Sequence and Effects of Glucocorticoid Receptor Nuclear Retention; An Aid to Understanding Nuclear Retention in Other Proteins?

By

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Abstract:

Corticosteroid ligands activate the glucocorticoid receptor (GR). GR plays a role in glucose homeostasis, adipogenesis, inflammation, and mood and cognitive functions. Understanding the interplay of diverse forms of receptor regulation (including post-translational modification, cofactor interactions, ligand binding, and receptor localization) and their effects is important for understanding and developing more effective treatment for a variety of conditions. Prior to ligand binding, the naïve GR is primarily cytoplasmic, residing in a chaperone complex containing heat-shock proteins and immunophilins. Upon ligand-binding, alterations to the complex allow the receptor to dimerize and import into the nucleus. Nuclear GR interacts with transcriptional regulatory sequences and recruits cofactors to regulate specific gene expression. Upon hormone withdrawal, the original chaperone complex is reassembled and the receptor is exported to the cytoplasm. Interestingly, while the import of GR into the nucleus occurs very rapidly ($t \frac{1}{2} = 5\text{ min}$), the re-export is significantly slower ($t \frac{1}{2} = 12-24\text{h}$).

Previous work by our lab and others has indicated the existence of a nuclear retention signal (NRS) within the GR. The NRS sequence of the GR, its interaction partners, and the role it might play in the activity of the receptor have not yet been fully defined. Work in the Hache lab indicates that mutation of the GR nuclear localization signal 1 (NL1) increases the export rate of nuclear GR to the cytoplasm, as well as compromising receptor import, suggesting that the NL1 overlapped an NRS sequence. In this work, I made a series of GR mutants, based on sequence from the SV40 large T
antigen NLS, which lacks nuclear retention activity. Using these mutants, I found that GR nuclear retention is influenced by both specific residues within the hinge region and the location of the sequence within the receptor, as reintroduction of the NLS sequence at the N-terminus of the receptor retention mutant failed to reconstitute the retention activity. Agonist liganded and hormone-withdrawn receptor mutants showed a similar decrease in retention. By contrast, antagonist-withdrawn GR mutants were retained in the nucleus, possibly due to altered receptor configuration and interactions.

Assays of GR-responsive promoter activation by receptor retention mutants showed that while no difference in the ability of retention mutants to activate transcription was seen at a simple promoter, activation of a complex promoter was compromised. This impaired transactivation for the SV506-523 mutant correlated with decreased histone H4 acetylation and PolII recruitment, while GR DNA-binding at the target promoter appeared to be unaffected. These results suggested that promoter-specific cofactor interactions might be implicated in GR nuclear retention. Loss of GR hinge interaction with Oct cofactors produced an incomplete loss of retention, suggesting overlapping signals, but not supporting Oct as a primary factor in GR retention.

The overlap between important residues in GR nuclear retention and localization signals and the lack of retention shown by the SV40 NLS suggested that retention might be intrinsic to the sequence of particular NLS. Preliminary results suggest that the KT511-512 residues of GR may be of general importance in protein nuclear retention, while the role of proline is likely more variable.

My research has focused on increasing our understanding of glucocorticoid receptor nuclear retention and its possible implications. I have determined that the
KT511-512 residues of GR play an important role in its retention, and possibly also figure in nuclear retention of other proteins. These residues are involved in interactions which affect promoter-specific histone acetylation and transcriptional activation in GR, suggesting a reason for the existence of nuclear retention.
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packages of food to scientific discussion. Impossible to adequately describe and still more impossible to replace.
List of Abbreviations:

AF-1, AF-2  activation function 1 and 2
Amp  ampicillin
AR  androgen receptor
c-hex  cycloheximide
ChIP  Chromatin Immunoprecipitation assay
CMV  cytomegalovirus
cort  cortisol
CRM-1  chromosomal region maintenance protein 1
DBD  DNA binding domain
dex  dexamethasone
DMEM  Dulbecco’s modified Eagle’s medium
ER  estrogen receptor
(s)FBS/FCS  (charcoal-stripped) fetal bovine serum / fetal calf serum
FL  Full-length
FLIP  fluorescence loss in photobleaching
FRAP  fluorescence recovery after photobleaching
GR  glucocorticoid receptor
GRE  glucocorticoid receptor response element
HRE  hormone response element
Impα / β  importin α / importin β
Kan  kanamycin
LBD  ligand binding domain
LMB  leptomycin B
luc  luciferase
MMTV  mouse mammary tumor virus
MR  mineralocorticoid receptor
NES  nuclear export signal
NF-1  nuclear factor 1
NL1, NL2  nuclear localization signal 1 and 2
NLS  nuclear localization signal
NR(S)  nuclear retention (signal)
Oct-1,-2  octamer transcription factor 1, 2
o/n  overnight
OTF  octamer transcription factor, aka Oct
PBS  phosphate-buffered saline
Pen  penicillin
Pol II  RNA polymerase II
PR  progesterone receptor
rluc  Renilla luciferase
SHR  steroid hormone receptor
Strep  streptomycin
SV40  simian virus 40 large T antigen
w/d  withdrawal
WT  wild-type
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**Introduction:**

*Overview of the endocrine system and the Hypothalamic-Pituitary-Adrenal (HPA) axis:*

The endocrine system is a regulator of metabolism, mood, and growth and development in the body. These functions are accomplished through the secretion of a variety of signalling molecules including hormones, which work to regulate signalling cascades and control gene transcription throughout the body. A part of the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, for example, is involved in adaptation to stressors, and affects mood, metabolism, homeostasis, and inflammatory/immune function (Charmandari 2004, Chrousos 2007). The core of the HPA axis forms a negative feedback loop, allowing auto-regulation. Briefly, the paraventricular nucleus in the hypothalamus secretes the peptide hormones vasopressin (AVP) and corticotrophin-releasing hormone (CRH). In turn, these stimulate the cleavage of POMC to ACTH, and the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH then increases the synthesis and release of cortisol from the adrenal glands (review in Papadimitriou 2009). Cortisol acts as a ligand for the glucocorticoid and mineralocorticoid receptors (GR and MR), transcription factors which form part of the steroid hormone receptor (SHR) family that are expressed in multiple locations, including the hypothalamus, where one role of cortisol is to decrease the production of AVP and CRH. Through its receptors, cortisol also decreases the cleavage of pro-opio-melanocortin (POMC) to ACTH, and by these two mechanisms negatively regulates its own production and the activity of the HPA axis.
The glucocorticoid receptor (GR), is the best studied of the two receptors for corticosteroids, and is involved in much more than the negative regulation of the HPA axis. As a ubiquitously expressed transcription factor, GR is involved directly or indirectly with the activation and repression of many genes – for example, up to 20% of the genome is reported to be influenced by GCs in leukocytes (Galon et al 2002). Natural and synthetic corticosteroid hormones, including cortisone and dexamethasone, are frequently prescribed for inflammation, autoimmune diseases, and mood disorders, as well as therapy for certain types of cancer such as leukemia (Smoak 2004, Bledsoe 2002). Glucocorticoids also play a role in metabolic homeostasis and adipogenesis (Charmandari 2004), and use of glucocorticoids as therapy can produce side effects including increased abdominal fat, osteoporosis, muscle wasting, diabetes, and mood disorders (Vegiopulous 2007, Chrousos 2007, Bledsoe 2002). Because of its wide range of activity, it is necessary that GR activity be strictly regulated within the cell. In order to optimize the use of corticosteroid therapies, and to better diagnose and treat disorders involving GR, it is important to understand how this regulation is accomplished and how it can be affected. Understanding this regulation may also lead to insights into the regulation of other steroid hormone receptors.

A significant amount of knowledge has already been garnered on the subject of the glucocorticoid receptor, at least in part due to the fact that both the positive and negative effects of glucocorticoids (GCs) have been recognized for over half a century. The first case of hypercortisolism identified predates the second World War (Cushing 1932), and the anti-inflammatory effects of glucocorticoids were being exploited in the treatment of rheumatoid
arthritis, tuberculosis and skin diseases in the 1950s (Gjorup 1956, Fernandez 1955, Aguet and Favez 1956). It was not until the early 1970s, however, that the characterization of the receptor involved in mediating GC activity began (Wira and Munck 1970). Data rapidly accumulated regarding receptor localization, specificity of steroid interaction, estimated number of binding sites present per cell, and yielding the first indications of the glucocorticoid receptor’s function as a transcription factor within a few years (Ballard 1972, Baxter 1972). This led to the cloning of the cDNA for the rat and human receptors in 1984-85 (Meisfeld 1984, Weinberger 1985), the first cDNA cloned of a steroid hormone receptor. A wide range of research on the structure, function, and interactions of GR has been published since, improving our ability to predict and control its actions in vivo, particularly in therapeutic uses. Current research focuses on two main priorities: on the development of new ligands which can maximize the anti-inflammatory activity of GR without producing negative side effects, in order to improve the efficacy and usefulness of GC therapy (Stahn 2007, McMaster 2007), and on the understanding of the activity of the receptor as a transcription factor on a genome-wide scale, and detailing the dynamics of its behaviour and interactions within the cell.

The Glucocorticoid Receptor Protein:

GR belongs to the steroid hormone receptor (SHR) family, a subset of the nuclear receptor superfamily, alternatively designated as NR3C1 (Robinson-Rechavi 2003). The SHRs also include, roughly in order of their similarity to GR, the mineralocorticoid, progesterone, androgen, estrogen and thyroid hormone receptors, with GR, PR, AR, and MR further forming the oxosteroid receptor subclass, with natural ligands based on a cholesterol ring structure. All members of the nuclear receptor family share a similar modular structure,
consisting of N-terminal, DNA-binding (DBD), hinge, and ligand-binding (LBD) domains (Giguere 1986). Steroid hormone receptors are found in vertebrates down to teleost fish, and their function is conserved across species (Hollenberg 1985). They are best known in their genomic function as nuclear transcription factors, but multiple SHRs have also been found to have rapid, non-genomic signalling roles within the cytoplasm (Lu 2004, Watson 2005, Haller 2008). Sequence conservation varies by domain, from the highly conserved DBD (85% and up for GR of different species) to the variable N-terminal domain (ranging from 17 to 84% between species for GR) (Stolte et al 2006).

