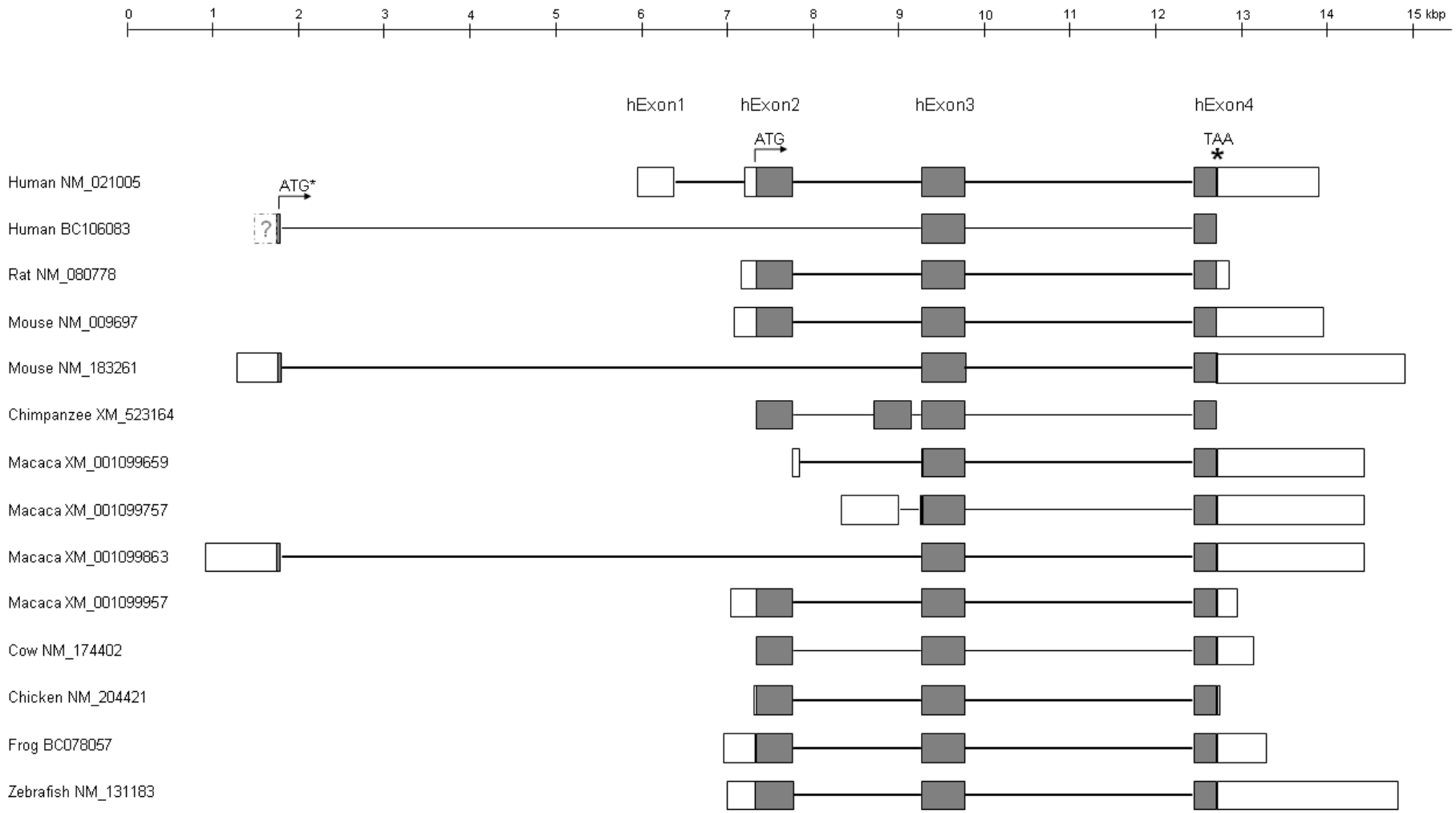

Figure 20: Coup-TFII exon usage of alternative transcripts of nine different species, Homo Sapiens, Rattus norvegicus, Mus musculus, Pan troglodytes, Macaca mulatta, Bos Taurus, Gallus gallus, Xenopus tropicalis with highlighted translated region relative to the human genomic sequence of Coup-TFII, dotted box in the alternative human transcript BC106083 correspond to our predicted first exon, scale in kbp.

Results

Prediction and identification of a new human Coup-TFII first exon

To investigate the 5' heterogeneity of the human Coup-TFII, alternative transcription start sites were localised in the human genomic sequence of Coup-TFII. Species alignments of known sequences of alternative transcripts from nine species and a human sequence submitted to the NCBI database by the mammalian gene collection were used to predict a new human Coup-TFII first exon. Figure 20 shows the species alignment of alternative transcripts with highlighted coding sequences and predicted human first exon. To confirm the existence of the alternative human first exon, PCR forward primer within this exon was combined with a reverse primer in the common exon 3. The 3' boundary of the new exon 1 was located by sequencing of cloned PCR products from total human cDNA. The start of the transcribed region was identified by sequencing cloned nested PCR products from the 5'RACE reaction. The new human Coup-TFII exon was named exon 1A, showed 98.3% homology with the corresponding exons of mouse and macaque and spliced directly to the common exon 3 (Figure 21A, B).

Identification of a new Coup-TFII isoform

The complete sequence of the new alternative Coup-TFII transcript was analysed with respect to alternative translation start sites and an alternative ATG start codon was identified in exon 1A. This start codon was in frame with the stop codon located in exon 4. Figure 21C highlighted the coding regions of the alternative Coup-TFII transcript and showed that this transcript can be translated into a new Coup-TFII protein isoform. Comparison of the amino acid sequences of the classical form and the isoform of the Coup-TFII receptor demonstrated that the isoform lacks the N-terminal region including the DNA binding domain and has a 14 amino acid replacement instead (Figure 22). The other typical structures such as the hinge region, ligand binding domain and C-terminal region with the ligand-inducible activation function (AF-2) are unmodified in the Coup-TFII isoform (Figure 22).

A)

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Coup-TFII exon 1A          AGGATCACTCCAGACATCTCCTACCTACGGTTTGGGGTTTTTTTT-CTTAAAGGCGAGGCTTGCATTCCTCAGCAGCTATGTACAAAGCTCCCTGAAAC-
Macaque XM_001099863       AGGATCACTCCAGACATCTCCTACCTACGGTTTGGGGTTTTTTTT-CTTAAAGGCGAGGCTTGCATTCCTCAGCAGCTATGTACAAAGCTCCCTGAAAC-
Mouse NM_183261            AGGATCACTCCAGACAGCTCCTACCTACGGTTTGGGGTTTTTTTTTCTTAAAGGCGAGGCTTGCATTCCTCAGCAGCTATGTACAAAGCTCCCTGAAGCT

Coup-TFII exon 1A          --CT-TGTCTCTCTAAAGTTAGTGTGCAGGGTTTTCCAA-GGCTGAGAGAGCCTAATACATGGGGAAGCACTTCCTTGAGGTGGAAGATCTCTCCCTTCA
Macaque XM_001099863       --CT-TGTCTCTCTAAAGTTAGTGTGCAGGGTTTTCCAA-GGCTGAGAGAGCCTAATACATGGGGAAGCACTTCCTTGAGGTGGAAGATCTCTCCCTTCA
Mouse NM_183261            GTCTCTCTCTCTCTAAAGTTAGTGTGCAGGCTTTTCCAACGGCTGAGAGCGCCTGGTACACAGGGAAGCAGTTCCTTGAGGTGGAAGATCTCTTCTTTCA

Coup-TFII exon 1A          CCTTTCCTCTTTTTCCCTGCAGGCTAGTGCCTACTTTTT-ATCAGTTTGCACAATCGCTTAGATAAACACCGAGGAGGAGATTCTCTTTAATTATCAAAG
Macaque XM_001099863       CCTTTCCTCTTTTTCCCTGCAGGCTAGTGCCTACTTTTT-ATCAGTTTGCACAATCGCTTAGATAAACACCGAGGAGGAGATTCTCTTTAATTATCAAAG
Mouse NM_183261            CCTTT---CTTTTTCCCTGCAGACTAATGCCTACTTTTTTATCAGTTTGCACAATCGCTTAGATAAACACCGAGGAGGAGAGTCTCTTTAATTATCAAAG

Coup-TFII exon 1A          ACACATCTTTTCAGGGGGCCAACAAAGCATTTATTTCACCCGCCAAACTAAAGGAGAGTTATTCCAGTTTAGGAGGAAGATGCAAGCGGTTTGGGACCTT
Macaque XM_001099863       ACACATCTTTTCAGGGGGCCAACAAAGCATTTATTTCACCCGCCAAACTAAAGGAGAGTTATTCCAGTTTAGGAGGAAGATGCAAGCGGTTTGGGATCTT
Mouse NM_183261            ACACATCTTTTCAGGGGGCCAACAAAGCATTTATTTCACCCGCCAAACTAAAGGAGAGTTATTCCAGTTTAGAAGGAAGATGCAAGCGGTTTGGGACCTT

Coup-TFII exon 1A          GAACAAGGCAAATATGGTTTTG
Macaque XM_001099863       GAACAAGGCAAATATGGTTTTG
Mouse NM_183261            GAACAAGGCAAATATGGTTTTG
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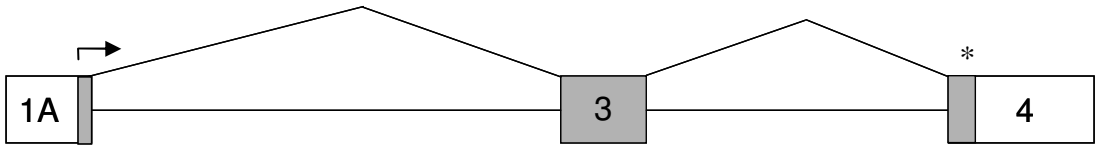
B)



C)

Alternative exon 1A spliced directly to Exon3

AGGATCACTCCAGACATCTCCTACCTACGGTTTGGGGTTTTTTTTCTTAAAGGCGAGGCTTGCATTCCTCAGCAGCTATGTACAAAGCTCCCTGAAACCTTGTCTCTCT*AAAGTTAGTGTGCAGGGTTTTCC*AAGGCTGAGAGAG
CCTAATACATGGGGAAGCACTTCCTTGAGGTGGAAGATCTCTCCCTTCACCTTTCCTCTTTTTCCCTGCAGGCTAGTGCCTACTTTTTATCAGTTTGCACAATCGCTTAGATAAACACCGAGGAGGAGATTCTCTTTAATTATCA
AAGACACATCTTTTCAGGGGGCCAACAAAGCATTTATTTCACCCGCCAAACTAAAGGAGAGTTATTCCAGTTTAGGAGGAAG**ATGCAAGCGGTTTGGGACCTTGAACAAGGCAAATATGGTTTTG**

Common exon 3

CGGTGCAGAGGGGCAGGATGCCGCCGACCCAGCCGACCCACGGGCAGTTCGCGCTGACCAACGGGGATCCCC*TCAACTGCCACTCGTACCTGTCCGG*ATATATTTCCCTGCTGTTGCGCGCGGAGCCCTATCCCACGTCGCGCTT
CGGCAGCC*AATGCATGCAGCCCAACAAC*ATCATGGGTATCGAGAACATTTGCGAACTGGCCGCGAGGATGCTCTTCAGCGCCGTCGAGTGGGCCCGGAACATCCCCTTCTTCCCCGACCTGCAGATCACGGACCAGGTGGCCCTG
CTTCGCCTCACCTGGAGCGAGCTGTTTGTGTTGAATGCGGCGCAGTGCTCCATGCCCCTCCACGTCGCCCCGCTCCTGGCCGCCGCCGGCCTGCATGCTTCGCCCATGTCCGCCGACCGGGTGGTCGCCTTTATGGACCACATAC
GGATCTTCCAAGAGCAAGTGGAGAAGCTCAAGGCGCTGCACGTTGACTCAGCCGAGTACAGCTGCCTCAAGGCCATAGTCCTGTTCACCTCAG

Common exon 4

ATGCCTGTGGTCTCTCTGATGTAGCCCATGTGGAAAGCTTGCAGGAAAAGTCTCAGTGTGCTTTGGAAGAATACGTTAGGAGCCAGTACCCCAACCAGCCGACGAGATTCGGAAAGCTTTTGCTTCGCCTCCCTTCCCTCCG*CAC
***CGTCTCCTCCTCAGTCA*TAGAGCAATTGTTTTTCGTCCGTTTGGTAGGTAAAACCCCCATC*GAAACCCTCATCCGGGATATG*TTACTGTCCGGCAGCAGTTTTAACTGGCCGTATATGGCAATTCAATAA**ATAAATAAAATAAGA
AGGGGGAGTGAAACAGAGAAAGAAAAGGCAAAAGACTGGTTTGTTTGCTTAATTTCCTTCTGTTAAGAAAGGATATAAAAGGATGTTACAAGTTTGCTAAAAGAAGAGAGGGGAAGAATTTAATGGACTGTGAATTTCAAAAAAA
AAAAAAAAGACTGTCAAATGAACTTTTACAGAATGCATTAAAAAAAAAAAAAAACTCCTGTGTCGGTCAGAACAACTTGCTACTTATCATTTTTGTATAAAAAGGAAATTAGTCTTTTTCTTTTTTTGGTAAATTTTTGAAAAAT
ATTGCTAAAAGTGCATTTAAGGAGATTGGGAGACAATTAGCAGAATGGAGAAAGTAAGTCTTTTTTTTTTCCAAATTATTAATTGTCCTGTGTCTATGTACCTCTAGCTGTTCTTTTTTGTACTTTTCTGGTTCCAAACCAGTTT
ATTCTGTGGTTCTATAATAAGTTTTGATATAATCTTGGCTTCTTAAAAACTGTGTATCATTAAAATATATGTTCTGCAAGAATTAAAACTGAGTCCATGAAAATACCATAGGAAGACATAAAACTTTAAAAGGCAACTCAAAGAT
GATGGAAACGCACTTACAAGTGGTGACCAAAATTTTTAGGTGAAGTCGAGCACTCTAATTAGAGAACTGGAGGAACCACATATAACACTTAACTTCCCCTACCCTGCCCCTCCCCAAAAGAAACCATGACAAACCTAGCTTTTAA
AAAATATTTTAAGAAAGAGAATGAACTGTGGAATTTATTGGCAGCCAAGGAATGTGTCCAAGACACATGCTGAGGTTTTGAATAAAAAGTGAACTTTTGTAATTTGAATTGGGTCCCGCTTAGTTCTTGAATTGTTATGAAAATC
CTATATCTGTTTGTATATTTGCAAACCCTTTGTATTATAATTGTTGATATTTTCCCTTTTTAAAAAATACCATTGAAATCAGCATGACAAAAATAACACTGTTGGCACTTA

Figure 21: A) mRNA sequence analysis of newly identified human exon 1A compared to known first exons of macaque and mouse demonstrating the high sequence homology of 98.3% and a common alternative translational start site (arrow) B) schematic illustration of alternative splicing from exon 1A directly to exon 3 and further exon 4, highlighted coding region with indicated start and stop sites. C) mRNA sequence of the newly identified alternative Coup-TFII transcript with highlighted complete coding region.

A)

B)

amino acids	function
1-79	AF-1, ligand independent activation function
79-144	DBD, binding to response elements on the DNA
145-195	hinge region
195-414	LBD and AF-2 (ligand dependent activation function)
363-370	ninth hepted repeat, essential for dimerisation with other NR
394-401	AF-2 core domain, important for transactivation and release of co-repressors
148-401	active repression
402-414	transrepression

Figure 22: A) Coup-TFII and Coup-TFII isoform amino acid alignment, showing the typical five domains of nuclear receptor; the N-terminal region with a ligand-independent activation function (AF-1), the DNA binding domain (DBD), a hinge region, the ligand binding domain (LBD) and the C-terminal region with a ligand-inducible activation function (AF-2). Functional domains of the Coup-TFII isoform lack the N-terminal region including the DBD but containing the complete LBD that harbours multiple functions like nuclear translocation, dimerisation and active repression domain B) tabular overview of amino acids with corresponding functions in Coup-TFII (Achatz et al., 1997).

Tissue distribution of both Coup-TFII forms

Human cDNA, reverse transcribed from blood cells, organ and brain tissue biopsies was used to investigate the expression pattern of both receptor forms. The classical Coup-TFII was detected in all analysed samples (Figure 23). The isoform of the receptor was not detectable in any of the blood cells and only three tissues, thyroid gland, liver and heart muscle showed a weak expression. In contrast we found a relative strong expression of the isoform in all brain regions ranging from amygdala, hippocampus, cingulate gyrus, inferior prefrontal gyrus to nucleus accumbens.

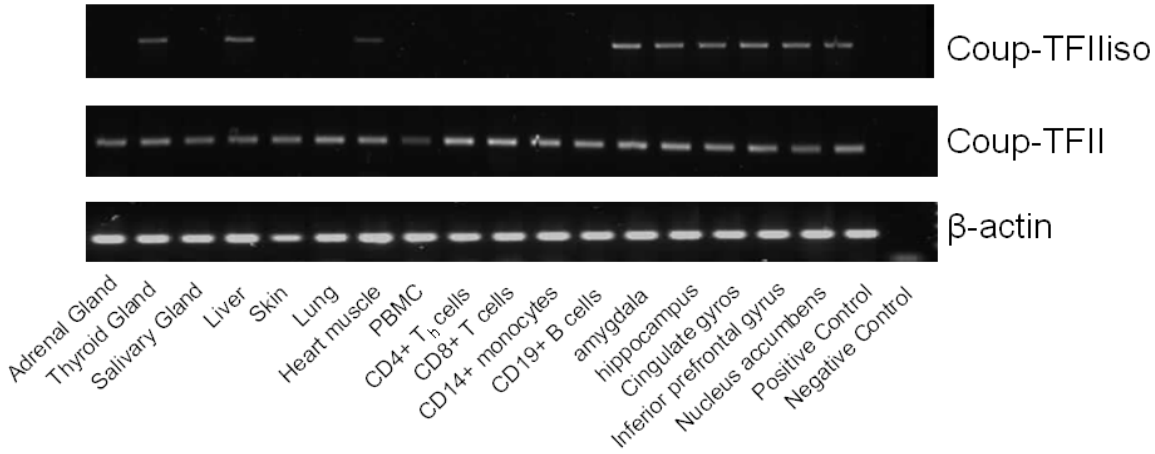


Figure 23: Tissues distribution of Coup-TFII and Coup-TFII isoform. Products of classical detection PCR are shown in different tissues. Total human cDNA and water were used as positive and negative controls, respectively. β-actin was used as housekeeping gene to control for the cDNA quality.

Intracellular localisation of both Coup-TFII forms

Different cell lines, CHO K1, MCF-7 and HEK 293T were transfected with Coup-TFII and Coup-TFIIiso n-terminal fused to GFP and receptor localisation analysed using fluorescence microscopy. In all three cell lines we identified the classical form of the receptor only in the nucleus and not in the cytoplasm (Figure 24). While less stringent, the localisation of the Coup-TFII isoform was similar in all three cell lines and detectable in both compartments, the nucleus and the cytoplasm (Figure 24).

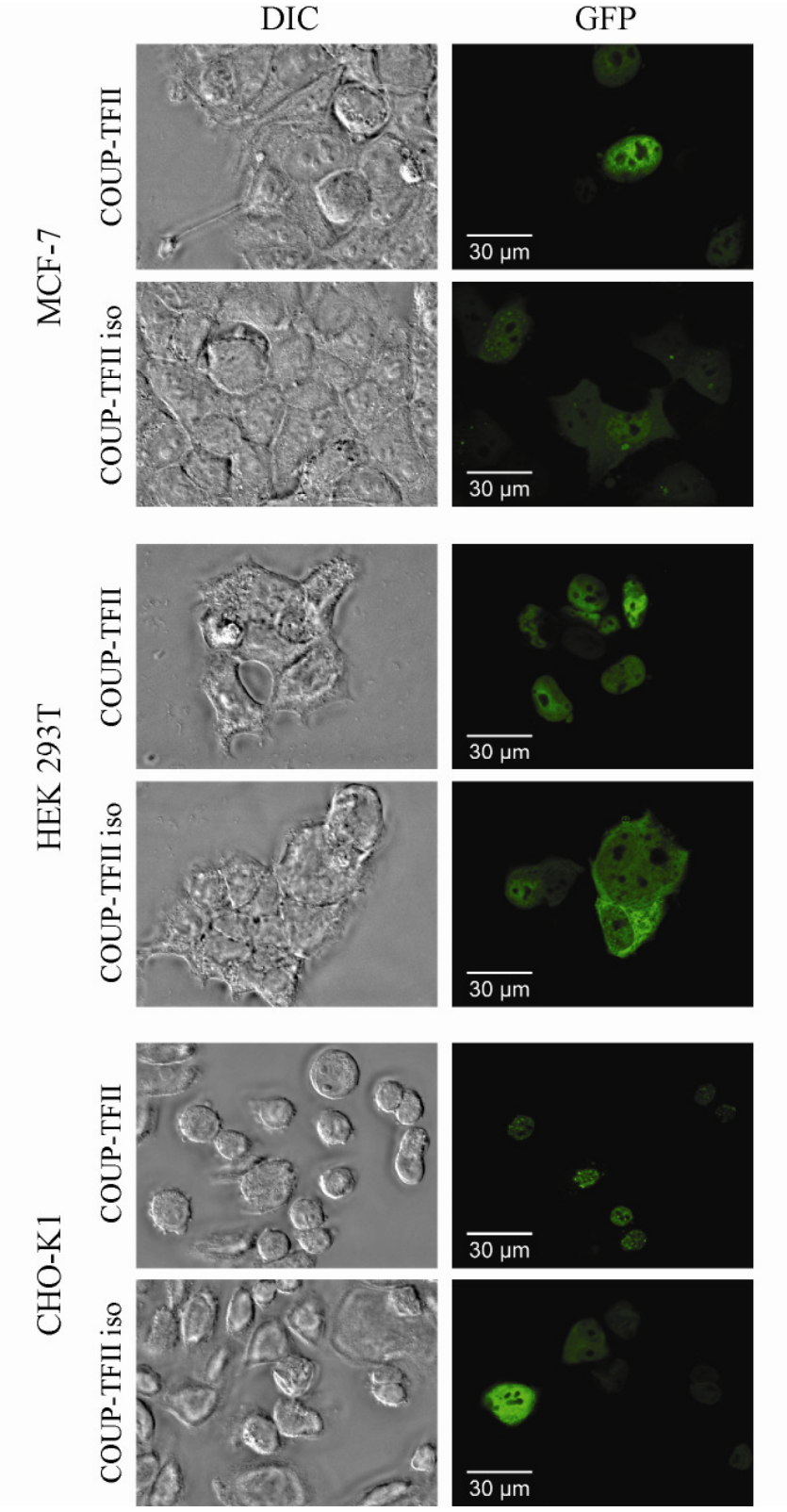


Figure 24: Intracellular localisation of Coup-TFII and Coup-TFIIiso in different cell lines transfected with GFP-fusion protein. Images were processed using ImageJ (NIH, Maryland USA), as follows. DIC images were pseudo-flat field corrected (kernel = 150 pixels) and median-filtered (kernel = 3x3 pixels). Fluorescence images were first background subtracted to remove artefactual horizontal striping typical of apotome acquisitions, then median-filtered (kernel = 3x3 pixels).

Cortisol and LPS dependent relative expression of both Coup-TFII forms

Coup-TFII showed after cortisol, LPS and combined cortisol/LPS treatment an increased expression in THP-1 monocytes. In LPS stimulated THP-1 macrophages the gene expression was decreased whereas the presents of cortisol could restore and even increase the Coup-TFII transcription (Figure 25A). In THP-1 monocytes and LPS treated macrophages Coup-TFIIiso was not at all detectable. However, in THP-1 macrophages after cortisol treatment the Coup-TFIIiso expression was slightly increased (Figure 25B).

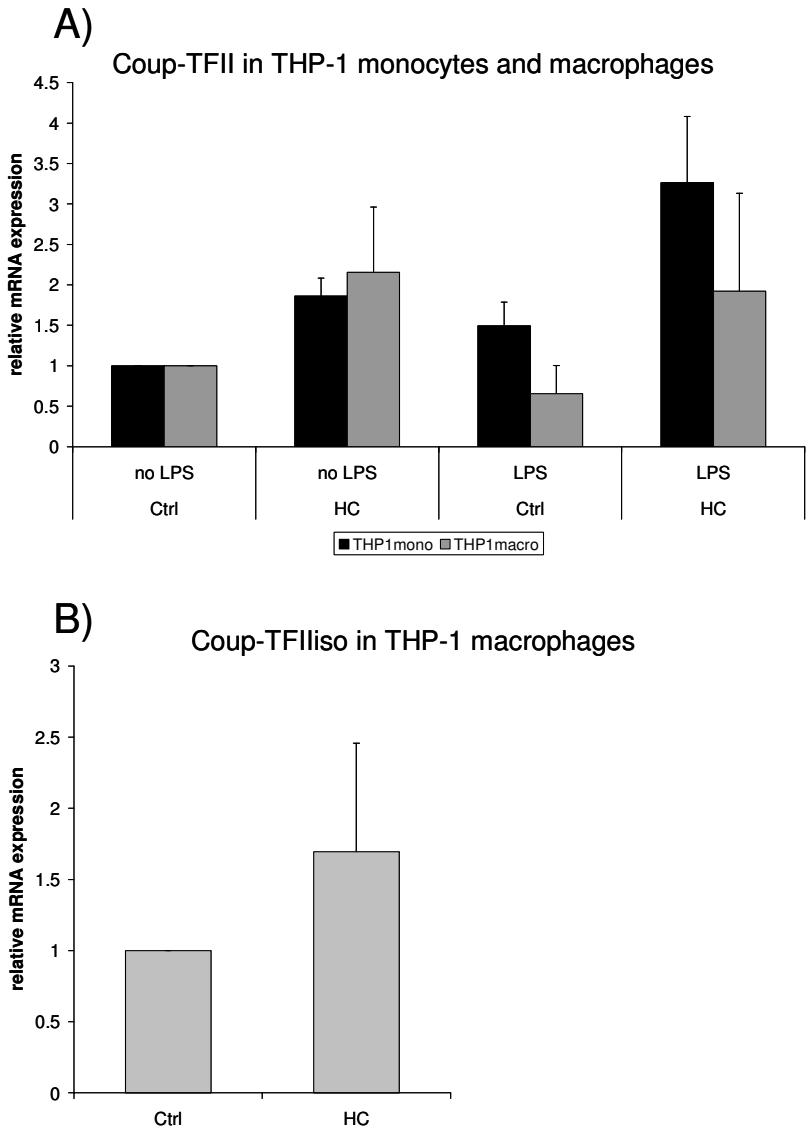


Figure 25: Relative mRNA expression of A) Coup-TFII in THP-1 monocytes and macrophages and B) Coup-TFIIiso in THP-1 macrophages after 6 hour stimulation with cortisol and/or LPS

Discussion

We identified a new protein isoform of Coup-TFII only containing hinge region and LBD. The lack of the complete DBD implicates the loss of gene transcription regulation via direct binding to response elements on the DNA. However, Coup-TF also regulates gene expression via other mechanisms. The molecular basis of Coup-TF actions is intensively studied and well characterised. Coup-TF can repress gene transcription via four mechanisms, the competition for occupancy of DNA binding sites; the DNA-binding-independent competition for RXR, an universal heterodimeric partner of other nuclear receptors; active repression and transrepression (Tsai&Tsai 1997; Park *et al.* 2003; Zhang&Dufau 2004). Positive regulation of gene transcription is realised via three mechanisms, direct activation by binding to DNA response elements; indirect activation by acting as an accessory to other transcription factors and activation through protein-protein interaction with DNA-bound factors (Park *et al.* 2003; Zhang&Dufau 2004). For realisation of Coup-TFII molecular effectiveness, different receptor domains are important. Achatz et al. analysed in detail the effects of internal and terminal deletions on the receptor activity and characterised the minimal domain required for active repression (Achatz *et al.* 1997). While this study inferred a stabilising function of the DBD, Leng et al demonstrated that RXR-bound Coup-TF is sufficient for transrepression without binding to cognate response elements thus inhibiting nuclear receptor-mediated transcription (Leng *et al.* 1996).

The herein newly described Coup-TFII isoform showed a different expression pattern from the classical form of the receptor. The isoform was not at all expressed in blood cells, whereas the classical form of the receptor was expressed in PBMC including different subtypes. Previous studies found that Coup-TFII expression is correlated with the expression of Coup-TFI in immune cells (Schote *et al.* 2007), giving evidence for heterodimeric regulation of genes important for the immune system such as MHC class I (Smirnov *et al.* 2001). Several lymphocyte derived cell lines such as Jurkat and Daudi cells were analysed and did not express the isoform either (data not shown). In THP-1 cells the expression of Coup.TFIIiso was only detectable in the differentiated macrophages with a slight increase after cortisol treatment. From these data we conclude that the Coup-TFII isoform can be regulated by other nuclear receptors such as GR but has no own direct regulatory function in the immune systems. However, both forms were highly expressed in

brain tissues, which could provide evidence for a physiological function of the isoform in differentiation processes in adult brains. The classical form of the receptor is known to be involved in tissue development and differentiation, and seems to play an important role in proliferation in mammalian epithelium and tissue homeostasis (Suzuki *et al.* 2000). Investigations in mice with genetic ablation of Coup-TFII resulted in early embryonic lethality showing the importance of Coup-TFII for organogenesis and cardiovascular development (Pereira *et al.* 2000). It has been demonstrated that Coup-TFII plays a role in motor-neuron development in chicken embryos and was found in a variety of other regions of the nervous system (Lutz *et al.* 1994). However, none of these studies analysed the expression of Coup-TFII in adult brains and investigated the possible impact of a Coup-TFII isoform on physiological functions in the nervous system. Further studies have to focus on the importance of both receptor forms in the brain especially since an interaction of Coup-TFII with the glucocorticoid receptor, a receptor important for the regulation of a wide range of processes in the brain, has been found (De Martino *et al.* 2004b).