In humans, at least, GR occurs as multiple isoforms, whose exact roles are still not completely defined (Duma 2006, Chrousos 2005). The receptor isoform commonly referred to as GR, the one which our lab studies, is actually the GRα-A. This is the most abundant isoform, the first one to have been identified, and the one which is thought to mediate most of the effects of GCs. All isoforms of GR are coded for by one gene, which is made up of nine exons (Lu 2004). The first exon does not code for part of the finished protein, but produces different untranslated regions from three different proximal promoters, one of which also has three alternative splicing options, leading to multiple possible mRNAs (Yudt 2002). There are also 5 possible open reading frames in exon 1, one of which has been shown to produce a peptide which influences the transcriptional regulation of GR (Diba 2001). GR also has two different potential start sites, at M1 and M27, the latter of which was found to be more efficient at transcriptional activation of a reporter gene (Yudt 2002). At the C-terminal end of the gene, alternative splicing of exon 9 leads to the most commonly known alternate form of GR, known as GRβ. Although the two forms differ only from aa 727 on, with the shorter GRβ form having 15 unique residues at the C-terminus and resulting
in a truncation of helix 12, this form lacks ligand binding capability, and functions as a dominant-negative repressor of the GRα form (Charmandari 2004). Although this was previously thought to be its only significant role in the cell, GRβ has also recently been shown to have some ability to activate transcription, from a set of target genes differing from those of GRα, suggesting it has a specific role to play in the cell (Kino 2009). Other known isoforms of GR include GRγ, which has a single amino acid insertion between the zinc fingers in the DNA-binding domain (DBD) due to inclusion of 3 bp from the intron between exons 3 and 4. As well, minor forms include GR-low sensitivity, a form which lacks exon 2, GR-P, which retains the intron between exons 7 and 8 and lacks correct exons 8 and 9, and GR-A, which lacks exons 5-7. Most of these alternative GR variants have been implicated in glucocorticoid resistance and can be involved in disease states – for example, the GRγ isoform has been linked to GC resistance in childhood acute lymphoblastic leukemia – but little else is known about any unique properties they may have, or whether they participate in the normal functioning of the cell (Duma 2006).

Besides the variant GR isoforms, a number of spontaneously occurring mutations and polymorphisms have also been identified in vivo, the majority of which occur within the ligand-binding domain (Review in Bray 2003, Derijk 2009). These manifest as either a partial glucocorticoid resistance due to altered receptor levels, hormone binding, or transactivational capability, or as glucocorticoid hypersensitivity. Glucocorticoid resistance has been associated with hypertension, anxiety, chronic fatigue and depression, but also with lower cholesterol levels, increased insulin sensitivity, and lower risk of depression (Chrousos 2007). Effects of glucocorticoid hypersensitivity may include increased body
Figure 1: The glucocorticoid receptor, HPA axis, and metabolism
Simplified diagram of how the Hypothalamic-Pituitary-Adrenal axis and GR activity interact with metabolic pathways and homeostasis. Stressors lead to increased HPA axis activity and cortisol production. Increased CRH also leads to increased energy expenditure, and increased insulin. GR activation by cortisol increases lipid and protein metabolism and decreases inflammation. Decreased HPA axis activity, on the contrary, leads to decreased net energy expenditure, increased adipogenesis and glucose sensitivity. Cort-cortisol, GR-glucocorticoid receptor, ACTH-adrenocorticotropic hormone, NPY-neuropeptide Y, POMC-pro-opio-melanocortin, MC4R-melanocortin 4 receptor, CRH-corticotropin-releasing hormone, TRH-thyrotropin-releasing hormone, MCH-melanocortin hormone, GLUT4-glucose transporter 4.
Food intake
CRH, POMC, ACTH, Cort, GR, insulin, leptin, orexin, TRH, ghrelin, NPY, MCH, serotonin

Glycolysis, gluconeogenesis
Glucose uptake and catabolism

Lipolysis, proteolysis

Glycogen and protein synthesis, GLUT4 translocation, inflammation

Adipose tissue

Energy expenditure

Stress, fasting, exercise, trauma

Adrenal gland
Pituitary gland
HPA Axis
Hypothalamus

Cort, GR
ACTH → POMC → NPY

MC4R

CRH, TRH

MCH, orexin

ghrelin

serotonin

insulin

Glucose sensitivity

Food intake Energy expenditure

Glycolysis, gluconeogenesis

7
fat, lower bone density, and higher incidence of coronary artery disease, similar to what can occur upon long-term glucocorticoid therapy (Reviewed in Nicolaides 2009) (figure 1).

Structure of the Glucocorticoid Receptor:

The glucocorticoid receptor, like other steroid hormone receptors, is a modular protein comprising N-terminal, DNA-binding, hinge, and ligand-binding domains (Giguere 1986, Danielsen 1986, reviewed in Robinson-Rechavi 2003) (figure 2). In the GR, the N-terminal domain is approximately 440 amino acids in size (domain sizes vary slightly between species) and exhibits less conservation between species than other domains in the protein. It has not yet been successfully crystallized. Results from protein structure-prediction programs, in conjunction with the difficulty of crystallization, suggest that the N-terminal domain is largely unstructured. It contains what is known as the activation function 1 (AF-1) domain, which functions as a cofactor binding site (Hollenberg 1988, Dahlman-Wright 1994), as well as the majority of the identified sites of post-translational modification in the protein. This includes two of three potential SUMOylation sites (Le Drean 2002), a candidate PEST motif for ubiquitylation (Wallace 2001), and at least 7 serine/threonine phosphorylation sites, several of which are hyperphosphorylated upon agonist treatment of the receptor, and have been shown to affect GR activity and regulation (Wang 2002, 2007, Blind 2008, Ismaili 2004, reviewed in Lu 2004). Interestingly, post-translational modification sites, especially phosphorylation, have been found to be common in unstructured regions of proteins, and there is some evidence suggesting that they may cluster there for the purpose of inducing the specific structure necessary for interacting with other proteins, while retaining the flexibility to interact with a variety of partners under different
The DNA-binding domain or DBD is the best-conserved region of GR, with respect both to similarity within the family and between species, with the crystal structure determined in 1990 (Hard 1990). It is roughly 65 amino acids long, and contains two zinc fingers, each a loop formed by the coordination of a zinc atom with 4 cysteine residues, which interact specifically with DNA at GR binding sites known as glucocorticoid response elements (GREs), with one zinc finger binding the major, and one the minor groove of the DNA (Hard 1990). These GREs are imperfect palindromic sequences, with a consensus sequence of GGTACAnnnTGTTCT (Scheidereit C 1986, Jantzen K 1987). Each half-site is capable of binding a GR monomer, and paired half-sites of binding GR homodimers (Luisi 1991). Specificity is conferred by conserved residues in the knuckle (P-box) of the first finger and the tips of the fingers (Danielsen 1989, Umesono 1989, Hard 1990), and mutation of these can alter the preference of the receptor for particular sites (Necela 2004) – for instance, mutation of these residues in AR to GR sequence produces a receptor incapable of activating androgen receptor-specific target genes but which can still activate general SHR response elements. (Verrijdt 2006) Conversely, the exact sequence and spacing of the GRE half-sites has been recently shown to play a role in determining the recruitment of cofactors and the activation of target genes by GR. This occurs independently of the actual affinity of the receptor binding to the site, and presumably affects cofactor affinity through allosteric alteration of the GR conformation (Meijsing 2009, Chandra 2008). Export of GR and other steroid receptors has been found to be mediated by a sequence within the spacer sequence between the two Zn fingers, with two Phe residues crucial for this function (Black 2001). Finally, the GR DNA-binding domain also contains a dimerization domain in its second Zn
finger, which is involved in DNA-dependent homodimerization between GR DBDs (Dahlman-Wright 1991, Luisi 1991).

The 35 amino acid hinge region of GR is sometimes grouped with the DNA binding domain in descriptions of the receptor domains. The sequence is not as well conserved as the DBD, and it is thought to have a flexible structure. While it is positioned to act as a hinge between the N and C-terminal portions of the receptor, it participates in the solution homodimerization of the receptor (Savory 2001) and also contains the major, hormone independent, nuclear localization signal (NL1) of the receptor (Rusconi 1987, Picard 1987). The NL1 is a basic bipartite NLS, containing a cluster of lysine and arginine residues, three of which are thought to undergo acetylation (Ito 2006, Nader 2009). The corresponding lysine residues in the androgen and progesterone receptor have been shown to be acetylated and sumoylated, respectively. Mutation of the AR hinge lysines has been shown to delay import and cause protein misfolding and aggregation (Thomas 2004), while sumoylation of PR hinge lysines has been shown to decrease nuclear retention and transactivational ability of the receptor (Man 2006). Early work on the GR receptor predicted that the hinge region could contain multiple activities (Miesfield 1987, Godowski 1987). This seems to be borne out by work in our lab implying that the hinge region also contains a nuclear retention activity for GR (Carrigan et al 2007), and by work in our lab and others identifying cofactor interactions within the hinge region (De Martino 2004, Prefontaine 1998, Kullmann 1998, Yoshikawa 2008, Nader 2009).