We showed that in human breast cancer, human embryonic kidney and Chinese hamster ovarian cell lines both receptor forms have a different cellular localisation. Coup-TFII was in all cell lines located in the nucleus, which is in line with findings of other groups and the functionality as transcription factor (Tsai&Tsai 1997; Suzuki *et al.* 2000; Park *et al.* 2003; Zhang&Dufau 2004). Coup-TFIIiso was found in the cytoplasm and nucleus independently from the cell lines used. A more general function as competitor for protein binding and translocation into the nucleus can be assumed. Studies investigating the function of the Coup-TFII ligand binding domain revealed three major findings. Firstly, the nuclear translocation sequence in the LBD is crucial for the transport into the nucleus (Achatz *et al.* 1997). Secondly, the LBD of the Coup-TFII isoforms harbors an intact dimerisation domain that was shown to be involved in the interaction with other nuclear receptors such as the RXR, VDR, GR and ER (Cooney *et al.* 1993; Klinge *et al.* 1997; De Martino *et al.* 2004b). Thirdly, interactions with corepressors such as NCoR and SMRT are realised as well in the LBD and potentiate Coup-TFs silencing effects (Shibata *et al.* 1997; Zhang&Dufau 2004).

Summarised, we demonstrated that a truncated form of the Coup-TFII receptor exists and that it is differentially expressed in human tissues with highest levels in human brain. Cellular localisation in different cell lines showed that the isoform is not restricted to the nucleus but located in the cytoplasm as well. We postulate that the Coup-TFII isoform compete with the function of other nuclear receptors and coregulators and has therewith an

indirect influence on the gene regulation. A direct repressor function of the isoform could also be possible if the stabilisation due to the DBD is less important or mediated via LBD-LBD interaction with other factors. Further more, functions of the Coup-TFII isoform on the gene regulation, interaction with other receptors as well as physiological implications in adult brains have to be investigated.

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Chapter 6:

General discussion

General discussion

NRs are key regulators in neuroendocine and immune systems. Due to their potential as transcription factors they control a wide variety of cell and tissue specific processes. In this thesis we investigated the transcription of NR throughout the immune system (Chapter 2) and analysed the variety of transcribed sequences (Chapter 3-5).

In the first part of this thesis we investigated human PBMCs and four important immune cell types; CD4 helper T cells, CD8 cytotoxic T cells, CD19 B cells and CD14 monocytes. We demonstrated for the first time that NRs are ubiquitously expressed in human immune cells and that NRs such as CAR, FXR and TLX, that normally have a more restricted expression profile, are also detectable in these cells (Chapter 2). In this study we adapted normalisation techniques to determine the most reliable housekeeping gene for immune cells. We performed a housekeeping gene plot using three genes from different functional classes to correctly normalise mRNA expression values between NRs across multiple cell types. We could show that the stability of housekeeping genes is dependent upon cell type and may compromise their suitability as standards for normalisation between different cell types or tissues. In immune cells, we found the most stable housekeeping gene to be GAPDH but for other tissues our suggests are that at least three genes should be trsted for their suitability as standards in quantitative mRNA measurements. NRs and in particular Coup-TFI and Coup-TFII, showed a coregulation pattern throughout the different immune cells. For some NRs mRNA expression was confirmed at the protein level and different protein bands corresponding to protein isoforms could be detected in a cell type specific manner. Interestingly, for Coup-TF we detected two bands but only one protein form was known. However, for other NRs such as GR a wide variety of different isoforms are known and correlated with a differential immune regulation (Hagendorf *et al.* 2005). This regulation is partially caused by changes in receptor crosstalk and signalling pathways and is currently under investigation.

Possible splice variants for GR and Coup-TFII were investigated in Chapters 3 to 5. We analysed their tissue specific distribution, underlaying mechanisms and possible implication in the immune regulation.

For GR so far 5 different 3' transcripts and 13 different 5' transcript variants based on 9 alternative first exons are known (Turner&Muller 2005; Zhou&Cidlowski 2005; Presul *et al.* 2007). In Chapter 3 we summarise the state of the art of GR alternative first exon usage and their tissue specificity. All the 5' variants contain the same splice acceptor site in exon

2 and vary in the splice donor site in the alternative first exons. Figure 26 provides an overview of the human alternative first exons. In the introduction we showed that the regulation of alternative splicing, the effects of transcription factors, and the actions of alternative transcripts are still under debate. We and other suggest that each of the alternative transcript variants has its own promoter region and therewith a set of specific transcription factor binding sites, which are involved in the cell and tissue specific gene regulation (Breslin&Vedeckis 1998; Breslin *et al.* 2001; Nunez&Vedeckis 2002; Turner&Muller 2005). Due to a high degree of interspecies genomic homology the transcriptional control mechanism should be similar in all vertebrates.

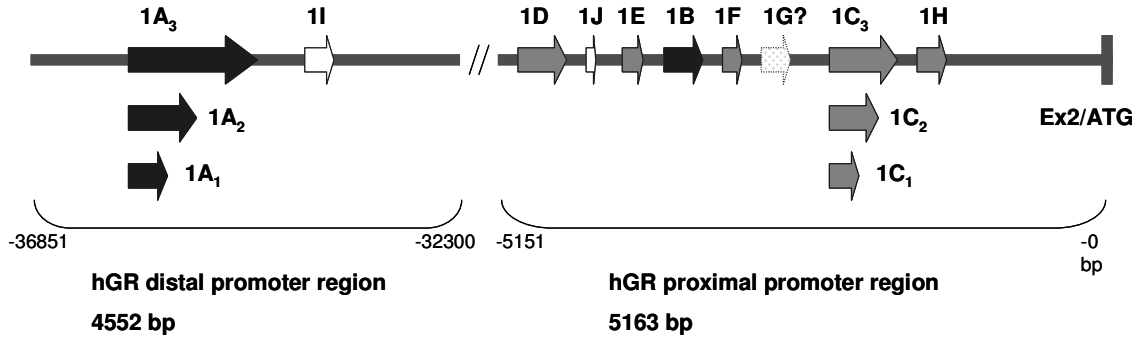


Figure 26: Mapping of the alternative first exon of the hGR. Black filled arrows indicate exons published by (Breslin *et al.* 2001), grey arrows shows exons identified by (Zong *et al.* 1990; Turner&Muller 2005), white indicated exons published by (Presul *et al.* 2007). Homologue of exon 1G exist in the rat but could not yet be amplified in human. The drawing is not to scale but the length of the proximal and distal promoter regions is indicated with the ATG start codon in exon 2 set to 0bp.

Different transcription factors have been shown to be associated with alternative first exons and their promoter usage (Chapter 3). The proximal promoter of exon 1-A has to date received more attention because of its role in GR autoregulation. This promoter contains GRE like half sites that are bound by GRβ but not GRα (Breslin *et al.* 2001). The examination of exon 1-B and 1-C promoter regions were performed on approximately 1kbp fragments (Breslin&Vedeckis 1998; Nunez&Vedeckis 2002). In this region numerous SP-1, AP2 and YY-1 transcription factor binding sites were identified. All the transcription factors were found to play an essential role in the activation of GR transcription and influence tissue specific GR actions (Breslin&Vedeckis 1998; Nunez&Vedeckis 2002). However, at the time these studies were performed exons 1-D to 1-I were not known and cloned regions includes exon 1-D, 1-E, 1-F and 1-G as well as their promoter regions. As such, the promoter activity can not be assigned definitively to the alternative first exons 1-B or 1-C. The analysis of promoter 1-F revealed a binding site

for nerve growth factor-inducible protein (NGFI-A) (McCormick *et al.* 2000; Weaver *et al.* 2004). The NGFI-A was shown to alter the expression of rat GR exon 1_7, human homologous to the human exon 1-F, in response to maternal care and influence GR expressions in rat hippocampus (McCormick *et al.* 2000; Weaver *et al.* 2007). Studies analysing the individual promoter regions for each first exon and the associated transcription factor binding sites needs to be performed to identify future applications for diagnosis, prognosis and treatment of GR associated disorders.

Not only transcript variants but protein isoforms are also important for the NR diversity. So far, 16 protein isoform have been described for the human GR but functions of each of these are not yet clearly understood. The work of the Cidlowski group demonstrated that for each GRα and GRβ transcript variant, 8 alternative translational initiations can occur (Yudt *et al.* 2003; Zhou&Cidlowski 2005). These correspond each to a methionin in the final protein sequence coded by different ATGs. All 8 splice forms of, for example GRα, are functional receptors that contain an identical intact LBD, only varying in the N-terminal A/B region. All GRα isoforms induce reporter gene expression in a dose-dependent manner, but show partially higher or lower transcriptional activities then the wild-type GRα (Duma *et al.* 2006; Lu&Cidlowski 2006). In Chapter 4 we identified a new GR protein isoform showing that other naturally occurring isoforms are possible, despite the already characterised protein variants. We identified a GRα protein isoform with a deletion in exon 2, between amino acids 313 and 338. This deletion occurs in the N-terminal transactivation domain (amino acids 1 to 420) before the DBD. Previous studies showed that exon 2 modifications as well as the complete removal of that exon in GR exon 2 knockout (GR2KO) mice result in an altered GC-induced gene transcription pattern. In GR2KO mice some GC responsive genes such as IL-7 receptor α-chain and glutamine synthetase showed a normal whereas other GR targets showed a reduced (*GILZ*) or no response (*FKBP51*) to GCs (Mittelstadt&Ashwell 2003). These data suggest, although aberrant, the C-terminal half of the GR contains a considerable amount of ligand-regulated transcriptional activity. Another important change introduced through the deletion in exon 2 is the modification of possible phosphorylation sites. The status of GR phosphorylation alters its transcriptional activity, decreases GR half-life by promoting a rapid turn over and influence target gene transcription dependent on the promoter context of the target gene (Duma *et al.* 2006). A closer look into the sequence to identify possible alternative transcription and translation start sites and their analysis in different tissues is necessary to

identify the multiply of possible receptor variants and therewith explain some of the specific receptor functions.

From our studies on the GR we know that alternative promoter usage and species homologies can provide information on the use of alternative first exons. In Chapter 5 we investigated the genomic sequence of Coup-TFII given in the database for transcriptional start sites (DBTSS) and found an alternative promoter region (ref. name WMD05712) 7718bp upstream of the normally used transcription start. Subsequently, we performed an alignment of the Coup-TFII reference mRNA sequences from nine different species and identified a new Coup-TFII alternative first exon that splices to the common exon 3. We suggest that the Coup-TFII alternative transcript has its own promoter region which is independent of the normal promoter. We performed an *in silico* analysis on the region 1559bp in front of the new first exon 1A (alternative promoter 1) and compared it with the promoter region, 4385bp upstream of the start of exon 1 (alternative promoter 2). We found that the binding sites of most common transcription factors are differentially distributed throughout both promoter regions. For example, the TATA binding sites occur twice as frequently, the CREB site almost ten times more often in the alternative promoter 1 (Table 9). The distribution of NFkappaB, OCT-1 and C/EBPdelta is equal between both promoters whereas the AP-1 sites were nearly twice as often in the alternative promoter 2 (Table 9). Unfortunately, the genomic data for other species were limited, making an interspecies comparison impossible for these regions. However, the alternative promoter region in front of exon 2 showed a high sequence homology in the analysed mammalian sequences as well as highly conserved binding sites for transcription factors. This highly conserved intronic sequence may play an important role in regulatory mechanisms controlling transcription and occurrence of splice events. A recent study investigated the impact of TFs as *trans*-acting factors on the regulation of genes. These *trans*-acting factors modify the expression of genes that are not in close proximity to their respective TF but are controlled by the binding of TF to response elements. Zhou *et al*. discovered that the number of TFs regulating a gene accounts for 8-14% of its expression variation, which is much higher than the impact of *cis*-elements. *Cis*-elements contain short consensus sequences that can act either as silencer or enhancer of transcription. These elements regulate the expression of genes located on the same chromosome and account only for 0.3-1.7% of the expression variation (Zhou *et al.* 2008). Taken these data into account we hypothesise that the alternative transcript is more strictly regulated than the classical transcript of Coup-TFII. We could confirm the tightly control of the Coup-TFII alternative

transcript with the performed tissue distributions and found that this receptor form is expressed in different brain areas including amygdala, hippocampus, cingulate gyrus, inferior prefrontal gyrus and nucleus accumbens but not at all in the cells of the immune system.