The C-terminal portion of the GR (to amino acid 777 in humans, 795 in rats) forms the ligand-binding domain (LBD) of GR. This region is highly-conserved, though not to the same extent as the DBD, and has a defined structure which has been crystallized in conjunction with agonist and antagonist molecules and with peptides of different co-factors,
allowing a detailed look at the relation between form and function. (Bledsoe 2002, Kauppi 2003, Kroe 2007). Briefly, it consists of a group of 12 α-helices, folded to form a hydrophobic pocket which allows the transcriptional regulatory potential of the receptor to be activated upon ligand binding. It also provides binding sites for chaperone proteins (Ricketson 2007), which maintain the naïve receptor in a conformation favourable to hormone binding and play a role in regulation of receptor transcriptional activation and mobility. Homo-and heterodimerization (with the closely related mineralocorticoid receptor, MR) (Savory 2001) and cofactor binding via the activation function 2 (AF-2) also occur within this region. A second, ligand-dependent nuclear localization signal (NL2), is also found in the LBD (Picard 1987, Rusconi 1987) and it is thought that nuclear export of GR may also be mediated at least partially through the LBD (Saporita 2003, our lab, unpublished observations). Changes in the conformation of the LBD caused by binding of specific ligands influence chaperone and cofactor binding, and through this, the activity of the protein (Garside 2004). The position of helix 12, especially, has been shown to be important in SHR activity. Co-crystallization and affinity experiments have shown that the structure of agonist ligands, when bound to GR, allows helix 12 positioning against the ligand-binding pocket so as to form an appropriate binding surface for coactivator proteins at the AF-2, whereas antagonist binding induces a different conformation and encourages interaction with co-repressors instead (Kroe 2007). Small changes in agonist structure can influence GR activity through modification of the positioning of helix 12, thereby altering the affinities of different coactivator/receptor interactions (Wang 2006) in a cell-type and receptor-specific manner. Current work on understanding the influences of GR ligands and co-factor interaction on gene activation profiles aims to maximize the anti-inflammatory potential of the receptor while minimizing the side effects, such as osteoporosis, immune suppression,
Figure 2: Modular diagram of the glucocorticoid receptor
GR α domain structure and approximate locations of interactions and signal sequences. DBD – DNA-binding domain, LBD-ligand-binding domain, AF-1,2 – activation function 1,2, NES – nuclear export signal, NL1,2 – nuclear localization signal 1,2, NRS – nuclear retention signal.
mood alteration and truncal obesity, resulting from GR’s influence on other target genes (Stahn 2007, McMaster 2007, Vegiopoulos 2007).

Mechanisms of GR Action and Regulation:

As GR has been associated with a wide variety of systems and functions in its role as a transcription factor, it is important that the active receptor be specifically regulated. This can occur in a variety of ways, including intracellular localization of the receptor, association of the receptor with different cofactors and combinations of cofactors, and post-translational modification of the receptor (Lu 2004, Collingwood 1999, McKenna 2002, Wang 2004, Glass 2000).

With regard to sub-cellular localization, steroid hormone receptors fall into two categories: those which are constitutively nuclear and those which are cytoplasmic or mostly cytoplasmic in the absence of hormone and translocate to the nucleus upon hormone binding. GR, along with the mineralocorticoid and androgen receptors, belongs to the latter group (Picard 1987, Htun 1996, Jenster 1993, Georget 1997, Fejes-Toth 1998, Nishi 2001), although the degree of cytoplasmic localization for the naïve receptors can be affected by cell type or level of expression (Sanchez 1990), while PR and ER are constitutively nuclear (Welshons 1984, Guiochon-Mantel 1989). Both the nuclear and cytoplasmic groups of receptors exhibit what appears to be a largely random, diffuse distribution in the absence of ligand which shifts to a punctate distribution within the nucleus in the presence of agonist (Htun 1996).

In most cell types, naïve GR resides mainly in the cytoplasm and associates with a chaperone complex containing heat shock proteins 90 and 50, p23, and the FK506-binding immunophilins FKBP51 or 52 (Pratt 1993, 1997, Cheung 2000). GR folding after translation
is aided by Hsp70, after which the protein is transferred to the Hsp90/p23/FKBP51 complex, which maintains it in a conformation competent to bind hormone (Pratt 2006). Upon hormone binding, it was originally believed that the receptor dissociated from the chaperone complex, exposing the receptor’s nuclear localization sequence and allowing import into the nucleus via interaction with the importin α/β complex (Pratt 1993, Tanaka 2003, Echeverria 2009). Evidence now suggests that GR import is more complex than previously thought. It is no longer clear that GR entirely dissociates from the chaperone complex – certainly the same elements are also present in the nucleus, and techniques such as immunofluorescence suggest GR associates with the chaperone complex in both compartments (Elbi 2004). As well, Hsp90 containing an NLS can ferry an NLS-deficient GR into the nucleus, suggesting that the two can associate while crossing the nuclear membrane (Kang 1994, Tago 2004). Further, disruption of Hsp90 interaction with GR in the nucleus can affect GR’s DNA-binding properties (Stavreva 2004), transcriptional activity, and ability to rebinding hormone and re-initiate transcription within the nucleus (Liu 1999, Rosenhagen 2003, Kang 1994). As well, p23 has been shown to be involved in GR release from the promoter and export of GR (Meijsing 2007, Tago 2004). In the cytoplasmic compartment, recent evidence suggests that upon hormone binding, the chaperone complex may be altered, with Hsp90 interaction becoming more dynamic, and the FKBP51 protein exchanged for the FKBP52 prior to import (Davies 2002). These changes would appear to allow recruitment of dynein, linkage of GR to microtubules, and transport to the nucleus along the cytoskeleton, presumably interacting with importins in order to transfer through the nuclear pore (Galigniana 2001, Pratt 2004). The chaperone complex only contains one binding site for immunophilin proteins, and under certain conditions, Cyp40 or PP5 take the place of the FKBP proteins
(Davies 2005). Once in the nucleus, GR can interact with a variety of transcriptional cofactors to modulate the activity of its complement of target genes.

After withdrawal of hormone, or dissociation of GR and hormone, the receptor is rapidly recycled back into the original (pre-hormone) chaperone complex. The half-life of receptor-steroid interaction is thought to be approximately 10 minutes, and recycling back into the original complex occurs within 1h, but may be faster. This allows the receptor to undergo hormone binding and activate transcription again repeatedly until the steroidal signal is spent, without having to be exported from and reimported into the nucleus in order to do so (Yang 1997, Haché 1999). This is significant for GR activity, since although the initial import into the nucleus occurs rapidly (10-15 min to completion) (Ogawa 1995), there is a considerable time lag between reassembly of the inactive GR-chaperone complex after hormone withdrawal (1h) versus export to the cytoplasm (12-24h) (Haché 1999). Observation of this delay led to studies on the possibility of GR being actively retained within the nucleus (figure 3).

Proteasomal activity and turnover of the receptor may also be involved in regulating GR’s activity, as proteasomal inhibition affects GR-mediated transactivation (Deroo 2002, Alarid 2006). It has been suggested that the cycling of GR on and off of the promoter requires proteasomal activity, and GR half-life or turnover time is halved in the presence of hormone, from 18-25h down to 9-11h. Phosphorylation has also been implicated in determination of GR stability (Kinyamu 2005, Stavreva 2004, Wallace 2001).

Within the nucleus, the activity of the receptor is affected by factors including cofactor interaction, post-translational modification, and promoter sequence. Earlier models, based on the results of immunofluorescence and immunoprecipitation assays, postulated a
Figure 3: Diagram of GR localization and cycling behaviour.

Ligand (a) enters the cell and interacts with the GR/immunophilin/chaperone complex, leading to alteration of GR configuration, more dynamic Hsp 90 interaction, and exchange of immunophilin FKP51 for FKBP52 (b), and import into nucleus using importin a/b via interaction with the nuclear pore (c). Once inside the nucleus, liganded GR interacts with cofactors (d) to control transcriptional activity at various genes. Ligand dissociates with a half-time of 10 minutes, and the original complex is reassembled (e) prior to export (f) via both CRM1 dependent and independent mechanisms. Back in the cytoplasm, the receptor then undergoes recycling or proteasomal degradation (g). GR interaction with a retention factor (h) in the presence or absence of hormone blocks export and may influence transcription of a subset of genes.
receptor that interacted stably with target promoters and recruited different cofactors, depending on the promoter, cofactor availability, and binding sites defined on GR by post-translational modification and ligand-induced configuration (Nagaich 2004, Deroo 2001). While this is still a fairly accurate perception of the regulation of GR transcription, it has been conclusively shown that the interactions of receptors and cofactors are very dynamic (Hager 2004, McNally 2000). Live cell experiments using fluorescently-tagged proteins have shown that while GR can interact specifically with a target promoter, it exchanges rapidly on and off the promoter, on a timescale of seconds (Metivier 2003). Indeed, most nuclear proteins, including receptors and cofactors, have been shown to be extremely mobile in the nucleus, even when they are presumably in contact with the nuclear architecture, and interacting with and affecting their partners (Defranco 2002, Hager 2004). In systems capable of tracking rapid movement within the cell, it has been shown that mobility of GR is affected in a ligand-specific manner, with various ligands differentially affecting the rate of movement of the protein (Schaaf 2003, 2005). These findings have led to what is called a “hit-and-run” model of promoter activation, where the receptor interacts briefly but frequently with the promoter, helping to increase the chances that the specific cofactors or complexes required will interact with the promoter and initiate transcription or repression (McNally 2000, Hager 2004). On a longer timescale, using chromatin immunoprecipitation assays, it has been shown that the dynamic interaction of receptors with the promoter also exhibits a longer cycle of on and off interactions that can extend from minutes to hours (Metivier 2003). This suggests that the variation seen between the cycle length of individual receptors and the general population may function in allowing a finer tuning of promoter activity in response to ligand signals.
As well as acting directly on GRE-containing promoters, GR can control transcription indirectly by interacting with other transcription factors such as NF-kB and either enhancing or repressing their ability to activate transcription (McKenna 2002, Valledor 2004, Smoak 2004). Interestingly, this function of GR may be more vital to survival than its direct interactions with target promoter, as experiments with transgenic mice have shown that a complete receptor knockout is not viable (the knockout pups die shortly after birth due to respiratory defects) (Cole 1995), whereas a mutant expressing a DNA-binding mutant of GR is viable and fertile (Reichardt 1998).

A wide variety of cofactors have been identified as interacting with the GR within the nucleus and it is by no means certain that the list is yet complete. Many interact with GR via the AF-1 and/or AF-2 sequences, although some have been reported as binding the hinge region and the DBD as well. One challenge in understanding GR regulation is to determine the roles, contexts and activities of all these factors.