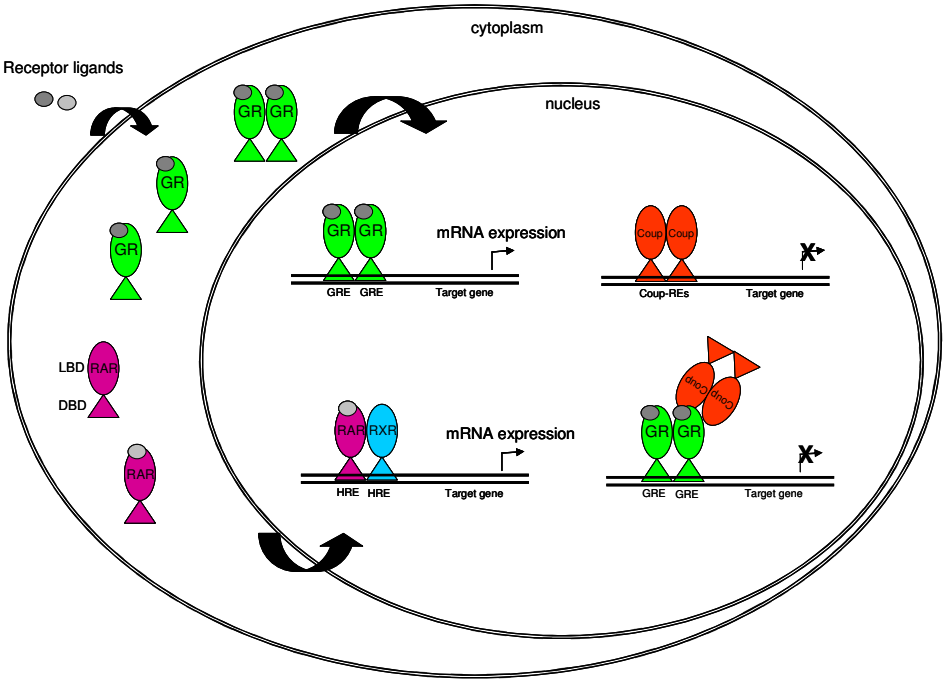
Table 9: Coup-TFII promoter analysis with regard to transcription factor binding sites

Transcription factor	AP[1]-1 infront of exon 1A (number of sites found in TRANSFAC search)	AP-2 infront of exon 1 (number of sites found in TRANSFAC search)
AP-1	8	15
C/EBPdelta	5	7
CREB	21	2
NFkappaB	2	2
Oct-1	8	8
TATA	12	6
Total	56	40

[1] alternative promoter

Further sequence investigations revealed a new Coup-TFII protein isoform translated from an alternative translation initiation site in the new first exon. No alternative protein isoforms are so far known for the Coup-TF orphan receptors. This newly identified Coup-TFII protein variant does not contain a DNA binding domain, which is exceptional for NRs. The lack of a DBD offers the possibility of new molecular functions for potentially many NRs. The Coup-TFII isoform may act as an ubiquitous heterodimerisation competitor for other NR in nucleus and cytoplasms. As such, its function could be that of a transcriptional repressor. Figure 27B shows, how the proposed molecular mechanisms of Coup-TFIIiso fits in the mechanism of NR action. We hypothesise that in the cytoplasm the Coup-TFII isoform binds either non ligated NRs and inhibit the cognate ligand binding or interacts with ligated NRs and suppresses their nuclear translocation. In the nucleus, the isoform can not bind to the DNA and regulate gene transcription via direct binding to response elements. Accordingly, it is available for interactions with other NRs, reduces their binding to cognate response elements and subsequently inhibits their target gene expression. No data are available for NR actions without the characteristic DBD, but without that domain the other functions mediated by the LBD seems to be favoured. Firstly, the nuclear translocation sequence in the LBD is crucial for the transport into the nucleus and enable the translocation of Coup-TFIIiso even without a DBD (Achatz *et al.* 1997). Secondly, the LBD of the Coup-TFII isoforms harbors an intact dimerisation

A)



B)

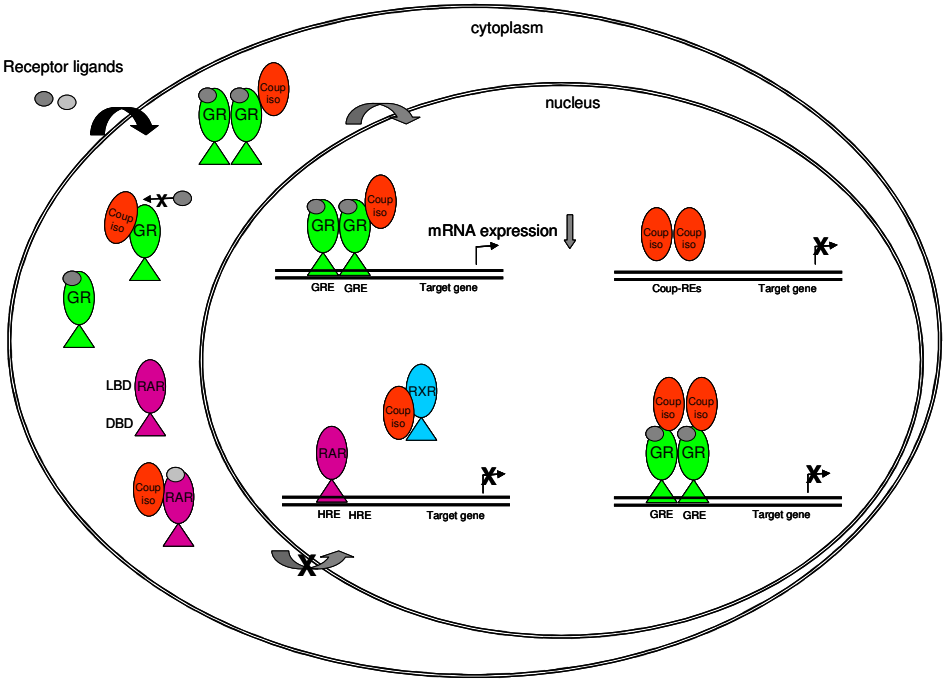


Figure 27: A) molecular mechanism of NRs in the nucleus after ligand binding. Transcription activation through GR homodimers and RAR-RXR heterodimers and transcription suppression via Coup-TF homodimers or protein-protein interactions with the DNA bound GR. B) proposed molecular mechanism of the new Coup-TFII isoform. In the cytoplasm Coup-TFIIiso can inhibit NR ligand binding and/or the nuclear translocation of ligated NR. In the nucleus four possible ways of suppressing mRNA expression are hypothesised: i) interaction with DNA bound homodimer complexes, ii) absense of Coup-TFIIiso DNA binding possibilities, iii) binding RXR and remove it for other interactions and iv) protein-protein interactions similar to the classical Coup-TF.

domain that was shown to be involved in the interaction with other nuclear receptors such as the RXR, VDR, GR and ER (Cooney *et al.* 1993; Klinge *et al.* 1997; De Martino *et al.* 2004b). Thirdly, interactions with corepressors such as NCoR and SMRT are realised as well in the LBD and potentiate Coup-TFs silencing effects (Shibata *et al.* 1997; Zhang&Dufau 2004).

The important role of Coup-TF in the mainly negative regulation of different pathways especially RXR and GR mediated signalling could be altered by the existence of new protein isoforms. The NRs investigated in this thesis, Coup-TFII and GR, are known to interact and influence each others activities as well as interacting with the common NR heterodimersiation partner RXR. RXR, together with GR, is involved in the suppression of several aspects of inflammation and as such contributes to the inhibition of various neurodegenerative diseases. The interaction of Coup-TFII with these pathways leads to a decrease of RXR and GR mediated gene transcription. If our mechanistic hypothesis is correct, the availability of the new Coup-TFII isoform in certain tissues such as the brain, could increase such downregulation (Figure 27). The downregulation of target gene expression can result in either immune activation or immune suppression dependent on the involved set of genes and effect in the end the regulation of neurodegenerative diseases. The impact of the new described Coup-TFII isoform on these signalling pathways needs to be further investigated and its own regulation has to be further validated.

Posttranslational modifications of the Coup-TFII have not been described in the literature. We analysed both forms to predict differences in posttranslational modifications using the NetPhos 2.0 software (http://www.cbs.dtu.dk/services/NetPhos/). We focused on potential phosphorylation sites and found in the Coup-TFII 9 serine, 6 threonine and 6 tyrosine and in Coup-TFIIiso 5 serine, 3 threonine and 5 tyrosine. The distribution of these sites is shown in Figure 28. The N-terminus of Coup-TFII has 8 modifiable amino acids, whereas the corresponding sequence from Coup-TFIIiso has only 1. This demonstrates a lower modification frequency in the isoform compared to the classical Coup-TFII receptor. It is known that many phosphorylation sites are located in the N-terminal region of NRs and that they influence the interaction with coregulators and the transcriptional activity of the NRs (Kumar&Thompson 2003; Weigel&Moore 2007). Due to the widely missing N-terminus including AF-1 domain and the absence of possible modifications sites in the Coup-TFII isoform, the interaction with coactivators might be reduced, which provides additional evidence for a more general repressor function of the Coup-TFII isoform.

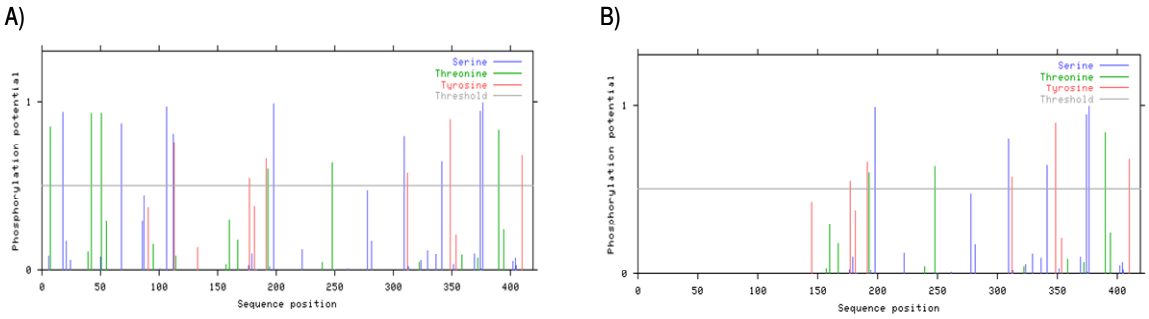


Figure 28: Net Phos 2.0 predicted phosphorylation sites for A) Coup-TFII and B) Coup-TFIIiso. * the only new phosphorylation site predicted in Coup-TFIIiso, which replace 8 possible phosphorylation sites in Coup-TFII.

As shown in the introduction, the immune and neuroendocrine systems are working together and influencing each others activities for example via the HPA axis. Especially in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease but also in others, the neuroendocrineimmune network is though to be an important regulatory factor. Recently, the analysis of GRα and GRβ expressions in the brain revealed that GRβ is present in very low amounts in the control human hippocampus, mRNA ratio of GRα to GRβ of 14500:1 (DeRijk *et al.* 2003). In human PBMCs all three GR mRNA isoforms were detected at relative levels of α/P/β = 1:0.25:0.001 (Hagendorf *et al.* 2005). Changes in the GRα to GRβ ratio towards higher GRβ levels may promote GR resistance, which is frequently observed in Alzheimer's disease or major depression. Another recent study demonstrated a differential expression profile of three GRα protein isoforms in rat hippocampus samples. GR-A, GR-C and GR-D were expressed in the rat brain over the course of postnatal development and maturation (Galeeva *et al.* 2006). The expression of different GR protein isoforms can be correlated with the HPA axis stress response that changes due to mild manipulations with pups in their neonatal life. However, the modified HPA axis function was not persistent in later life and the different GR isoforms did not show a differential expression in adult rat brains (Galeeva *et al.* 2006). These findings underline the importance of analysing different NR variants as cause or effect of certain disorders. In humans, the dramatic effects of cortisol changes in diverse psychological and immunological diseases are well described but the functional mechanisms are still not fully understood and are only recently investigated. Since human brain studies have many limitations, not least being access to such tissue, more effort has been put into the investigation of GR single nucleotide polymorphisms in peripheral tissues such as human

PBMCs and their clinical implications. Several GR polymorphisms were investigated and have been associated with GC resistance, sensitivity and interindividual variability of HPA axis activity (DeRijk *et al.* 2002). The common GR polymorphism *BclI* (rs41423247) located in intron 2, 646 nucleotides downstream from exon 2 was shown to be associated with increased GC sensitivity and increased susceptibility to develop major depression (van Rossum *et al.* 2003; van Rossum *et al.* 2006). The Asn363Ser polymorphism was shown to be associated with higher salivary cortisol levels after the Trier Social Stress Test and related to an increased response to a psychosocial stress procedure (Wust *et al.* 2004). Other studies demonstrated that the promoter associated polymorphism NR3C1-1 (rs10482605) and the in exon 2 located polymorphism Arg23Lys (rs6190) increase the genetic vulnerability for major depression (van West *et al.* 2006; Kumsta *et al.* 2008). Russcher *et al*. demonstrated that the reduced GC sensitivity associated with the Arg23Lys polymorphism is caused by an increased GR-A protein expression. Approximately 15% more GR-A than GR-B protein was detected in the carriers of the polymorphism whereas the total GR levels were not affected (Russcher *et al.* 2005). The polymorphism facilitates the expression of the less transcriptional active GR-A protein isoform and reduces accordingly the more active GR-B protein. This change in ratios is suggested to cause decreased GC sensitivity and resulted in a better metabolic health profile as well as increased chance of longevity (Russcher *et al.* 2005). As demonstrated above, polymorphisms only affect small parts of the genomic DNA sequence either in coding, non-coding or intergenic regions due to single nucleotide changes. However, they can have large effects on the protein sequence and therewith on protein structure and function or have consequences for gene splicing and transcription factor binding. Splice variants on the other hand are determined by changes in the transcription and translation of the genomic DNA and occur in a cell and tissue specific manner. Polymorphisms can influence these splice variants either by modifying the splice sites or by changing the binding sites for other cofactors and have therewith effects on the activity of receptor variants.

Other NRs are not yet that intensively studied, although there are some promising investigations. Recently, an association between the PPARγ polymorphisms Pro12Ala and exon 6 C478T with the earlier onset of dementia was made (Koivisto *et al.* 2006). Two polymorphisms *PvuII* (rs2234693) and *XbaI* (rs2077647) in the first intron of ERα were shown to increase the risk of Alzheimer's disease in women (Schupf *et al.* 2008). The common NURR1 polymorphism NI6P that displays a single base-pair insertion in intron 6

has been reported to be associated with an increased risk of Parkinson disease (Zheng *et al.* 2003). However, the four common polymorphisms *FokI* (rs10735810), *BsmI* (rs1544410), *ApaI* (rs7975232), and *TaqI* (rs731236) in the VDR were studied in patients with schizophrenia and could not be significantly associated with an increases risk (Handoko *et al.* 2006). In the dbSNP, 46 and 82 single nucleotide polymorphisms have been registered for human Coup-TFI and Coup-TFII, respectively. None of these has yet been associated with any disease and no information is available from the literature that this has been considered for investigations. Until now, not a single study has been performed, analysing Coup-TFs and their variants as important factors in neuroimmune diseases, although their complex regulation of neural specific genes and early brain development is well known (Lu *et al.* 1994; Lutz *et al.* 1994; Pereira *et al.* 2000; Suzuki *et al.* 2000). In line with the role of Coup-TFs in the brain, we observed Coup-TFII and Coup-TFIIiso as being highly expressed in the brain and hypothesise that these receptors have there an ubiquitous repressor function. In the immune system the Coup-TFII isoform was absent, suggesting in healthy subject no implications in the immune regulation. However, in the investigated THP-1 macrophages we could see, after cortisol stimulation, a slight increase in the expression of Coup-TFIIiso, which could provide initial evidence for an increased action in for example hypercortisolemic patients. Additionally, from the above mentioned studies on the GR it is known that a change in the ratios of different isoforms can increase the probability for certain disease. Variations in the Coup-TFII to Coup-TFIIiso ratio could also be involved in an increased susceptibility for diseases and needs to be further considered as a relevant factor in neuroendocine disorders.