Several identified cofactors of GR are capable of post-translationally modifying other proteins, and are thought to have a role in determining the configuration of the promoter and the activity of other proteins associated with either the promoter or the transcription complex (Li 2003). Histone acetyltransferases, or HATs, identified as interacting with GR include p300/CBP, P/CAF, and the p160 or SRC proteins SRC-1a and -1e, GRIP1/TIF2 and RAC3/ACTR (Eggert 1997 and refs, Li 2002, Glass 2000). The p160 proteins are thought to be recruited as platforms for other coactivators, and show cell-type, receptor- and promoter-specific preferences for their recruitment (Trousson 2007). In one example, on the MMTV promoter, SRC 1a, 1e, and 3 in Schwann cells and 1e, 3, and some 2 in astrocytes are preferentially recruited (Grenier 2006). Intracellular localization of the SRC proteins also varies, with only the SRC-1 showing hormone-induced transfer to the nucleus (Grenier
In another study, PR and GR showed differing recruitment of cofactors and specific histone modifications at the MMTV promoter under identical conditions (Wang 2005). Further, p300 has been shown to interact in a complex with GR and SRC-1 in a hormone-dependent manner, and its acetyltransferase activity is important for GR transactivation of target genes (Kinyamu 2004, Li 2002). Deacetylation of GR is also important for proper function. Members of the histone deacetylase (HDAC) group of proteins can also interact with GR, and their proper function is important for GR’s ability to affect NF-kB mediated transcription as well as activate some promoters and form stable complexes with Hsp90 (Ito 2006, Qiu 2006). The role of HDACs has traditionally been to repress transcription, mainly through regulating histone tail deacetylation and its effect on promoter configuration, with deacetylation generally signalling a more compact, less transcriptionally active form of chromatin (Shahbazian 2007, Aoyagi 2007). Some newer reports, however, provide evidence that HDACs can also play a role as activators (Qiu 2006). A similar case of a somewhat unexpected role for a cofactor is that of the ubiquitin (Ub) conjugating enzyme UBCH7, which has been shown to function as a coactivator for multiple steroid hormone receptors, including GR, which have an Ub site in the N-terminus (Verma 2004). Further, it is recruited in a steroid dependent fashion, and the ubiquitin-conjugating activity is necessary for proper receptor function. One recent hypothesis is that Ub modification of this type serves as an activator and a timer of sorts of the target protein, with the addition of the first Ub moiety serving to activate the protein, and a new Ub being added during each cycle of binding and release of the protein from the promoter. The increasing levels of ubiquitin are thought to slow transcriptional activation via steric effects, and eventually target the protein for degradation after a predetermined number of cycles (Lonard and O’Malley 2009).
With the PIAS protein family, which function in sumoylation, transactivation of the MMTV promoter by GR is increased in the presence of PIASxb and PIASy, while PIAS1 represses the same promoter, and PIAS3 has no effect with GR (Tirard 2004), although it has been reported to sumoylate progesterone receptor (PR) and decrease its nuclear retention (Man 2006). On the same promoter, transactivation by the closely related MR, however, is not affected by PIASy, while the other three isoforms are inhibitory (Tirard 2004).

A subset of the cofactors identified for GR do not appear to influence post-translational modification. However, several have been reported to show cell-type and/or gene specificity, and likely function to fine-tune GR activity in this manner. Mediator (MED) cofactors 1 and 14, for instance, are thought to serve as bridges between the polymerase complex and other cofactors, and have also been reported to interact with GR, via both its AF1 and AF2 regions, in a gene-specific manner (Chen 2007). Evaluation of several known GR-activated genes showed that ladinin-1 and IRF8 require both MED proteins, IGFBP1 requires MED14 but not MED1, and GILZ appears to be independent of both MED proteins (Chen 2006). The recruitment and role of specific co-activators by steroid hormone receptors can thus be seen as a highly complex system of regulation.

While most cofactors interact with GR via the AF1 and/or the AF2 regions, a subset of factors, primarily corepressors, have been reported to interact via the hinge region of the receptor. Rap46 interacts with the hinge region, and inhibits DNA binding and transcriptional activation by GR, although it does not affect the activities of GR which do not require direct DNA interaction (Schneikert 1999). HEXIM1 can also suppress GR-induced gene activation as a result of its interaction with the hinge region (Yoshikawa 2008). Oct1/2 and other POU-domain proteins have been shown to interact with the GR hinge region, likely in a complex with SRC-1 (Gonzalez 2001, Prefontaine 1998), and Oct-1 has
been shown to be required for the transcriptional activity of GR on the MMTV promoter (Prefontaine 1998). The related AR and PR receptors also interact both physically and functionally with Oct (Prefontaine 1999). COUP-TFI also interacts with the hinge region of GR, and thereby stimulates COUP-TFI-interacting promoters, while repressing GR-activated promoters (De Martino 2004 a,b). CLOCK, a circadian regulator, has also been shown to interact with GR via the hinge region; it acetylates several lysines in the NL1/hinge sequence, leading to repression of GR transactivation due to loss of DNA binding. Curiously, CLOCK activity has also been shown to increase repression at promoters negatively regulated by GR (Nader 2009).

Besides the previously mentioned post-translational modification of GR via phosphorylation and ubiquitylation, acetylation and sumoylation have also been reported to occur on the receptor, but have been studied less extensively. The most common, and varied, modification of GR is phosphorylation, for which a number of sites have been identified, 5 in human and 7-8 in rodents, mostly clustered in the N-terminal region (Webster 1997, Ismaili 2004, Wang 2002, 2007). Thus far, the regulation of GR activity through phosphorylation appears to be a very complex system which is still very incompletely defined. The lack of conservation of sites between species may account for differences between reports on the effects of phosphorylation on GR, and the difficulty in defining a phosphorylation ‘code’ as has been done for histone modifications (Webster 1997, Ismaili 2004, Lu 2004). Several different kinases (CDK2, p38 MAPK, JNK, cyclin A, E) have been identified as having the potential to phosphorylate GR, and the sites they modify overlap with each other (Ismaili 2004). Individual mutation of phosphorylation sites has been shown to have little effect on GR activity (Webster 1997), although mutation of multiple sites or of particular kinase genes does. Further, transcription from simple GREs appears to be more
dependent on GR phosphorylation status than is transcription from complex GREs (Lu 2004, Blind 2008). Studies with human GR show that S-211 and S-203 phosphorylation seem to be most strongly associated with receptor activation (Wang 2002). S211-P and S226-P kinetics are promoter dependent (Blind 2008) and the latter has also been implicated in export of GR from the nucleus post hormone withdrawal (Itoh 2002). Individual phospho-isoforms of GR have also been reported to selectively occupy different gene promoters (Lu 2004, Blind 2008). Lastly, in some reports, phosphorylation acts as a method of downregulating GR, helping to target Ub and degradation (Wallace 2001, Lu 2004).

Only one ubiquitylation site has been identified on GR, in the N-terminal region/DBD. A candidate PEST sequence was defined just N-terminal to the DBD (residues 407-426 in mouse), with ubiquitylation occurring at K426. Mutation of this residue to alanine was found to exert a similar effect to that of proteasome inhibition by MG132, leading to a block in hormone-induced downregulation of GR and an increase in transcriptional activation (Wallace 2001, Lu 2004).

Sumoylation (addition of the small, ubiquitin-like modifier, on lysine residues) of GR has been reported at 3 possible sites, two within the N-terminal region, corresponding with sumoylation sites reported for the androgen receptor (Le Drean 2002), and one site in the ligand-binding domain (Le Drean 2002, Tian 2002), although these may not be all the sites present (Tian 2002). Sumoylation was observed to occur in a ligand-enhanced fashion, and to enhance transcriptional activation at promoters containing a simple GRE. The estrogen receptor (Sentis 2005), and progesterone receptor (Man 2006) have also been reported to undergo sumoylation, but in the hinge region of the protein rather than the N-terminal sites reported for GR and AR, and mutation of this site has been reported to
decrease transcription from the ER. Ubc-9 and SUMO-1 have been reported to be involved in GR sumoylation as well as in that of other receptors.

Lysine residues are a target of acetylation as well as sumoylation, and this modification is shown to occur in GR as well as in other SHRs. Several lysines within the hinge region of GR, coinciding with the NL1 sequence, have been reported to undergo acetylation (Ito 2006, Nader 2009). This modification seems to be involved in decreasing GR-mediated transcription, possibly through decreasing the interaction of GR with the DNA (Nader 2009). It has been shown that acetylation of the GR hinge by the circadian factor CLOCK leads to repression of transcription from the MMTV promoter (Nader 2009), while failure of HDAC2 to deacetylate GR leads to insensitivity towards GC-induced gene expression (Ito 2006), and mutation of CLOCK leads to a Cushingoid syndrome in mice (Nader 2009). Acetylation of the corresponding sites is reported to occur in AR and ER, with ER acetylation reported to enhance receptor binding and transactivation (Kim 2006), while mutations of the AR sites was shown to delay receptor nuclear translocation and cause misfolding and aggregation (Thomas 2004).

Protein Localization and Nucleocytoplasmic Trafficking:

The net localization of a protein within the cell is influenced by multiple factors. These can include signal sequences within the protein that specify transport into or out of an organelle, availability of cofactors to produce or act upon post-translational modification of the target protein, cell-cycle dependent changes in the cellular environment, and presence of signal molecules or ligands for the target protein. The glucocorticoid receptor, as mentioned earlier, exchanges primarily between nucleus and cytoplasm, although a small portion is though to be membrane-bound (Bartholome 2004), which may function in non-genomic
pathways (Ismaili 2004, Haller 2008), and there is some evidence for a mitochondrial glucocorticoid receptor which affects apoptosis and neural plasticity through interaction with Bcl-2 (Cato 2001, Scheller 2003, Sionov 2006).

Transfer of proteins between the nucleus and cytoplasm occurs through nuclear pores, large (125MDa) multiprotein complexes forming a selectively permeable passage through the nuclear membrane of about 9 nm in diameter, which can expand to 40 nm in diameter to accommodate the transfer of large factors (Ohno 1998, Rout 2001, Pante 2002). The arrangement of the component proteins (nucleoporins or Nups) in the pore shows 8-fold symmetry, and various component Nups show specific preferences for the nuclear or the cytoplasmic face of the pore and undergo differing rates of exchange on and off of the pore (Rabut 2004). Fibrils extend from both ends of the pore, and form a basket-like structure on the nuclear side of the passage. Small molecules can passively diffuse through the pore, while larger molecules must be actively transported to traverse the pore. The size limit for molecules to be able to freely diffuse through the pore has variously been reported as between 20 and 70 kDa, possibly influenced by the conditions under which the assay was performed (refs in Picard 1987, Ohno 1998, Rout 2001, Tanaka 2003). Active transport of large molecules occurs via transport receptors such as the karyopherin proteins and is powered by a Ran-GTP cycle, which depends on the maintenance of a gradient of GDP in the cytoplasm and GTP in the nucleus. In the cytoplasm, the importin-cargo complex associates with the small protein Ran-GDP for transport across the nuclear pore. In the nucleus, a protein called Ran-GEF (guanidine exchange factor) catalyzes the exchange of GTP for the GDP of Ran, leading to the dissociation of the import complex. Ran-GTP, on the other hand, associates with the exportin-cargo complex and allows it to translocate to the cytoplasm, where Ran-GAP hydrolyzes Ran-GTP back to Ran-GDP and promotes

The karyopherins are a group of transport proteins which contain characteristic HEAT and ARM repeats, modules containing two or three helices respectively, joined by sharp turns, creating hairpin-like structures. The multiple HEAT/ARM repeats in karyopherins form a flexible spiral that can interact with a wide variety of cargo proteins as well as with Ran-GTP (Madrid 2006 and refs). Karyopherins are categorized as importins, exportins, and transportins, based on whether they act to shuttle cargo into or out of the nucleus, or perform both tasks. Importins have been thus far identified as the most numerous karyopherins, and are comprised of importin α and β forms. Importin α has 1 isoform in yeast and 7 in higher eukaryotes (Pemberton 1998, Nakielny 1999, Weis 2002), and binds to sequences of basic amino acids, the classical nuclear import signals exemplified in the SV40 large T antigen NLS (PKKKRKV). The importin α generally serves as an adaptor protein, which binds to cargo and then to an importin β through an N-terminal importin β binding (IBB) domain in order to be imported to the nucleus (Gorlich 1996, Weis 1996), but some recent evidence has suggested that it may be able to act independently to import cargo under certain conditions (Kotera 2005). The importin β family of karyopherins is somewhat more numerous than their α counterparts, having 8-9 members (Harel 2004). In the classical model of nuclear import, importin β interacts with the importin α and its cargo, and interacts with the nuclear pore, allowing import of the complex. However, again, like importin α, it has been shown that some cargos can interact with and be imported directly by importin β without having to utilize the importin α adaptor (Harel 2004, Palmeri 1999). Many proteins and RNAs which are imported into the nucleus have a preference for a particular importin,
while some cargoes are more promiscuous, and it has been speculated that the ability to interact with multiple importins is more common for essential nuclear proteins (Kohler 1999, Nadler 1997, Weis 2002). The glucocorticoid receptor is thought to import primarily via the common importin α/β pathway, with importin α binding at the basic NL1 sequence of GR (Savory 1999). The second NLS (NL2) of the GR thus far does not appear to resemble any known NLS, and contains an atypical signal sequence which is assembled as a consequence of receptor folding. However, the specific residues involved and the importin(s) it uses are thus far unidentified (Savory 1999), although importins 7, 8 and 13 have been suggested as candidates based on work in a yeast system, with importin 7 also being capable of importing the NL1 fragment in the same system (Freedman 2004, Tao 2006).

Cargo-importin specificity is determined by the sequence of nuclear localization signal sequences (NLSs) present on cargo proteins. Several types of NLS have been identified, including a variety of basic NLSs (Jans 1998, Leslie 2004, Guttinger 2004, Christophe 2000, Lee 2006). This appears to be the most common form of NLS (Lange 2007), consisting of a cluster of lysine and/or arginine residues, which interact with importin α/β complexes in order to enter the nucleus. The original (canonical) NLS identified of this category is that of the SV40 large T antigen with the sequence PKKKRKV (Kalderon 1984a,b). Many other proteins have been subsequently identified as having basic NLS, either monopartite, as in the SV40 sequence, or bipartite, containing an additional small basic cluster several residues upstream. Classical monopartite sequences may take the form B₄, P(B₃X), PXX(B₃X), or B₃(H/P), where B is a basic (K or R) residue, X is any residue, and letters in brackets may be in any order. Bipartite sequences tend to take the form BBX₁₀(B₃X₂) (Macara 2001). The hormone-independent NL1 of GR is a basic NLS which is
technically a bipartite NLS, but the main cluster of basic residues has the greatest effect on import, and the smaller upstream cluster cannot act as an NLS on its own (Sackey 1996, Tang 1997).

Although some nuclear export occurs via karyopherin family members known as transportins and exportins (Nakielny 1999, Nigg 1997, Macara 2001, Mingot 2004), transport out of the nucleus has also been shown to use unrelated proteins as export receptors (Khacho 2008, Hendriksen 2005, Holaska 2001). This may be partly due to the variety of cargoes which require export, comprising not only proteins, but also RNA molecules. With respect to proteins, although a few nuclear export signals have been identified, they are neither as well defined nor as common as those identified for import (Mingot 2004, Macara 2001, Khacho 2008, Connor 2003, Hakata 2003, Black 2001). Nuclear export of GR appears to be complex and is incompletely defined to date. It has been well established that GR undergoes nuclear export after hormone withdrawal. CRM1, also known as exportin 1, which binds to leucine-rich sequences, and is sensitive to leptomycin B (LMB) inhibition, was found to be partly responsible for this export (Liu 2000, Walther 2003). The DBD of GR has been proposed to mediate this activity (Black 2001, Saporita 2003), and a candidate leucine-rich region in the LBD has also been identified (Unpublished, Haché lab). However, the ability of GR to export to the cytoplasm appears to be partially independent of CRM1, and no other putative export sequence or receptor which operates under normal cellular conditions has been identified to date for GR (Walther 2003).

The re-export of GR to the cytoplasm under normal conditions is very slow, occurring over 12-24 h or even longer (Haché 1999). Although a rapid shuttling mechanism was originally thought to underlie this slow export, this hypothesis has since been disproven (Walther 2003). The rapid shuttling previously observed in heterokaryon experiments was
found to be an artifact due to the release of calreticulin from the ER during cell fusion, and does not seem to be relevant to normal cellular conditions (Holaska 2001, Walther 2003).

The slow transfer out of the nucleus shown by GR is not a general mechanism, however. Other proteins which export via a CRM1-dependent pathway can transfer rapidly to the cytoplasm (Hakata 2003), while GR appears to be retained in the nucleus.

Retention or sequestration of proteins in the nucleus or cytoplasm has been previously described, and proposed as a method of protein regulation (Xu 2004). Confining a protein to one compartment under particular conditions functions to limit its access to specific interaction partners and cellular components while increasing its access to others, thereby providing a mechanism for controlling aspects of protein turnover, post-secondary modification, and transcriptional activation (DeFranco 2002, Black and Paschal 2004). The Smad proteins 2 and 4, for instance, normally shuttle between nucleus and cytoplasm, but nuclear retention is induced by TGF-β, through decreasing intranuclear mobility and export rate of the Smads from the nucleus (Schmierer 2005). The interaction with and activation of mCAR in the nucleus by GRIP1 (which is also a GR cofactor) similarly increases nuclear retention of mCAR (Xia 2005). Cytoplasmic sequestration of p53 is thought to be mediated by GR (Sengupta 2004), while nuclear retention of phospho-p53 is induced by E2F1 in a cell-cycle dependent manner, allowing very specific regulation (Fogal 2005). Intriguingly, the progesterone receptor (PR), a SHR relative of GR, is thought to possess nuclear retention which is decreased by sumoylation of lysine residues in its hinge region. The protein PIAS3 is recruited to a PR-activated promoter in a hormone-dependent manner, and effects sumoylation of the PR hinge lysines, resulting in destabilization of DNA binding and nuclear retention of PR, and a consequent loss of transactivational ability (Man 2006). The significance of regulation by nuclear retention can also be seen in the fact that multiple
disease states have been associated with misregulated retention of proteins in specific cellular compartments. For example, a mutation of ataxin-1 resulting in its retention in the nucleus is associated with spinocerebellar ataxia, a neurodegenerative condition (Irwin 2005).

The presence of a nuclear retention activity in GR has been suggested previously, based on its native slow rate of export. Observation of an enhancement of GR export under a variety of circumstances suggests that the necessary signals or interactions for receptor export are at least partially blocked under normal cellular conditions. Conditions favourable to GR export include the G2 phase of the cell cycle (Hsu 1992), v-mos oncoprotein transformation of cells (Qi M 1989), and exposure to calreticulin (CRT) (Holaska 2001, Walther 2003). As well, GR export rate increases as a result of modifications including Hsp90 inhibition (Tago 2004), mutation of the GR NL1 signal (Savory 1999), or DBD (Walther 2003), phosphorylation of S226 by JNK (Itoh 2002), and addition of an exogenous (IκB) export sequence to GR (Liu 2000). Although the endogenous exposure to CRT has been shown to be an abnormal occurrence which is due to the release of CRT from endoplasmic reticulum upon breakdown of membrane integrity, and thus likely not part of the regular export machinery of the cell, this does not negate the observation that it does increase GR export, and therefore seems to possess an influence on GR nuclear retention (Walther 2003). The presence of GR nuclear retention has been linked to conditions which increase its interaction with the nuclear matrix of the cell, such as depletion of ATP (Tang 1996), proteasome inhibition (Deroo 2002, Schaaf 2003), or interaction with proteins such as hnRNP U via a nuclear matrix-targeting signal defined in the GR LBD (Tang 1998). It does not seem likely that a static interaction with the nucleus such as that seen with ATP depletion correlates with the high intranuclear mobility of GR observed in FRAP.
experiments, but an increase in transient interactions with a static nuclear structure could very well be involved in GR nuclear retention. This would correlate with time-lapse observations in live cells showing that the liganded receptor exchanges rapidly in and out of immobile nuclear subdomains (Schaaf 2005, Hager 2004).