Summary

In this thesis the ubiquitous expression of non-steroidal NRs in human immune cells was demonstrated for the first time and NRs that have normally a more restricted tissue distribution were detected. Correlation of NRs showed a coregulation of Coup-TFI and Coup-TFII. These orphan receptors are known to form heterodimers with each other and other NRs and repress gene transcription, although they had not previously been reported in immune cells. The expression of Coup-TFs in immune cells facilitates heterodimerisation with GR, regulation of target genes in immunity and the stress response. Since these functions can be impaired by Coup-TF and GR isoforms, alternative splicing was investigated. The GR, already well characterised, was a good paradigm for the

initial investigations of alternative splice events. We extended the use of GR alternative first exons to other species and provided evidence for a conserved and tightly controlled function of these 5´alternative transcript variants in the gene regulation. These splice forms are not translated into protein but might influence protein functions including protein-protein interactions by for example phosphorylation. Our investigations of the N-terminal transactivation domain in exon 2 of the GR showed that there are a number of potential phosphorylation sites which are important for the transactivation of GR and further interactions with other proteins. We found a GR variant that has a deletion in that domain suggesting an altered glucocorticoid-dependent transactivation profile. The GR variant was only expressed in some peripheral tissues but not in the immune cells which exclude its direct implication in the heterodimerisation of GR and Coup-TFII. Although, Coup-TFII is known to regulate complex processes, only one variant of this receptor had been previously described. We report a new human Coup-TFII alternative first exon that introduce an in frame alternative translation start site. This new Coup-TFII protein isoform (Coup-TFIIiso) had no DNA binding domain which excludes a function via direct binding to its response element. We suggest that this isoform can interact via its LBD with other ligated NRs such as the GR and repress their transcriptional activity. However, the tissue distribution of this Coup-TFII variant showed a strong expression in the brain and was absent in human immune cells suggesting that there will be no heterodimerisation of Coup-TFIIiso and the GR in the immune but maybe in the nervous system. The distribution also provides evidence for tightly controlled, cell type specific actions of this receptor variant, a mechanism we also observed for the GR. Stimulation of differentiated THP-1 cells, a model for macrophages, with stress hormones showed an increase of Coup-TFIIiso expression, providing evidence for a prominent role of this isoform only in stress pathophysiology rather physiology. We showed that NR variants are important for the understanding of the enormous regulatory potential of this receptor family and play a distinct role in the interaction with other factors and pathways. With the work presented in this thesis, another part in the puzzle of NR complexity has been added and further investigations will bring these findings together with possible therapeutic strategies developed for complex diseases such as stress related and neurodegenerative disorders.

Future perspectives

The major findings presented in this thesis were not described previously and could offer a completely new understanding of NR actions. To our knowledge no DBD deficient NR has previously been described and characterised. Further investigations are needed to determine the role and mechanism of Coup-TFIIiso, for which we have so far only hypothesis. The most interesting future investigations are based on the protein-protein-interactions of Coup-TFIIiso with other factors. As proposed, we would expect that the isoform interacts with NRs such as the GR but also with coregulators. To elucidate proteins involved in Coup-TFIIiso interactions co-immunoprecipitation is considered to be the gold standard assay. The protein of interest is isolated with a specific antibody and interaction partners which stick to this protein are subsequently identified by western blotting. Before finishing this thesis, we were able to produce in house mouse sera containing polyclonal antibodies directed against the 14 N-terminal amino acids of Coup-TFIIiso, and showed them to be specific in both western Blot and ELISA. This serum could be used for the Coup-TFIIiso specific coimmunoprecipitation. A polyclonal antibody against an internal amino acid sequence of all so far known Coup-TFs could be used as control. Interactions detected by this approach are considered to be real and would give evidence for the Coup-TFIIiso interaction partners. To determine the involved transcription factors important for the regulation of Coup-TFIIiso itself, reporter gene assays with the already mentioned 1559bp promoter region could be cloned and further investigated. The appropriate set of transcriptions factors can be analysed with real-time PCR in different tissues including immune cells and brain areas. Colocalisation of the truncated Coup-TF isoform in cell lines together with other NRs under treatment will open more insights into the intracellular localisation and might provide some proof for the hypothesis of ligand binding inhibition in the cytoplasm. The strong expression of Coup-TFIIiso we observed in human brain samples could be further quantified in brain samples from the Netherlands Brain Bank including control and depressed samples to determine the possible impact of Coup-TFIIiso in psychosocial diseases. However, a lot of fundamental research needs be performed on the Coup-TFII isoform before this knowledge can be applied to clinical studies. The investigations of GR variants are already beyond such teething troubles and are already intensely studied at our institute. Especially the different first exons, their promoter regions and regulations are in the focus of further projects that will give new insights in psychosocial diseases such as depression.

References

Achatz G., Holzl B., Speckmayer R., Hauser C., Sandhofer F.&Paulweber B. (1997). Functional domains of the human orphan receptor ARP-1/COUP-TFII involved in active repression and transrepression. *Mol Cell Biol* **17**(9): 4914-32.

Adamson K. A., Pearce S. H., Lamb J. R., Seckl J. R.&Howie S. E. (2004). A comparative study of mRNA and protein expression of the autoimmune regulator gene (Aire) in embryonic and adult murine tissues. *J Pathol* **202**(2): 180-7.

Akerblom I. E., Slater E. P., Beato M., Baxter J. D.&Mellon P. L. (1988). Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. *Science* **241**(4863): 350-3.

Aranda A.&Pascual A. (2001). Nuclear hormone receptors and gene expression. *Physiol Rev* **81**(3): 1269-304.

Arnold K. A., Eichelbaum M.&Burk O. (2004). Alternative splicing affects the function and tissue-specific expression of the human constitutive androstane receptor. *Nucl Recept* **2**(1): 1.

Auboeuf D., Dowhan D. H., Kang Y. K., Larkin K., Lee J. W., Berget S. M.&O'Malley B. W. (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**(8): 2270-4.

Auboeuf D., Honig A., Berget S. M.&O'Malley B. W. (2002). Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* **298**(5592): 416-9.

Bain D. L., Heneghan A. F., Connaghan-Jones K. D.&Miura M. T. (2006). Nuclear Receptor Structure: Implications for Function. *Annu Rev Physiol*.

Barish G. D., Downes M., Alaynick W. A., Yu R. T., Ocampo C. B., Bookout A. L., Mangelsdorf D. J.&Evans R. M. (2005). A Nuclear Receptor Atlas: Macrophage Activation. *Mol Endocrinol*.

Bas A., Forsberg G., Hammarstrom S.&Hammarstrom M. L. (2004). Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol* **59**(6): 566-73.

Beigneux A. P., Moser A. H., Shigenaga J. K., Grunfeld C.&Feingold K. R. (2000). The acute phase response is associated with retinoid X receptor repression in rodent liver. *J Biol Chem* **275**(21): 16390-9.

Beigneux A. P., Moser A. H., Shigenaga J. K., Grunfeld C.&Feingold K. R. (2002). Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. *Biochem Biophys Res Commun* **293**(1): 145-9.

Berrodin T. J., Marks M. S., Ozato K., Linney E.&Lazar M. A. (1992). Heterodimerization among thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, chicken ovalbumin upstream promoter transcription factor, and an endogenous liver protein. *Mol Endocrinol* **6**(9): 1468-78.

Bocquel M. T., Kumar V., Stricker C., Chambon P.&Gronemeyer H. (1989). The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell-specific. *Nucleic Acids Res* **17**(7): 2581-95.

Boeger H., Bushnell D. A., Davis R., Griesenbeck J., Lorch Y., Strattan J. S., Westover K. D.&Kornberg R. D. (2005). Structural basis of eukaryotic gene transcription. *FEBS Lett* **579**(4): 899-903.

Breslin M. B., Geng C. D.&Vedeckis W. V. (2001). Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. *Mol Endocrinol* **15**(8): 1381-95.

Breslin M. B.&Vedeckis W. V. (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**(5-6): 369-81.

Bruggemeier U., Kalff M., Franke S., Scheidereit C.&Beato M. (1991). Ubiquitous transcription factor OTF-1 mediates induction of the MMTV promoter through synergistic interaction with hormone receptors. *Cell* **64**(3): 565-72.

Brunner A. M., Yakovlev I. A.&Strauss S. H. (2004). Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol* **4**(1): 14.

Brzozowski A. M., Pike A. C., Dauter Z., Hubbard R. E., Bonn T., Engstrom O., Ohman L., Greene G. L., Gustafsson J. A.&Carlquist M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**(6652): 753-8.

Carlstedt-Duke J., Stromstedt P. E., Wrange O., Bergman T., Gustafsson J. A.&Jornvall H. (1987). Domain structure of the glucocorticoid receptor protein. *Proc Natl Acad Sci U S A* **84**(13): 4437-40.

Chambon P. (2005). The nuclear receptor superfamily: a personal retrospect on the first two decades. *Mol Endocrinol* **19**(6): 1418-28.

Charmandari E., Kino T., Ichijo T.&Chrousos G. P. (2008). Generalized glucocorticoid resistance: clinical aspects, molecular mechanisms, and implications of a rare genetic disorder. *J Clin Endocrinol Metab* **93**(5): 1563-72.

Chen F., Watson C. S.&Gametchu B. (1999). Multiple glucocorticoid receptor transcripts in membrane glucocorticoid receptor-enriched S-49 mouse lymphoma cells. *J Cell Biochem* **74**(3): 418-29.

Chen J. D. (2000). Steroid/nuclear receptor coactivators. *Vitam Horm* **58**: 391-448.

Chen N.&Napoli J. L. (2008). All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *Faseb J* **22**(1): 236-45.

Chen S., Wang J., Yu G., Liu W.&Pearce D. (1997). Androgen and glucocorticoid receptor heterodimer formation. A possible mechanism for mutual inhibition of transcriptional activity. *J Biol Chem* **272**(22): 14087-92.

Chiang J. Y. (2002). Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* **23**(4): 443-63.

Chisa J. L.&Burke D. T. (2007). Mammalian mRNA splice-isoform selection is tightly controlled. *Genetics* **175**(3): 1079-87.

Clark F.&Thanaraj T. A. (2002). Categorization and characterization of transcript-confirmed constitutively and alternatively spliced introns and exons from human. *Hum Mol Genet* **11**(4): 451-64.

Cooney A. J., Lee C. T., Lin S. C., Tsai S. Y.&Tsai M. J. (2001). Physiological function of the orphans GCNF and COUP-TF. *Trends Endocrinol Metab* **12**(6): 247-51.

Cooney A. J., Leng X., Tsai S. Y., O'Malley B. W.&Tsai M. J. (1993). Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. *J Biol Chem* **268**(6): 4152-60.

Cooney A. J., Tsai S. Y., O'Malley B. W.&Tsai M. J. (1992). Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D3, thyroid hormone, and retinoic acid receptors. *Mol Cell Biol* **12**(9): 4153-63.

Copik A. J., Webb M. S., Miller A. L., Wang Y., Kumar R.&Thompson E. B. (2006). Activation function 1 of glucocorticoid receptor binds TATA-binding protein in vitro and in vivo. *Mol Endocrinol* **20**(6): 1218-30.

Crofts L. A., Hancock M. S., Morrison N. A.&Eisman J. A. (1998). Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci U S A* **95**(18): 10529-34.

Czlonkowska A., Ciesielska A., Gromadzka G.&Kurkowska-Jastrzebska I. (2006). Gender differences in neurological disease: role of estrogens and cytokines. *Endocrine* **29**(2): 243-56.

De Bosscher K., Van Craenenbroeck K., Meijer O. C.&Haegeman G. (2008). Selective transrepression versus transactivation mechanisms by glucocorticoid receptor modulators in stress and immune systems. *Eur J Pharmacol*.

de Kloet E. R., Oitzl M. S.&Joels M. (1999). Stress and cognition: are corticosteroids good or bad guys? *Trends Neurosci* **22**(10): 422-6.

De Kloet E. R., Vreugdenhil E., Oitzl M. S.&Joels M. (1998). Brain corticosteroid receptor balance in health and disease. *Endocr Rev* **19**(3): 269-301.

de Kok J. B., Roelofs R. W., Giesendorf B. A., Pennings J. L., Waas E. T., Feuth T., Swinkels D. W.&Span P. N. (2005). Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest* **85**(1): 154-9.

De Martino M. U., Alesci S., Chrousos G. P.&Kino T. (2004a). Interaction of the glucocorticoid receptor and the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII): implications for the actions of glucocorticoids on glucose, lipoprotein, and xenobiotic metabolism. *Ann N Y Acad Sci* **1024**: 72-85.

De Martino M. U., Bhattachryya N., Alesci S., Ichijo T., Chrousos G. P.&Kino T. (2004b). The glucocorticoid receptor and the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II interact with and mutually affect each other's transcriptional activities: implications for intermediary metabolism. *Mol Endocrinol* **18**(4): 820-33.

Deluca H. F.&Cantorna M. T. (2001). Vitamin D: its role and uses in immunology. *Faseb J* **15**(14): 2579-85.

DeRijk R. H., Schaaf M.&de Kloet E. R. (2002). Glucocorticoid receptor variants: clinical implications. *J Steroid Biochem Mol Biol* **81**(2): 103-22.

DeRijk R. H., Schaaf M., Stam F. J., de Jong I. E., Swaab D. F., Ravid R., Vreugdenhil E., Cidlowski J. A., de Kloet E. R.&Lucassen P. J. (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**(1-2): 17-26.

Di Comite G., Grazia Sabbadini M., Corti A., Rovere-Querini P.&Manfredi A. A. (2007). Conversation galante: how the immune and the neuroendocrine systems talk to each other. *Autoimmun Rev* **7**(1): 23-9.

Doppler W., Windegger M., Soratroi C., Tomasi J., Lechner J., Rusconi S., Cato A. C., Almlof T., Liden J., Okret S.*, et al.* (2001). Expression level-dependent contribution of glucocorticoid receptor domains for functional interaction with STAT5. *Mol Cell Biol* **21**(9): 3266-79.

Ducouret B., Tujague M., Ashraf J., Mouchel N., Servel N., Valotaire Y.&Thompson E. B. (1995). Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinology* **136**(9): 3774-83.

Duma D., Jewell C. M.&Cidlowski J. A. (2006). Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. *J Steroid Biochem Mol Biol* **102**(1-5): 11-21.

Dussault I., Lin M., Hollister K., Fan M., Termini J., Sherman M. A.&Forman B. M. (2002). A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. *Mol Cell Biol* **22**(15): 5270-80.

Dzhagalov I., Giguere V.&He Y. W. (2004). Lymphocyte development and function in the absence of retinoic acid-related orphan receptor alpha. *Journal of Immunology* **173**(5): 2952-9.

Eisen L. P., Elsasser M. S.&Harmon J. M. (1988). Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. *J Biol Chem* **263**(24): 12044-8.

Eskandari F.&Sternberg E. M. (2002). Neural-immune interactions in health and disease. *Ann N Y Acad Sci* **966**: 20-7.

Espinas M. L., Roux J., Ghysdael J., Pictet R.&Grange T. (1994). Participation of Ets transcription factors in the glucocorticoid response of the rat tyrosine aminotransferase gene. *Mol Cell Biol* **14**(6): 4116-25.