While the extended nuclear retention of GR following dissociation of steroid is now generally accepted, the mechanism that prevents rapid export and/or determines slow export remains to be elucidated. Similarly, the imperative for the slow nuclear export of GR is not yet understood. Under some of the conditions of decreased nuclear retention described, a decrease in transactivational activity and an increase in GR turnover has been noted (Liu 2000, Carrigan 2007). Investigating the mechanism and function of GR nuclear retention should allow for a better and more complete understanding of GR function, and aid in controlling and predicting its activity.

Project background and goals:

Previous work in the Haché laboratory yielded the observation that mutation of the core lysines of the GR NL1 produced a receptor that was not only compromised for nuclear import, but which also showed an increased rate of nuclear export in both the presence and absence of hormone (Savory 1999)(fig 4). Further, the addition of a 26 amino acid fragment of the GR hinge region, containing the NL1, was capable of decreasing the export rate of a heterologous shuttling protein which incorporated strong NLS and NES sequences, while still retaining the nuclear localization and nuclear mobility observed in the same protein without the addition of the GR hinge (Walther 2003). These findings led to the theory that GR nuclear retention is largely controlled via the hinge region, by a signal sequence at least partially overlapping with the NL1. Due to the function of GR as a transcription factor
within the nucleus, it is likely that retention serves in some way to augment the ability of GR to perform its tasks efficiently (Cadepond 1992, Hsu 1992, Liu 2000). This also raised the question of what precisely determined whether a protein containing a basic NLS sequence would exhibit retention, and whether nuclear retention might be a general mechanism for the regulation of nuclear proteins and transcription factors.

My project goal was therefore to characterize the GR nuclear retention signal sequence and its activity. More specifically, I set out to define the specific residues involved in retention and determine whether nuclear localization and retention activity could be separated, in order to examine the role of retention in GR function, and to look at the possibility of my findings on retention being generally applicable to other nuclear proteins.
Figure 4: The NL1- mutation affects GR export both in the presence of hormone and after hormone withdrawal.
A) Diagrams of full length (FL) GFP-GR constructs of the WT and NL1- receptors. The NL1- mutation (K513-515N) produces a receptor which has a decreased nuclear localization and an increased export rate relative to the WT receptor in both B) FRAP assays with live cells in the presence of agonist and C) In cells fixed at certain time points after agonist withdrawal.
Materials and Methods:

Plasmids: The NES-GST-GFP-NLS plasmid was previously produced in the Haché lab. Oligonucleotides with compatible restriction sites on their termini were produced commercially (Invitrogen), annealed in coding and noncoding-strand pairs to produce double-stranded DNA sequences with XhoI sticky restriction site ends and ligated into NES-GST-GFP-NLS at the XhoI restriction site situated in the linker DNA sequence N-terminal of the GFP, as described below for linker tailing, to produce plasmids with GR hinge insertions 500-525, 525-500, GR alanine-scanning mutations RKT510-512AAA, LE507-508AA, IKG516-518AAA, and NLS plasmids IFNgR, LXR, and Rac1. The GFP-GR and GFP-GR_{NL1} plasmids were derived from those described in (Savory et al 1999) by digestion of the plasmid to remove GR N-terminal residues 20-153, and ligation of residues 1-153 in their place, originating from PCR amplification of 6RGR sequence, and the C500Y plasmid was produced by site-directed mutagenesis of the WT GFP-GR. The nSV40 and nNL1 plasmids were derived from the GFP-GR_{NL1} plasmid by the insertion of annealed commercial oligonucleotides, as described in linker tailing, into a unique XhoI site between GFP and GR sequence. The full-length LE-AA mutant, and the I516R and SV511-517 plasmids were derived from GFP-GR using PCR-based site-directed mutagenesis as described below. The SV506-523 plasmid was made in multiple steps. A BssHII site was introduced at GR residue 506 through site-directed mutagenesis. The sequence between this site and an existing PstI site at residue 523 was digested out, and replaced with SV40 large T antigen sequence of residues 122-139, including the SV40 NLS using linker tailing. In order to have a useful enzyme site for this ligation, the last two residues of the SV40 sequence
were altered. The final step was therefore PCR-based site-directed mutagenesis to return these to SV40 sequence and produce the final plasmid.

All plasmids made underwent restriction digestion and sequencing, in order to verify sequence, orientation, and frame of the mutants. The MMTV reporter has previously been used in our lab (Giffin W, 1994), as has the CMV reporter control, originally from Promega. The GRE luciferase reporter was ordered from Stratagene. Primer and oligonucleotide sequences used in this thesis can be found in Appendix A.

**Linker Tailing:** Used to insert a piece of DNA into a vector, either cut from another vector, or ordered as oligonucleotides for the top and bottom strand. If the latter, equal amounts (about 20 ug) of each oligo were mixed, and annealed to form double-stranded DNA, by heating the mixture to 80°C for 2 minutes, then cooling at 4°C. 10ug of the vector desired was digested with appropriate restriction enzymes for 1-2h at 37°C, then gel purified using Qiagen’s gel purifying kit. At this point, vector and insert were dephosphorylated and phosphorylated, respectively, if necessary to prevent religation of the vector, and repurified. Equal amounts of vector and insert (usually 1 ug) were mixed and ligated using T4 DNA ligase at 15°C overnight. Subsequently, the ligase was heat-inactivated, excess oligonucleotide/insert removed, and the ligated vector was precipitated, washed, and resuspended in TE buffer. Plasmid was electroporated into DH5α bacteria and amplified, then sequenced to verify the insertion.

**Site-directed Mutagenesis:** The PfuTurbo Quikchange protocol was used. Briefly, commercially produced complementary oligonucleotides containing the desired mutation in the middle of the sequence were used as primers for PCR amplification of desired expression
plasmids, using Pfu Turbo polymerase, and elongation cycles long enough to transcribe the entire plasmid, approximately 2 min/ kb. Template DNA was subsequently digested with DpnI, and the PCR product containing the mutation was gel purified and amplified, then sequenced to verify presence of the mutation.

_Tissue Culture:_ Cos 7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) from Gibco, supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, and plasmocin (Invivogen) used according to the manufacturer’s directions. Penicillin and streptomycin were added to cells plated for experiments (final concentration of 0.068 mg/mL penicillin and 0.134 mg/mL streptomycin). Cells were kept in 37°C humidified incubators at 5% CO₂. Cos 7 cells stably expressing the MMTV promoter had previously been produced in our lab, and were used for the ChIP assays described. These were grown with the addition of 250 ug/mL hygromycin (Roche) in order to select for cells expressing MMTV.

_Confocal Microscopy and FRAP/FLIP:_ Cos7 cells for live-cell confocal microscopy were plated on lysine-coated 40mm round coverslips in 60 mm round dishes, and grown in DMEM supplemented with 10% FBS, non-essential amino acids, and sodium pyruvate. On day 2, cells were transfected with 0.5 ug DNA for GFP-tagged constructs, using 8 uL Lipofectamine (Invitrogen) per dish for the transfection, following manufacturer’s recommendations. Transfection was stopped after 16 h, and the media changed to phenol-red free DMEM with 10% sFBS supplemented with non-essential amino acids and sodium pyruvate. 8 hours later, cells were rinsed twice with 1x PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂PO₄•7H₂O, 1.5 mM KH₂PO₄) and then cultured in serum-free media overnight to synchronize them in G₀. Prior to microscopy, cells were treated for 1-2 h with 1 uM
cortisol to induce nuclear localization of GR, and for 1h with cycloheximide at 20 ug/ml to prevent any de novo protein synthesis during experiments. During FRAP/FLIP, cells were maintained at 37°C in circulating phenol-red free media, containing cortisol and cycloheximide at the above concentrations, using the Bioptechs FCS2 chamber system. Microscopy was performed using a Bio-Rad MRC 1024 confocal microscope, and Bio-Rad LaserSharp software package, and using Zeiss LSM510 microscope and Pascal software package.

For FRAP assays, binucleate cells were selected, and one nucleus of the binucleate cell was photobleached using 100% laser power from an Ar laser for 5-10 passes. Each cell was photographed before and after photobleaching, and at regular intervals of 1-2 minutes for up to 2 hours afterwards, to monitor the transfer of fluorescence between nuclei (see figure 5a). The ImageJ program from NIH was used to quantify and compare the fluorescence of the bleached and unbleached nuclei over the course of the experiment after subtracting for background fluorescence, in order to determine nuclear export and re-import of fluorescent protein over time.

FRAP assays were performed on 2-3 cells per construct per experiment, N of at least 3 experiments per final graph. Standard deviation and SEM were calculated and ANOVA used to determine significance.

Localization, Withdrawal, and Hormone Response Assays: Cos7 cells were seeded onto 22 mm square poly-L-lysine coated coverslips. On day 2, cells were transfected with 0.3 ug DNA for GFP-tagged constructs and 2.7 uL Lipofectamine (Invitrogen) per coverslip. After 16 hours, transfection was stopped, and the media changed to phenol-red free media with 10% sFBS.
Localization and hormone response assays: On day 4, cells were treated with hormone as described in individual experiments. At the indicated timepoints post hormone treatment, coverslips with cells were rinsed with 1x PBS and fixed in 4% paraformaldehyde for 30 minutes at 4 °C, rinsed again twice with 1x PBS, and mounted on slides for viewing. DAPI-containing mounting medium (Vectashield, Vector Laboratories, Burlington, CA) was used to allow visualization of nuclei, and coverslip edges were sealed with nail polish. Slides were blinded before microscopy to avoid introducing a bias in the analysis. Cells were imaged on a Nikon TE300 fluorescence microscope and scored into 5 categories according to the localization within the cell of the GFP-labeled protein of interest. These categories are N (completely/almost completely nuclear), N>C (more nuclear than cytoplasmic), N=C (uniformly distributed fluorescence between nucleus and cytoplasm), C>N (more cytoplasmic than nuclear fluorescence), and C (very little or no fluorescence visible in nucleus). See (Figure 5b) for images of representative cells of each category. In analyses with only 3 categories, N>C also comprises N, and C>N includes C. 100-200 cells were scored per coverslip, and final graphs have an N of at least 3 experiments, with duplicate coverslips in each experiment.

Hormone withdrawal assays: Cells were treated with 1 uM hormone on day 4 for 1-4h as indicated, long enough to maximize nuclear localization of the transfected construct. Hormone withdrawal was initiated by washing cells for 5x 5 minutes with PBS containing 10% BSA (bovine serum albumin, tissue culture grade from Sigma-Aldrich), then 1x 5 minutes with serum-free, phenol-red free medium, and finally incubated in serum-free medium for the indicated withdrawal time. Fixing, mounting and scoring were performed in the same way as for the localization and hormone response assays.
Figure 5: FRAP assays and examples of fluorescence localization
A) Diagram of binucleate FRAP assay procedure. A binucleate cell expressing GFP-tagged protein of interest is selected (a) One nucleus is selectively photobleached (b), then the cell is monitored and photographed at interval for a given period. Movement of the non-photobleached GFP from the donor to the acceptor nucleus indicates transfer (c) through export and re-import of the receptor. B) Representative images of fluorescence localization in cells during hormone withdrawal assays, with N=nuclear and C=cytoplasmic localization.
A  Schematic of FRAP assays:

Select binucleate cell expressing GFP-GR, hormone treated if necessary.

Photobleach N2

Fluorescence transfer indicates export and re-import.

B  Localization categories for withdrawal assays:

N

N>C

N=C

C>N

C
Transcription Assays: Cos7 cells were seeded into 12-well dishes, and transfected the following day. Each well was transfected with 500 ng total DNA and 1.5 µL FuGene 6 (Roche) as per manufacturer’s directions. Triplicate wells were transfected for each condition in each experiment. The 500 ng DNA was made up of 250 ng luciferase-tagged reporter construct (MMTV-luc or GRE-luc), 25 ng CMV-renilla luciferase internal control, between 6 ng and 50 ng plasmid expressing GR wild type or mutant DNA, and sufficient stuffer DNA (parent plasmid GFP-C1, originally from Clontech) to complete the 500 ng total. 16 h post-transfection (day 3), media was changed, and on day 4 cells were treated with hormone as indicated in individual experiments and harvested. Lysis and analysis were performed using the Promega dual-luciferase kit components. 150 µL of lysis buffer was used per well, with 20 minutes lysis at room temperature. Analysis of luciferase levels was performed using 20 µL of this lysate in a Lumistar luminometer. The internal control readout (CMV-renilla luc) was used to normalize values for the reporter. Values for triplicate wells were averaged, and the fold increase in transcription in the presence versus the absence of hormone was calculated. ANOVA was used to determine significant differences between constructs. A minimum of three experiments were performed for each condition.

Western Blotting: For Western blotting to verify protein expression and size, cells were seeded in 60mm dishes, and transfected on day 2 with 0.5 µg DNA and 8µL Lipofectamine following the manufacturer’s protocol. Transfection was stopped and media changed to 10% sFBS-containing phenol-red free DMEM after 16 h. On day 4, cells were treated with hormone if needed, scraped and harvested in 1 mL 1x PBS, then pelleted by centrifugation. Cell pellets were lysed in lysis buffer (150 mM NaCl, 50 mM Tris pH7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol, protease inhibitor (Roche) and 0.5% NP-
40) for 30 minutes on ice, centrifuged on high for 15 minutes to remove cell debris, and the supernatant was collected. Bradford assay was used to determine protein concentration. Equal amounts of protein (generally 50 ug) were loaded onto SDS-PAGE gel and separated by electrophoresis in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), then transferred to PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 10% v/v methanol) and blocked with PBS-T (1X PBS with 0.05% Tween-20) containing 5% skim milk powder. Primary antibodies used were: FiGR monoclonal (in house-produced antibody to the BuGR epitope of GR), GFP JL-8 monoclonal (Living Colors, BD Bioscience). HRP-tagged secondary antibody and Western Lightning™ ECL reagents were used to amplify and visualize the signal on photographic film.

**Chromatin Immunoprecipitation (ChIP) Assays:** Cos7 cells stably transfected with the MMTV promoter were used to study GR interaction with the MMTV GRE. Cells were seeded onto 10cm plates on day 1 and transfected the next day with 1.5 ug of wild-type or mutant GR, using 24 uL Lipofectamine, following the manufacturer’s protocol. Media was replaced after 16 h with phenol-red-free DMEM containing 10% sFBS, supplemented with non-essential amino acids, penicillin and streptomycin. The next day, cells were treated +/- 10 uM dexamethasone for indicated times. To harvest, cells were rinsed twice with serum-free media and scraped into 15 mL tubes in a total of 5 mL media per sample. Formaldehyde was added to a final concentration of 1% and incubated 10 minutes at room temperature to cross-link proteins to DNA. The formaldehyde was removed with 2 rinses with 1x PBS, and nuclei were isolated using sequential incubation with Buffer I (0.25% Triton-X 100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5) and Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5). The nuclei were lysed and DNA sheared via
sonication in Sonication Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl pH 8.1, 1 mM DTT, 20 mM sodium molybdate and 1x protease inhibitor). Centrifugation was used to separate lysate with desired elements from cell debris, and the lysate was precleared by incubation with protein A sepharose beads in Dilution Buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 1 mM DTT, 20 mM sodium molybdate, and 1x protease inhibitor). 10% input samples were taken and their crosslinking reversed by overnight incubation at 65°C in Extraction Buffer (1% SDS, 0.1 M NaHCO₃). The remainder of the lysate had the protein of interest immunoprecipitated out overnight using polyclonal antibody (polyclonal GR M-20, Santa Cruz) and collected using protein A Sepharose beads. Beads were rinsed in buffers TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), TSE II (As for TSE I but with 500 mM NaCl), B III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and three times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The immunoprecipitated DNA-protein complexes were extracted from the beads using buffer E (1% SDS, 0.1 M NaHCO₃) and the crosslinking reversed by overnight incubation at 65°C. The DNA fragments in input and IP samples were then isolated using a PCR purification kit (Qiagen), and amplified by PCR of the MMTV promoter sequence, then subsequently separated and imaged by agarose gel electrophoresis with ethidium bromide to determine whether GR or other protein of interest was interacting with the MMTV promoter.
Results:

Specific residues in the isolated GR hinge region influence the rate of export of a shuttling protein.

Initial work on GR nuclear retention in the Haché lab showed that nuclear retention activity was present in a 26-residue fragment of the hinge region, and that it overlapped significantly with the GR NL1 lysine core at K513-515 (Walther RF, PhD thesis, University of Ottawa, 2003, Carrigan 2007). However, mutation of these residues impacted NL1 activity as well as GR nuclear retention, making it impossible to examine the relative contribution of each activity to GR function. Therefore, my first task was to determine whether GR NL1 activity and retention activity could be separated within the hinge region, in order to produce a receptor which lacked nuclear retention without significantly compromising its nuclear localization function. In order to accomplish this, I made a series of alanine-scanning mutations within the GR hinge region, and took advantage of a system previously developed in our lab (Walther 2003, figure 5A in Materials and Methods) to study the effect of these mutations on GR nuclear retention. This involves FRAP assays, performed as described in the Materials and Methods section, using live, binucleated cells, which have been transfected with the NES-GST-GFP-NLS expression plasmid. The product of this plasmid is a 55kDa protein, too large to diffuse through the nuclear pore without active transport. It can be visually tracked due to its GFP moiety, and contains a GST portion which serves to add bulk and can be used as an epitope for immunoprecipitation and Western blotting. The Rev NES is a short, leucine rich-sequence which allows the protein to export continuously. The SV40 NLS sequence, similarly, is a strong basic NLS, which
allows the protein to import continuously. Together, the two nuclear transport sequences allow the protein product to shuttle continuously between the nucleus and cytoplasm, although the net localization is nuclear (see diagram, figure 6a). This makes it ideal for the binucleate FRAP assay which our lab uses to study protein trafficking. A cloning site situated between the GST and GFP moieties was used to clone in the protein or sequence of interest. The previous studies described above showed that cloning a 26-residue fragment of the WT GR hinge into this site decreased the rate of transfer of the protein product between nuclei of binucleated cells in FRAP assays, whereas the use of the NL1- GR hinge did not affect the shuttling rate of the protein. The alanine-scanning mutations were made within the hinge region of GR in the context of this plasmid, in order to further define residues which might be involved in retention. The initial mutation made in the hinge region was in the NL1 core at K513-515N, therefore I made my mutations on either side of this site, to produce mutants LE507-509AA, RKT510-512AAA, and IKG516-518AAA (Figure 6b). These mutants were then tested for the presence of nuclear retention activity in FRAP assays. Controls were constructs using the same backbone, with the DNA sequence for the wild-type hinge inserted in forward (500-525) and reverse (525-500) directions, to give WT GR or a reverse, non-specific peptide sequence, respectively. These controls were previously used and verified in our lab, showing that the addition of the 500-525 hinge fragment increased the nuclear retention of the construct, whereas the inverted oligonucleotide did not (Walther RF Ph.D. Thesis, University of Ottawa, 2003, Carrigan 2007). As these mutants do not contain the LBD of GR, required for hormone response, and are constitutively nuclear due to the presence of the SV40 NLS, they do not require treatment with hormone prior to FRAP. Cycloheximide was added 1h prior to FRAP, in order to prevent de novo protein synthesis.
Figure 6: Alanine-scanning mutations made in the NLS-GST-GRhinge-GFP-NES construct.
A) Modular diagram of the NLS-GST-hinge-GFP-NES shuttling construct, with the amino acid sequence of the GR hinge insertion expanded below. Yellow rectangle indicates NL1 sequence, underlined residues in blue represent the location of the original NL1- (K513-515N) mutation, and underlined clusters of residues in red show the locations of alanine-scanning mutations tested in this thesis. B) Nucleotide sequence of the various GR hinge controls and mutants used, with amino acid sequence below. Alanine-scanning mutations marked in red. The inverted oligo control is essentially the reverse complementary strand of the WT.
B