Fondell J. D., Brunel F., Hisatake K.&Roeder R. G. (1996). Unliganded thyroid hormone receptor alpha can target TATA-binding protein for transcriptional repression. *Mol Cell Biol* **16**(1): 281-7.

Franchimont D. (2004). Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. *Ann N Y Acad Sci* **1024**: 124-37.

Francis D., Diorio J., LaPlante P., Weaver S., Seckl J. R.&Meaney M. J. (1996). The role of early environmental events in regulating neuroendocrine development. Moms, pups, stress, and glucocorticoid receptors. *Ann N Y Acad Sci* **794**: 136-52.

Fukuen S., Fukuda T., Matsuda H., Sumida A., Yamamoto I., Inaba T.&Azuma J. (2002). Identification of the novel splicing variants for the hPXR in human livers. *Biochem Biophys Res Commun* **298**(3): 433-8.

Gabrielsson B. G., Olofsson L. E., Sjogren A., Jernas M., Elander A., Lonn M., Rudemo M.&Carlsson L. M. (2005). Evaluation of reference genes for studies of gene expression in human adipose tissue. *Obes Res* **13**(4): 649-52.

Galeeva A., Ordyan N., Pivina S.&Pelto-Huikko M. (2006). Expression of glucocorticoid receptors in the hippocampal region of the rat brain during postnatal development. *J Chem Neuroanat* **31**(3): 216-25.

Geng C. D., Pedersen K. B., Nunez B. S.&Vedeckis W. V. (2005). Human glucocorticoid receptor alpha transcript splice variants with exon 2 deletions: evidence for tissue- and cell type-specific functions. *Biochemistry* **44**(20): 7395-405.

Geng C. D.&Vedeckis W. V. (2004). Steroid-responsive sequences in the human glucocorticoid receptor gene 1A promoter. *Mol Endocrinol* **18**(4): 912-24.

Geng C. D.&Vedeckis W. V. (2005). c-Myb and members of the c-Ets family of transcription factors act as molecular switches to mediate opposite steroid regulation of the human glucocorticoid receptor 1A promoter. *J Biol Chem* **280**(52): 43264-71.

Giguere V. (1999). Orphan nuclear receptors: from gene to function. *Endocr Rev* **20**(5): 689-725.

Glass C. K. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr Rev* **15**(3): 391-407.

Glass C. K.&Ogawa S. (2006). Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol* **6**(1): 44-55.

Goidin D., Mamessier A., Staquet M. J., Schmitt D.&Berthier-Vergnes O. (2001). Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal Biochem* **295**(1): 17-21.

Gray N. K.&Wickens M. (1998). Control of translation initiation in animals. *Annu Rev Cell Dev Biol* **14**: 399-458.

Guo G. L., Lambert G., Negishi M., Ward J. M., Brewer H. B., Jr., Kliewer S. A., Gonzalez F. J.&Sinal C. J. (2003). Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* **278**(46): 45062-71.

Gustafsson J. A. (2005). Steroids and the scientist. *Mol Endocrinol* **19**(6): 1412-7.

Haarman E. G., Kaspers G. J., Pieters R., Rottier M. M.&Veerman A. J. (2004). Glucocorticoid receptor alpha, beta and gamma expression vs in vitro glucocorticod resistance in childhood leukemia. *Leukemia* **18**(3): 530-7.

Hagendorf A., Koper J. W., de Jong F. H., Brinkmann A. O., Lamberts S. W.&Feelders R. A. (2005). Expression of the human glucocorticoid receptor splice variants alpha, beta, and P in peripheral blood mononuclear leukocytes in healthy controls and in patients with hyper- and hypocortisolism. *J Clin Endocrinol Metab* **90**(11): 6237-43.

Hamid Q. A., Wenzel S. E., Hauk P. J., Tsicopoulos A., Wallaert B., Lafitte J. J., Chrousos G. P., Szefler S. J.&Leung D. Y. (1999). Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* **159**(5 Pt 1): 1600-4.

Hammad H., de Heer H. J., Soullie T., Angeli V., Trottein F., Hoogsteden H. C.&Lambrecht B. N. (2004). Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. *Am J Pathol* **164**(1): 263-71.

Han S. N., Leka L. S., Lichtenstein A. H., Ausman L. M.&Meydani S. N. (2003). Effect of a therapeutic lifestyle change diet on immune functions of moderately hypercholesterolemic humans. *J Lipid Res* **44**(12): 2304-10.

Handoko H. Y., Nancarrow D. J., Mowry B. J.&McGrath J. J. (2006). Polymorphisms in the vitamin D receptor and their associations with risk of schizophrenia and selected anthropometric measures. *Am J Hum Biol* **18**(3): 415-7.

Handschin C.&Meyer U. A. (2003). Induction of drug metabolism: the role of nuclear receptors. *Pharmacol Rev* **55**(4): 649-73.

Handschin C.&Meyer U. A. (2005). Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. *Arch Biochem Biophys* **433**(2): 387-96.

Hard T., Kellenbach E., Boelens R., Maler B. A., Dahlman K., Freedman L. P., Carlstedt-Duke J., Yamamoto K. R., Gustafsson J. A.&Kaptein R. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* **249**(4965): 157-60.

Hastings M. L., Milcarek C., Martincic K., Peterson M. L.&Munroe S. H. (1997). Expression of the thyroid hormone receptor gene, erbAalpha, in B lymphocytes: alternative mRNA processing is independent of differentiation but correlates with antisense RNA levels. *Nucleic Acids Res* **25**(21): 4296-300.

Heck S., Bender K., Kullmann M., Gottlicher M., Herrlich P.&Cato A. C. (1997). I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. *Embo J* **16**(15): 4698-707.

Heck S., Kullmann M., Gast A., Ponta H., Rahmsdorf H. J., Herrlich P.&Cato A. C. (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *Embo J* **13**(17): 4087-95.

Heneka M. T.&Landreth G. E. (2007). PPARs in the brain. *Biochim Biophys Acta* **1771**(8): 1031-45.

Herman J. P., Patel P. D., Akil H.&Watson S. J. (1989). Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. *Mol Endocrinol* **3**(11): 1886-94.

Hollenberg S. M.&Evans R. M. (1988). Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell* **55**(5): 899-906.

Hollenberg S. M., Weinberger C., Ong E. S., Cerelli G., Oro A., Lebo R., Thompson E. B., Rosenfeld M. G.&Evans R. M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318**(6047): 635-41.

Horwitz K. B., Jackson T. A., Bain D. L., Richer J. K., Takimoto G. S.&Tung L. (1996). Nuclear receptor coactivators and corepressors. *Mol Endocrinol* **10**(10): 1167-77.

House A. E.&Lynch K. W. (2008). Regulation of alternative splicing: more than just the ABCs. *J Biol Chem* **283**(3): 1217-21.

Huang H. D., Horng J. T., Lin F. M., Chang Y. C.&Huang C. C. (2005). SpliceInfo: an information repository for mRNA alternative splicing in human genome. *Nucleic Acids Res* **33**(Database issue): D80-5.

Imai E., Miner J. N., Mitchell J. A., Yamamoto K. R.&Granner D. K. (1993). Glucocorticoid receptor-cAMP response element-binding protein interaction and the response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids. *J Biol Chem* **268**(8): 5353-6.

Ishunina T. A.&Swaab D. F. (2008). Estrogen receptor-alpha splice variants in the human brain. *Gynecol Endocrinol* **24**(2): 93-8.

Jensen E. V.&Jacobson H. I. (1962). Basic guides to the mechanism of estrogen action. *Recent Prog Horm Res* **18**: 387-414.

Jiang G., Nepomuceno L., Hopkins K.&Sladek F. M. (1995). Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol Cell Biol* **15**(9): 5131-43.

Johnson J. M., Castle J., Garrett-Engele P., Kan Z., Loerch P. M., Armour C. D., Santos R., Schadt E. E., Stoughton R.&Shoemaker D. D. (2003). Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302**(5653): 2141-4.

Jones D. C., Manning B. M.&Daynes R. A. (2002). A role for the peroxisome proliferator-activated receptor alpha in T-cell physiology and ageing immunobiology. *Proc Nutr Soc* **61**(3): 363-9.

Kan Z., Rouchka E. C., Gish W. R.&States D. J. (2001). Gene structure prediction and alternative splicing analysis using genomically aligned ESTs. *Genome Res* **11**(5): 889-900.

Kassel O.&Herrlich P. (2007). Crosstalk between the glucocorticoid receptor and other transcription factors: Molecular aspects. *Mol Cell Endocrinol*.

Khorasanizadeh S.&Rastinejad F. (2001). Nuclear-receptor interactions on DNA-response elements. *Trends Biochem Sci* **26**(6): 384-90.

Ki S. H., Cho I. J., Choi D. W.&Kim S. G. (2005). Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. *Mol Cell Biol* **25**(10): 4150-65.

Kim M. S., Shigenaga J., Moser A., Feingold K.&Grunfeld C. (2003). Repression of farnesoid X receptor during the acute phase response. *J Biol Chem* **278**(11): 8988-95.

Kimura K., Wakamatsu A., Suzuki Y., Ota T., Nishikawa T., Yamashita R., Yamamoto J., Sekine M., Tsuritani K., Wakaguri H.*, et al.* (2006). Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes. *Genome Res* **16**(1): 55-65.

King L. B., Vacchio M. S., Dixon K., Hunziker R., Margulies D. H.&Ashwell J. D. (1995). A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. *Immunity* **3**(5): 647-56.

Kliewer S. A., Umesono K., Heyman R. A., Mangelsdorf D. J., Dyck J. A.&Evans R. M. (1992a). Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc Natl Acad Sci U S A* **89**(4): 1448-52.

Kliewer S. A., Umesono K., Mangelsdorf D. J.&Evans R. M. (1992b). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**(6359): 446-9.

Klinge C. M., Silver B. F., Driscoll M. D., Sathya G., Bambara R. A.&Hilf R. (1997). Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. *J Biol Chem* **272**(50): 31465-74.

Koivisto A. M., Helisalmi S., Pihlajamaki J., Hiltunen M., Koivisto K., Moilanen L., Kuusisto J., Helkala E. L., Hanninen T., Kervinen K.*, et al.* (2006). Association analysis of peroxisome proliferator-activated receptor gamma polymorphisms and late onset Alzheimer's disease in the Finnish population. *Dement Geriatr Cogn Disord* **22**(5-6): 449-53.

Kornberg R. D.&Lorch Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**(3): 285-94.

Kota B. P., Huang T. H.&Roufogalis B. D. (2005). An overview on biological mechanisms of PPARs. *Pharmacol Res* **51**(2): 85-94.

Kumar R.&Thompson E. B. (2003). Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. *Mol Endocrinol* **17**(1): 1-10.

Kumar R., Volk D. E., Li J., Lee J. C., Gorenstein D. G.&Thompson E. B. (2004a). TATA box binding protein induces structure in the recombinant glucocorticoid receptor AF1 domain. *Proc Natl Acad Sci U S A* **101**(47): 16425-30.

Kumar S., Tamura K.&Nei M. (2004b). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**(2): 150-63.

Kumsta R., Moser D., Streit F., Koper J. W., Meyer J.&Wust S. (2008). 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*.

Kurihara I., Lee D. K., Petit F. G., Jeong J., Lee K., Lydon J. P., Demayo F. J., Tsai M. J.&Tsai S. Y. (2007). COUP-TFII Mediates Progesterone Regulation of Uterine Implantation by Controlling ER Activity. *PLoS Genet* **3**(6): e102.

Kushner I. (1993). Regulation of the acute phase response by cytokines. *Perspect Biol Med* **36**(4): 611-22.

Kushner I. (1998). Semantics, inflammation, cytokines and common sense. *Cytokine Growth Factor Rev* **9**(3-4): 191-6.

Ladias J. A.&Karathanasis S. K. (1991). Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. *Science* **251**(4993): 561-5.

Lai M., McCormick J. A., Chapman K. E., Kelly P. A., Seckl J. R.&Yau J. L. (2003). Differential regulation of corticosteroid receptors by monoamine neurotransmitters and antidepressant drugs in primary hippocampal culture. *Neuroscience* **118**(4): 975-84.

Laudet V.&Gronemeyer H. (2002). The nuclear receptor facts book. *London: Academic Press*.

Lee K., Jeong J., Tsai M. J., Tsai S., Lydon J. P.&DeMayo F. J. (2006). Molecular mechanisms involved in progesterone receptor regulation of uterine function. *J Steroid Biochem Mol Biol* **102**(1-5): 41-50.

Lee Y. F., Lee H. J.&Chang C. (2002). Recent advances in the TR2 and TR4 orphan receptors of the nuclear receptor superfamily. *J Steroid Biochem Mol Biol* **81**(4-5): 291-308.

Leng X., Cooney A. J., Tsai S. Y.&Tsai M. J. (1996). Molecular mechanisms of COUP-TF-mediated transcriptional repression: evidence for transrepression and active repression. *Mol Cell Biol* **16**(5): 2332-40.

Liang F., Holt I., Pertea G., Karamycheva S., Salzberg S. L.&Quackenbush J. (2000). An optimized protocol for analysis of EST sequences. *Nucleic Acids Res* **28**(18): 3657-65.

Liu D., Diorio J., Tannenbaum B., Caldji C., Francis D., Freedman A., Sharma S., Pearson D., Plotsky P. M.&Meaney M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* **277**(5332): 1659-62.

Liu W., Wang J., Sauter N. K.&Pearce D. (1995). Steroid receptor heterodimerization demonstrated in vitro and in vivo. *Proc Natl Acad Sci U S A* **92**(26): 12480-4.

Livak K. J.&Schmittgen T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**(4): 402-8.

Lopes da Silva S.&Burbach J. P. (1995). The nuclear hormone-receptor family in the brain: classics and orphans. *Trends Neurosci* **18**(12): 542-8.

Lu N. Z.&Cidlowski J. A. (2006). Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol* **16**(6): 301-7.

Lu X. P., Salbert G.&Pfahl M. (1994). An evolutionary conserved COUP-TF binding element in a neural-specific gene and COUP-TF expression patterns support a major role for COUP-TF in neural development. *Mol Endocrinol* **8**(12): 1774-88.

Lundell K., Thulin P., Hamsten A.&Ehrenborg E. (2007). Alternative splicing of human peroxisome proliferator-activated receptor delta (PPAR delta): effects on translation efficiency and trans-activation ability. *BMC Mol Biol* **8**: 70.

Lutz B., Kuratani S., Cooney A. J., Wawersik S., Tsai S. Y., Eichele G.&Tsai M. J. (1994). Developmental regulation of the orphan receptor COUP-TF II gene in spinal motor neurons. *Development* **120**(1): 25-36.

Mader S., Kumar V., de Verneuil H.&Chambon P. (1989). Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* **338**(6212): 271-4.

Mader S., Leroy P., Chen J. Y.&Chambon P. (1993). Multiple parameters control the selectivity of nuclear receptors for their response elements. Selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors. *J Biol Chem* **268**(1): 591-600.

Mangelsdorf D. J.&Evans R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**(6): 841-50.

Mangelsdorf D. J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P.*, et al.* (1995). The nuclear receptor superfamily: the second decade. *Cell* **83**(6): 835-9.

Marcus S. L., Capone J. P.&Rachubinski R. A. (1996). Identification of COUP-TFII as a peroxisome proliferator response element binding factor using genetic selection in yeast: COUP-TFII activates transcription in yeast but antagonizes PPAR signaling in mammalian cells. *Mol Cell Endocrinol* **120**(1): 31-9.

Marin R., Guerra B., Alonso R., Ramirez C. M.&Diaz M. (2005). Estrogen activates classical and alternative mechanisms to orchestrate neuroprotection. *Curr Neurovasc Res* **2**(4): 287-301.

Mathieu C., van Etten E., Decallonne B., Guilietti A., Gysemans C., Bouillon R.&Overbergh L. (2004). Vitamin D and 1,25-dihydroxyvitamin D3 as modulators in the immune system. *J Steroid Biochem Mol Biol* **89-90**(1-5): 449-52.

Matsuzaki S., Canis M., Pouly J. L., Botchorishvili R., Dechelotte P. J.&Mage G. (2006). Differential expression of genes in eutopic and ectopic endometrium from patients with ovarian endometriosis. *Fertil Steril* **86**(3): 548-53.

McCormick J. A., Lyons V., Jacobson M. D., Noble J., Diorio J., Nyirenda M., Weaver S., Ester W., Yau J. L., Meaney M. J.*, et al.* (2000). 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol Endocrinol* **14**(4): 506-17.

McGrath J. J., Feron F. P., Burne T. H., Mackay-Sim A.&Eyles D. W. (2004). Vitamin D3-implications for brain development. *J Steroid Biochem Mol Biol* **89-90**(1-5): 557-60.

McKay L. I.&Cidlowski J. A. (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev* **20**(4): 435-59.

McKenna N. J., Lanz R. B.&O'Malley B. W. (1999a). Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**(3): 321-44.

McKenna N. J.&O'Malley B. W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**(4): 465-74.

McKenna N. J., Xu J., Nawaz Z., Tsai S. Y., Tsai M. J.&O'Malley B. W. (1999b). Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol* **69**(1-6): 3-12.

Metivier R., Gay F. A., Hubner M. R., Flouriot G., Salbert G., Gannon F., Kah O.&Pakdel F. (2002). Formation of an hER alpha-COUP-TFI complex enhances hER alpha AF-1 through Ser118 phosphorylation by MAPK. *Embo J* **21**(13): 3443-53.

Miesfeld R., Okret S., Wikstrom A. C., Wrange O., Gustafsson J. A.&Yamamoto K. R. (1984). Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. *Nature* **312**(5996): 779-81.

Mignone F., Gissi C., Liuni S.&Pesole G. (2002). Untranslated regions of mRNAs. *Genome Biol* **3**(3): REVIEWS0004.

Mitchell J. B., Betito K., Rowe W., Boksa P.&Meaney M. J. (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**(3): 631-9.

Mittelstadt P. R.&Ashwell J. D. (2003). Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression. *Mol Endocrinol* **17**(8): 1534-42.

Miyake J. H., Wang S. L.&Davis R. A. (2000). Bile acid induction of cytokine expression by macrophages correlates with repression of hepatic cholesterol 7alpha-hydroxylase. *J Biol Chem* **275**(29): 21805-8.

Nembaware V., Lupindo B., Schouest K., Spillane C., Scheffler K.&Seoighe C. (2008). Genome-wide survey of allele-specific splicing in humans. *BMC Genomics* **9**: 265.

Newton C. R., Graham A., Heptinstall L. E., Powell S. J., Summers C., Kalsheker N., Smith J. C.&Markham A. F. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* **17**(7): 2503-16.

Nishimura M., Naito S.&Yokoi T. (2004). Tissue-specific mRNA expression profiles of human nuclear receptor subfamilies. *Drug Metab Pharmacokinet* **19**(2): 135-49.

Nobukuni Y., Smith C. L., Hager G. L.&Detera-Wadleigh S. D. (1995). Characterization of the human glucocorticoid receptor promoter. *Biochemistry* **34**(25): 8207-14.

Nuclear Receptors Nomenclature Committee (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**(2): 161-3.

Nunez B. S., Geng C. D., Pedersen K. B., Millro-Macklin C. D.&Vedeckis W. V. (2005). Interaction between the Interferon Signaling Pathway and the Human Glucocorticoid Receptor Gene 1A Promoter. *Endocrinology* **146**(3): 1449-57.

Nunez B. S.&Vedeckis W. V. (2002). Characterization of promoter 1B in the human glucocorticoid receptor gene. *Mol Cell Endocrinol* **189**(1-2): 191-9.

Obexer P., Certa U., Kofler R.&Helmberg A. (2001). Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. *Oncogene* **20**(32): 4324-36.

Ou X. M., Storring J. M., Kushwaha N.&Albert P. R. (2001). Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. *J Biol Chem* **276**(17): 14299-307.

Park J. I., Tsai S. Y.&Tsai M. J. (2003). Molecular mechanism of chicken ovalbumin upstream promoter-transcription factor (COUP-TF) actions. *Keio J Med* **52**(3): 174-81.

Pascussi J. M., Busson-Le Coniat M., Maurel P.&Vilarem M. J. (2003). Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. *Mol Endocrinol* **17**(1): 42-55.

Patchev V. K., Hayashi S., Orikasa C.&Almeida O. F. (1995). Implications of estrogen-dependent brain organization for gender differences in hypothalamo-pituitary-adrenal regulation. *Faseb J* **9**(5): 419-23.

Pedersen K. B., Geng C. D.&Vedeckis W. V. (2004). Three mechanisms are involved in glucocorticoid receptor autoregulation in a human T-lymphoblast cell line. *Biochemistry* **43**(34): 10851-8.

Pedersen K. B.&Vedeckis W. V. (2003). Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. *Biochemistry* **42**(37): 10978-90.

Pepin M. C., Pothier F.&Barden N. (1992). Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. *Nature* **355**(6362): 725-8.

Pereira F. A., Tsai M. J.&Tsai S. Y. (2000). COUP-TF orphan nuclear receptors in development and differentiation. *Cell Mol Life Sci* **57**(10): 1388-98.

Petit F. G., Jamin S. P., Kurihara I., Behringer R. R., DeMayo F. J., Tsai M. J.&Tsai S. Y. (2007). Deletion of the orphan nuclear receptor COUP-TFII in uterus leads to placental deficiency. *Proc Natl Acad Sci U S A* **104**(15): 6293-8.

Pfahl M. (1993). Nuclear receptor/AP-1 interaction. *Endocr Rev* **14**(5): 651-8.

Pickering B. M.&Willis A. E. (2005). The implications of structured 5' untranslated regions on translation and disease. *Semin Cell Dev Biol* **16**(1): 39-47.

Piotrowski P., Burzynski M., Lianeri M., Mostowska M., Wudarski M., Chwalinska-Sadowska H.&Jagodzinski P. P. (2007). Glucocorticoid receptor beta splice variant expression in patients with high and low activity of systemic lupus erythematosus. *Folia Histochem Cytobiol* **45**(4): 339-42.

Poola I.&Speirs V. (2001). Expression of alternatively spliced estrogen receptor alpha mRNAs is increased in breast cancer tissues. *J Steroid Biochem Mol Biol* **78**(5): 459-69.

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**(1-2): 79-90.

Pruitt K. D., Tatusova T.&Maglott D. R. (2005). NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **33**(Database issue): D501-4.

Purdom E., Simpson K. M., Robinson M. D., Conboy J. G., Lapuk A. V.&Speed T. P. (2008). FIRMA: a method for detection of alternative splicing from exon array data. *Bioinformatics*.

Purton J. F., Monk J. A., Liddicoat D. R., Kyparissoudis K., Sakkal S., Richardson S. J., Godfrey D. I.&Cole T. J. (2004). Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. *J Immunol* **173**(6): 3816-24.

Riecher-Rossler A.&de Geyter C. (2007). The forthcoming role of treatment with oestrogens in mental health. *Swiss Med Wkly* **137**(41-42): 565-72.

Riggs K. A., Wickramasinghe N. S., Cochrum R. K., Watts M. B.&Klinge C. M. (2006). Decreased chicken ovalbumin upstream promoter transcription factor II expression in tamoxifen-resistant breast cancer cells. *Cancer Res* **66**(20): 10188-98.

Ris-Stalpers C., Verleun-Mooijman M. C., de Blaeij T. J., Degenhart H. J., Trapman J.&Brinkmann A. O. (1994). Differential splicing of human androgen receptor pre-mRNA in X-linked Reifenstein syndrome, because of a deletion involving a putative branch site. *Am J Hum Genet* **54**(4): 609-17.

Ritchie H. H., Wang L. H., Tsai S., O'Malley B. W.&Tsai M. J. (1990). COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily. *Nucleic Acids Res* **18**(23): 6857-62.

Rochette-Egly C. (2005). Dynamic combinatorial networks in nuclear receptor-mediated transcription. *J Biol Chem* **280**(38): 32565-8.

Rogatsky I., Luecke H. F., Leitman D. C.&Yamamoto K. R. (2002). Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci U S A* **99**(26): 16701-6.

Ruberte E., Friederich V., Chambon P.&Morriss-Kay G. (1993). Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development* **118**(1): 267-82.

Russcher H., van Rossum E. F., de Jong F. H., Brinkmann A. O., Lamberts S. W.&Koper J. W. (2005). Increased expression of the glucocorticoid receptor-A translational isoform as a result of the ER22/23EK polymorphism. *Mol Endocrinol* **19**(7): 1687-96.

Sabatino L., Casamassimi A., Peluso G., Barone M. V., Capaccio D., Migliore C., Bonelli P., Pedicini A., Febbraro A., Ciccodicola A., *et al.* (2005). A novel peroxisome proliferator-activated receptor gamma isoform with dominant negative activity generated by alternative splicing. *J Biol Chem*.

Sadovsky Y., Webb P., Lopez G., Baxter J. D., Fitzpatrick P. M., Gizang-Ginsberg E., Cavailles V., Parker M. G.&Kushner P. J. (1995). Transcriptional activators differ in their responses to overexpression of TATA-box-binding protein. *Mol Cell Biol* **15**(3): 1554-63.

Sakai D. D., Helms S., Carlstedt-Duke J., Gustafsson J. A., Rottman F. M.&Yamamoto K. R. (1988). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. *Genes Dev* **2**(9): 1144-54.

Savkur R. S., Wu Y., Bramlett K. S., Wang M., Yao S., Perkins D., Totten M., Searfoss G., 3rd, Ryan T. P., Su E. W.*, et al.* (2003). Alternative splicing within the ligand binding domain of the human constitutive androstane receptor. *Mol Genet Metab* **80**(1-2): 216-26.

Schote A. B., Turner J. D., Schiltz J.&Muller C. P. (2007). Nuclear receptors in human immune cells: Expression and correlations. *Mol Immunol* **44**(6): 1436-45.

Schulman I. G., Chakravarti D., Juguilon H., Romo A.&Evans R. M. (1995). Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation. *Proc Natl Acad Sci U S A* **92**(18): 8288-92.

Schupf N., Lee J. H., Wei M., Pang D., Chace C., Cheng R., Zigman W. B., Tycko B.&Silverman W. (2008). Estrogen receptor-alpha variants increase risk of Alzheimer's disease in women with Down syndrome. *Dement Geriatr Cogn Disord* **25**(5): 476-82.

Shibata H., Nawaz Z., Tsai S. Y., O'Malley B. W.&Tsai M. J. (1997). Gene silencing by chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is mediated by transcriptional corepressors, nuclear receptor-corepressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT). *Mol Endocrinol* **11**(6): 714-24.

Smirnov D. A., Hou S., Liu X., Claudio E., Siebenlist U. K.&Ricciardi R. P. (2001). Coup-TFII is up-regulated in adenovirus type 12 tumorigenic cells and is a repressor of MHC class I transcription. *Virology* **284**(1): 13-9.

Smirnov D. A., Hou S.&Ricciardi R. P. (2000). Association of histone deacetylase with COUP-TF in tumorigenic Ad12-transformed cells and its potential role in shut-off of MHC class I transcription. *Virology* **268**(2): 319-28.

Smith C. L.&O'Malley B. W. (2004). Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* **25**(1): 45-71.

Sobczak K.&Krzyzosiak W. J. (2002). Structural determinants of BRCA1 translational regulation. *J Biol Chem* **277**(19): 17349-58.

Strahle U., Schmidt A., Kelsey G., Stewart A. F., Cole T. J., 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**(15): 6731-5.

Straub R. H. (2007). The complex role of estrogens in inflammation. *Endocr Rev* **28**(5): 521-74.

Stromstedt P. E., Poellinger L., Gustafsson J. A.&Carlstedt-Duke J. (1991). The glucocorticoid receptor binds to a sequence overlapping the TATA box of the human osteocalcin promoter: a potential mechanism for negative regulation. *Mol Cell Biol* **11**(6): 3379-83.

Sultan M., Schulz M. H., Richard H., Magen A., Klingenhoff A., Scherf M., Seifert M., Borodina T., Soldatov A., Parkhomchuk D.*, et al.* (2008). A Global View of Gene Activity and Alternative Splicing by Deep Sequencing of the Human Transcriptome. *Science*.

Suzuki T., Moriya T., Darnel A. D., Takeyama J.&Sasano H. (2000). Immunohistochemical distribution of chicken ovalbumin upstream promoter transcription factor II in human tissues. *Mol Cell Endocrinol* **164**(1-2): 69-75.

Takamoto N., Kurihara I., Lee K., Demayo F. J., Tsai M. J.&Tsai S. Y. (2005). Haploinsufficiency of chicken ovalbumin upstream promoter transcription factor II in female reproduction. *Mol Endocrinol* **19**(9): 2299-308.

Takeo J., Hata J., Segawa C., Toyohara H.&Yamashita S. (1996). Fish glucocorticoid receptor with splicing variants in the DNA binding domain. *FEBS Lett* **389**(3): 244-8.

Tenbaum S.&Baniahmad A. (1997). Nuclear receptors: structure, function and involvement in disease. *Int J Biochem Cell Biol* **29**(12): 1325-41.

Thanaraj T. A. (1999). A clean data set of EST-confirmed splice sites from Homo sapiens and standards for clean-up procedures. *Nucleic Acids Res* **27**(13): 2627-37.

Tissing W. J., Lauten M., Meijerink J. P., den Boer M. L., Koper J. W., Sonneveld P.&Pieters R. (2005). Expression of the glucocorticoid receptor and its isoforms in relation to glucocorticoid resistance in childhood acute lymphocytic leukemia. *Haematologica* **90**(9): 1279-81.

Tissing W. J., Meijerink J. P., Brinkhof B., Broekhuis M. J., Menezes R. X., den Boer M. L.&Pieters R. (2006). Glucocorticoid-induced glucocorticoid receptor expression and promoter-usage is not linked to glucocorticoid resistance in childhood ALL. *Blood*.

Tonko M., Ausserlechner M. J., Bernhard D., Helmberg A.&Kofler R. (2001). Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. *Faseb J* **15**(3): 693-9.

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**(9): 4350-4.

Trotter K. W.&Archer T. K. (2007). Nuclear receptors and chromatin remodeling machinery. *Mol Cell Endocrinol* **265-266**: 162-7.

Tsai S. Y.&Tsai M. J. (1997). Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. *Endocr Rev* **18**(2): 229-40.

Tsuritani K., Irie T., Yamashita R., Sakakibara Y., Wakaguri H., Kanai A., Mizushima-Sugano J., Sugano S., Nakai K.&Suzuki Y. (2007). Distinct class of putative "non-conserved" promoters in humans: comparative studies of alternative promoters of human and mouse genes. *Genome Res* **17**(7): 1005-14.

Turner J. D.&Muller C. P. (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**(2): 283-92.

Turner J. D., Schote A. B., Macedo J. A., Pelascini L. P.&Muller C. P. (2006). Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage? *Biochem Pharmacol* **72**(11): 1529-37.

Tzameli I., Chua S. S., Cheskis B.&Moore D. D. (2003). Complex effects of rexinoids on ligand dependent activation or inhibition of the xenobiotic receptor, CAR. *Nucl Recept* **1**(1): 2.

Uht R. M., Anderson C. M., Webb P.&Kushner P. J. (1997). Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* **138**(7): 2900-8.

van Neerven S., Kampmann E.&Mey J. (2008). RAR/RXR and PPAR/RXR signaling in neurological and psychiatric diseases. *Prog Neurobiol*.

van Rossum E. F., Binder E. B., Majer M., Koper J. W., Ising M., Modell S., Salyakina D., Lamberts S. W.&Holsboer F. (2006). Polymorphisms of the glucocorticoid receptor gene and major depression. *Biol Psychiatry* **59**(8): 681-8.

van Rossum E. F., Koper J. W., van den Beld A. W., Uitterlinden A. G., Arp P., Ester W., Janssen J. A., Brinkmann A. O., de Jong F. H., Grobbee D. E.*, et al.* (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**(5): 585-92.

van West D., Van Den Eede F., Del-Favero J., Souery D., Norrback K. F., Van Duijn C., Sluijs S., Adolfsson R., Mendlewicz J., Deboutte D.*, et al.* (2006). Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. *Neuropsychopharmacology* **31**(3): 620-7.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A.&Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**(7): RESEARCH0034.

Voegel J. J., Heine M. J., Zechel C., Chambon P.&Gronemeyer H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *Embo J* **15**(14): 3667-75.

Wang K.&Wan Y. J. (2008). Nuclear receptors and inflammatory diseases. *Exp Biol Med (Maywood)* **233**(5): 496-506.

Wang L. H., Tsai S. Y., Cook R. G., Beattie W. G., Tsai M. J.&O'Malley B. W. (1989). COUP transcription factor is a member of the steroid receptor superfamily. *Nature* **340**(6229): 163-6.

Wang Z.&Burge C. B. (2008). Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *Rna* **14**(5): 802-13.

Warnmark A., Treuter E., Wright A. P.&Gustafsson J. A. (2003). Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. *Mol Endocrinol* **17**(10): 1901-9.

Warnmark A., Wikstrom A., Wright A. P., Gustafsson J. A.&Hard T. (2001). The N-terminal regions of estrogen receptor alpha and beta are unstructured in vitro and show different TBP binding properties. *J Biol Chem* **276**(49): 45939-44.

Weaver I. C., Cervoni N., Champagne F. A., D'Alessio A. C., Sharma S., Seckl J. R., Dymov S., Szyf M.&Meaney M. J. (2004). Epigenetic programming by maternal behavior. *Nat Neurosci* **7**(8): 847-54.

Weaver I. C., D'Alessio A. C., Brown S. E., Hellstrom I. C., Dymov S., Sharma S., Szyf M.&Meaney M. J. (2007). The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J Neurosci* **27**(7): 1756-68.

Webster J. I., Tonelli L.&Sternberg E. M. (2002). Neuroendocrine regulation of immunity. *Annu Rev Immunol* **20**: 125-63.

Webster N. J., Green S., Jin J. R.&Chambon P. (1988). The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* **54**(2): 199-207.

Weigel N. L.&Moore N. L. (2007). Steroid receptor phosphorylation: a key modulator of multiple receptor functions. *Mol Endocrinol* **21**(10): 2311-9.

Whitney K. D., Watson M. A., Goodwin B., Galardi C. M., Maglich J. M., Wilson J. G., Willson T. M., Collins J. L.&Kliewer S. A. (2001). Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. *J Biol Chem* **276**(47): 43509-15.

Wilkie G. S., Dickson K. S.&Gray N. K. (2003). Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. *Trends Biochem Sci* **28**(4): 182-8.

Woerly G., Honda K., Loyens M., Papin J. P., Auwerx J., Staels B., Capron M.&Dombrowicz D. (2003). Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J Exp Med* **198**(3): 411-21.

Wrange O.&Gustafsson J. A. (1978). Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. *J Biol Chem* **253**(3): 856-65.

Wu S. Y., Thomas M. C., Hou S. Y., Likhite V.&Chiang C. M. (1999). Isolation of mouse TFIID and functional characterization of TBP and TFIID in mediating estrogen receptor and chromatin transcription. *J Biol Chem* **274**(33): 23480-90.

Wust S., Van Rossum E. F., Federenko I. S., Koper J. W., Kumsta R.&Hellhammer D. H. (2004). Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. *J Clin Endocrinol Metab* **89**(2): 565-73.

Xing W., Danilovich N.&Sairam M. R. (2002). Orphan receptor chicken ovalbumin upstream promoter transcription factors inhibit steroid factor-1, upstream stimulatory factor, and activator protein-1 activation of ovine follicle-stimulating hormone receptor expression via composite cis-elements. *Biol Reprod* **66**(6): 1656-66.

Xu L., Glass C. K.&Rosenfeld M. G. (1999). Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* **9**(2): 140-7.

Yanai K., Hirota K., Taniguchi-Yanai K., Shigematsu Y., Shimamoto Y., Saito T., Chowdhury S., Takiguchi M., Arakawa M., Nibu Y.*, et al.* (1999). Regulated expression of human angiotensinogen gene by hepatocyte nuclear factor 4 and chicken ovalbumin upstream promoter-transcription factor. *J Biol Chem* **274**(49): 34605-12.

Yang-Yen H. F., Chambard J. C., Sun Y. L., Smeal T., Schmidt T. J., Drouin J.&Karin M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**(6): 1205-15.

Yang Q.&Gonzalez F. J. (2004). Peroxisome proliferator-activated receptor alpha regulates B lymphocyte development via an indirect pathway in mice. *Biochem Pharmacol* **68**(11): 2143-50.

Yau J.&Seckl J. R. (2000). Antidepressant actions on glucocorticoid receptors. Encyclopedia of Stress. G. Fink. San Diego, Academic Press**:** 212-222.

Yau J. L., Hibberd C., Noble J.&Seckl J. R. (2002). The effect of chronic fluoxetine treatment on brain corticosteroid receptor mRNA expression and spatial memory in young and aged rats. *Brain Res Mol Brain Res* **106**(1-2): 117-23.

Yen P. M., Ikeda M., Wilcox E. C., Brubaker J. H., Spanjaard R. A., Sugawara A.&Chin W. W. (1994). Half-site arrangement of hybrid glucocorticoid and thyroid hormone response elements specifies thyroid hormone receptor complex binding to DNA and transcriptional activity. *J Biol Chem* **269**(17): 12704-9.

Yudt M. R.&Cidlowski J. A. (2002). The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. *Mol Endocrinol* **16**(8): 1719-26.

Yudt M. R., Jewell C. M., Bienstock R. J.&Cidlowski J. A. (2003). Molecular origins for the dominant negative function of human glucocorticoid receptor beta. *Mol Cell Biol* **23**(12): 4319-30.

Zhang T., Haws P.&Wu Q. (2004a). Multiple variable first exons: a mechanism for cell- and tissue-specific gene regulation. *Genome Res* **14**(1): 79-89.

Zhang Y.&Dufau M. L. (2004). Gene silencing by nuclear orphan receptors. *Vitam Horm* **68**: 1-48.

Zhang Z., Burch P. E., Cooney A. J., Lanz R. B., Pereira F. A., Wu J., Gibbs R. A., Weinstock G.&Wheeler D. A. (2004b). Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. *Genome Res* **14**(4): 580-90.

Zheng K., Heydari B.&Simon D. K. (2003). A common NURR1 polymorphism associated with Parkinson disease and diffuse Lewy body disease. *Arch Neurol* **60**(5): 722-5.

Zhong H.&Simons J. W. (1999). Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res Commun* **259**(3): 523-6.

Zhou J.&Cidlowski J. A. (2005). The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. *Steroids* **70**(5-7): 407-17.

Zhou L., Ma X.&Sun F. (2008). The effects of protein interactions, gene essentiality and regulatory regions on expression variation. *BMC Syst Biol* **2**(1): 54.

Zong J., Ashraf J.&Thompson E. B. (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**(10): 5580-5.

Annexes

Publications, presentations and meetings attended

Andrea B. Schote, Jonathan D. Turner, Jang Schiltz, Claude P. Muller, Nuclear receptors in human immune cells: expression and correlation, Molecular Immunology, 2006, Vol 44 (6) pp 1436-45

Jonathan D. Turner, **Andrea B. Schote**, Joana Macedo, Laetitia Pelascini, Claude P. Muller, Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage?, Biochemical Pharmacology, 2006, Vol 72 (11) pp 1529-37

Jonathan D. Turner, **Andrea B. Schote**, Marc Keipes, Claude P. Muller, A new transcript splice variant of the human glucocorticoid receptor. Identification and tissue distribution of hGR313–338, an alternative exon 2 transactivation domain isoform , Annals of the New York Academy of Science, 2007, Vol 1095 pp 334–341

Anja M. Billing, Fred Fack, Jenny Renaut, Christophe M. Olinger, **Andrea B. Schote**, Jonathan D. Turner, Claude P. Muller, Proteomic analysis of the cortisol mediated stress response in THP-1 monocytes using DIGE Technology, Journal of Mass Spectrometry, 2007, Vol 42(11) pp 1433-44

Andrea B. Schote, Jonathan D. Turner, Claude P. Muller, Nuclear receptors and their interactions: Estrogen and cortisol dependent gene regulation via a hybrid ERE-GRE in MCF7 cells, 2nd IRTG Summerschool 2008, Psychobiology of stress: Steroid Hormone Actions, Leiden, Netherlands, oral presentation and abstract

Andrea B. Schote, Nuclear receptors in the field of psychoneuroendocrinology: GR, ER and Coup-TF variants and possible interactions, 1th IRTG Summerschool 2007, Frontiers in Psychobiology and Stress Research, Trier, Germany, oral presentation

Andrea B. Schote, Jonathan D. Turner, Claude P. Muller, Receptor promiscuity, ER and GR together at hybrid ERE-GRE binding sites?, 39th Annual ISPNE Conference 2008, Dresden, Germany, poster and abstract

Andrea B. Schote, Jonathan D. Turner, Claude P. Muller, Receptor promiscuity, ER and GR together at hybrid ERE-GRE binding sites, Apoptosis World 2008: From mechanisms to applications, Luxembourg, Luxembourg, poster and abstract

Andrea B. Schote, Jonathan D. Turner, Jang Schiltz, Claude P. Muller, Nuclear receptors in human immune cells: Expression and Correlation, Nuclear receptors 2006, Stockholm, Sweden, poster and abstract

Andrea B. Schote, Jonathan D. Turner, Jang Schiltz, Claude P. Muller, Nuclear receptors in human immune cells: Expression and Correlation, 37th International Society of Psychoneuroendocrinology Annual Meeting 2006, HORMONES & BRAIN, From Cloning to Clinic, Leiden, Netherlands, poster and abstract

Jonathan D. Turner, **Andrea B. Schote**, Joana Macedo, Laetitia Pelascini, Claude P. Muller, Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage?, 37th International Society of Psychoneuroendocrinology Annual Meeting 2006, HORMONES & BRAIN, From Cloning to Clinic, Leiden, Netherlands, poster and abstract

Workshop 2008, Glucocorticoid-programming of Sickness Behaviour, Trier, Germany

Cell Signalling World 2006, Signal Transduction Pathways as therapeutic targets, Luxembourg, Luxembourg,

5th Trier Summer School of Psychobiology 2006, Pathogeneic mechanisms of stress-related and genetically influenced psychoses, Trier, Germany,

4th Trier Summer School of Psychobiology 2005, Psychobiology of Pain, Luxembourg, Luxembourg

1st international Alfried Krupp Kolleg Symposioum 2004, Stress, Behaviour, Immune Response, Greifswald, Germany

Annual Belgian Immunological Society Meeting 2004, Brussels, Belgian

Immunology lectures, journal club, writing school and department seminars, 2004-2008, Institute of Immunology, Laboratoire National de Santé, Luxembourg, Luxembourg

Curriculum vitae

Andrea Schote-Frese, née Schote

In der Olk 9
54332 Wasserliesch
Tel.:0049 162 6606941
email: Andrea.Schote-Frese@gmx.de

Date of birth: 07.11.1979
Place of birth: Prenzlau
Marital status: married
Nationality: German

Education and Qualification

from Jan. 2009	Postdoctoral position *Leiden and Amsterdam Center for Drug Research, Leiden, Netherlands*
2008	Assistance researcher *Institute of Immunology, Laboratoire National de Santé, Luxembourg, Luxembourg*
2004-2008	PhD in the Institute of Immunology, Laboratoire National de Santé and student in the graduate school of Psychobiology Trier Thesis: Nuclear receptors , their variants and implications in neuro-endocrine-immune regulations *Institute of Immunology, Laboratoire National de Santé, Luxembourg, Luxembourg*
1999 – 2004	Degree in biology as Diplom Biologin (final grade „A") Main focus: Applied microbiology and biotechnology, biochemistry and immunology Thesis: "Characterisation of cytochrom P450 proteins in yeasts of the species *Candida* and *Trichosporon* with special consideration of alkanhydroxylating forms" *Ernst-Moritz-Arndt-University, Greifswald, Germany*
1992 – 1999	A-level/Abitur (final grade „A") *Städtisches Gymnasium, Prenzlau, Germany*

Other skills and interests

Languages	German, English, French (basic level)
IT knowledge	MS Office, Internet, Vector NTI, TRANSFAC professional 10.1
Hobbies	karate, literature, dancing, travelling

Erklärung

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

Luxemburg,

Andrea Schote-Frese