Nucleotide sequences of hinge mutants:

WT GR
(500-525) 5’ TC GAG TGT CTT CAG GCT GGA ATG AAC CT T G A GA C G A AAA ACA
C L Q A G M N L E A R K T
AAG AAA AAA ATC AAA GGG ATT CAG CAA GCC ACT GCA GGA G 3’
K K K I K G I Q Q A T A G

Scrambled
(525-500) 5’ TC GAG TCC TGC AGT GGC TTG CTG AAT CCC TTT GAT TTT TTT CTT
SC S G LL LN PF DF FL
TGT TTT TCG TGC TTC AAG GTT CAT TCC AGC CTG AAG ACA G 3’
C F S C F K V H S S L K T

LE mut
(507-508 LE-AA) 5’ TC GAG TGT CTT CAG GCT GGA ATG AAC GCA CGA AAA ACA
C L Q A G M N A A K R K T
AAG AAA AAA ATC AAA GGG ATT CAG CAA GCC ACT GCA GGA G 3’
K K K I K G I Q Q A T A G

RKT mut
(510-512 RKT-AAA) 5’ TC GAG TGT CTT CAG GCT GGA ATG AAC CT T G A GA C G A GCG GCC
C L Q A G M N L E A AAA
GCT AAG AAA AAA ATC AAA GGG ATT CAG CAA GCC ACT GCA GGA G 3’
A K K K I K G I Q Q A T A G

IKG mut
(516-518 IKG-AAA) 5’ TC GAG TGT CTT CAG GCT GGA ATG AAC CT T G A GA C G A GCG GCC
C L Q A G M N L E A R K
ACA AAG AAA AAA GCC GCG GCA ATT CAG CAA GCC ACT GCA GGA G 3’
T K K K A A A I Q Q A T A G
during the time of the assay, which could have the potential to confuse the results. The constructs were expressed in Cos7 cells, and FRAP was performed using a confocal microscope as described in Materials and Methods. Each cell was tracked over 30 minutes post-FRAP, for 2-3 cells per construct per experiment, with experiments performed at minimum in triplicate. Neither the IKG516-518AAA mutation (32.4 +/- 4.0% recovery) nor the RKT510-512AAA (47.2 +/- 10.3% recovery) mutation had any significant effect on the export rate of the protein compared to the 37.5 +/- 8.8% recovery for WT hinge 500-525 after 30 minutes, although the RKT510-512 mutant appeared to have an increased rate of transfer between nuclei. However, the LE507-509AA (46.2 +/- 5.5% recovery) produced a protein which showed more transfer between nuclei than the wild-type hinge after 30 minutes, at a rate similar to that of the reverse DNA control sequence (45.3 +/- 4.1% recovery for inverted control 525-500) (Fig 7).

Analysis of the initial rates of transfer, over the first 5 minutes post-photobleaching was also performed, in order to verify that the final results were consistent with the initial transfer rates, and that incomplete or inconsistent photobleaching were not affecting the results. These initial rates showed that the LE507-509AA mutant (4.01 +/- 0.43 % return per minute) had a phenotype which was not significantly different from the inverted hinge control (3.83 +/- 0.52 % return per minute). The RKT-AAA mutation had a lower initial return rate of 2.46 +/- 0.25% per minute, close to that of the wild-type hinge (2.66 +/- 0.31 % per minute), and the IKG-AAA mutation was even slower than the wild-type, at 1.91 +/- 0.05% return per minute (fig. 8).

The LE-AA mutation therefore showed a phenotype similar to the retention-deficient control both in the initial rate of return post-FRAP and the degree of transfer between nuclei
Figure 7: Mutation of residues 507-08 or 510-512 in the GR hinge sequence can affect the rate of export of shuttling protein NLS-GST-hinge-GFP-NES.

A) Representative images of FRAP results of alanine-scanning mutations, pre-bleach, immediately post-bleach, and 30 minutes post bleach. Controls are WT and inverted hinge oligonucleotide sequence, as described in figure 6. B) Graph of fluorescence return to the bleached (acceptor) nucleus over time, expressed as a percentage of the total fluorescence in the two nuclei. Analysis of significance (p<0.05) using ANOVA shows that at 30 min post-bleach, the LE mutation is different from all constructs except the inverted oligo control, while the RKT mutation is not significantly different from the WT hinge.
A

(inverted oligo)

WT  Control  LE-AA  RKT-AAA  IKG-AAA

Pre-bleach

Bleach

t = 30min

B

% flou, acceptor nucleus

wt hinge  inverted oligo  507-08 LE-AA  510-12 RKT-AAA  516-18 IKG-AAA

time (min) post-FRAP

52
Figure 8: Initial rates of return of shuttling constructs can also be affected by mutations in the GR hinge region.

Graph of the average percentage fluorescence increase per minute in the acceptor nucleus over the first 5 minutes post-bleach. Matching symbol pairs are not significantly different from each other, but are different from other symbols with a p<0.05 using ANOVA.
after 30 min post-FRAP. Due to this, and to the fact that, unlike the RKT sequence, the LE sequence does not overlap with the basic residues of the NL1, and thus would seem less likely to affect the NL1 activity, the LE-AA mutation was selected as a candidate for decreasing retention in the context of the full-length GR receptor.

The isolated hinge region fails to predict the retention behaviour of the full–length GR.

In order to evaluate the relevance of the LE 507-508 residues on the nuclear retention of the full-length GR protein, this mutation was made in the context of a GFP-tagged full-length GR (fig. 9a), using site-directed mutagenesis. Cells were treated with 1uM cortisol, in addition to cycloheximide, for 1hour prior to microscopy. FRAP assays were performed as described in Materials and Methods, and constructs were monitored for 1h post FRAP. The GFP-tagged WT receptor and the GFP-NL1- mutant, which was previously determined to lack retention, and which showed an N=C distribution in the presence of hormone (figure 4), were used, respectively, as positive and negative controls for nuclear retention. As previously observed, the NL1- mutation (K513-515N) showed a pronounced decrease in nuclear retention, with a recovery of 23 +/-3% total fluorescence in the bleached nucleus after one hour post-FRAP versus a recovery of  8 +/- 2% for the wild-type receptor. However, the full-length GR with the LE-AA mutation failed to show a significant decrease in nuclear retention relative to the wild-type receptor, having a recovery of only 10 +/- 1% at 1 hour post-FRAP (fig. 9 b,c).

In order to assess the behaviour of the LE-AA mutation in the absence of hormone, with respect to the WT receptor, the rate of return of receptor to the cytoplasm following
hormone withdrawal was measured. In these assays, cells containing the receptor were treated with hormone for a minimum of 1h, in order to translocate most or all of the receptor into the nucleus. Hormone was then withdrawn, and the localization of the receptor was observed at several time points over a 24 hour period. Previous work had shown that the NL1- receptor could be observed to export much more rapidly than the wild-type in these assays, as well as in FRAP assays, suggesting that the effect of this mutation on GR nuclear retention and export was not dependent on the presence of hormone (Savory 1999). When the LE-AA mutant was examined in withdrawal assays, it did not export any more rapidly than the wild-type receptor, and indeed was almost slower than the wild-type receptor at exporting. The percentage of cells containing the WT receptor in a mostly nuclear (N+N>C) localization dropped from 90 +/- 8% to 22 +/-8% of cells over 24h withdrawal, and in the same period, the LE-AA receptor dropped from being mostly nuclear in 94 +/--2% of cells initially to 37 +/-- 12% of cells after 24h of hormone withdrawal.(fig 9d). This seems to indicate that any role the LE507-508 residues play in GR nuclear retention may be a minor or adjunct one. Although there is still the possibility that they may play a role in nuclear retention in conjunction with other residues, alone, they do not show any significant effect on the nuclear retention of the full-length GR receptor.

**GR hinge alone does not completely reconstitute retention:**

The LE-AA mutation, while sufficient to disrupt nuclear retention in the context of the hinge fragment, was not sufficient to influence retention in the full-length GR. Two possibilities therefore suggest themselves. First, mutation of the LE may not be sufficient by
Figure 9: The LE507-508AA mutant is not sufficient to affect nuclear retention in the context of the FL GR receptor.
A) Schematic of the FL receptor construct containing the LE-AA mutation. B,C) Graph and representative images of FRAP results comparing export of the LE-AA mutant to WT and NL1- GFP-GR in the presence of agonist hormone. D) Graph of receptor nuclear localization over time following agonist withdrawal, comparing export of the LE-AA mutant to WT and NL1- GFP-GR.
A. GFP-GR (LE-AA)  

B. Before bleach, bleach, t=2h images for WT GR, NL1-GR, LEAmut GR.

C. Graph showing % nuclear fluorescence over time (min) for GR WT, NL1-GR K513-515N, GR 507-08 LE-AA.

D. Graph showing % cells with N+N>C localization over time post hormone-withdrawal (1uM cortisol) for WT, NL1-, LE507-508 AA.
itself to produce a noticeable decrease in retention of the full-length receptor, and so a larger mutation within the hinge would be necessary to see an effect. Second, retention activity may also be present in regions of the full-length GR outside of the hinge region, paralleling the dual NLS sequences found in GR. I turned to the SV40 NLS in order to produce glucocorticoid receptor mutants that were compromised for nuclear retention but which were able to localize to the nucleus like the WT GR, in order to distinguish between the two possibilities.

The SV40 large T antigen NLS sequence was used in the NES-GST-hinge-GFP-NLS construct. It is a basic NLS, like that of GR, having the sequence PKKKRKV. Although the SV40 NLS contains a triple lysine cluster like that of the GR NL1 which was shown to affect retention of GR, the SV40 sequence does not appear to cause nuclear retention of the control construct. The 7-amino acid sequence of the SV40 NLS, shown above, was cloned into the GFP-tagged NL1- GR, at the N-terminus of the GR, to produce the nSV40 construct (fig 10a). It was anticipated that this would produce a receptor which was localized to the nucleus due to an exposed NLS, but would not have increased nuclear retention when compared to the NL1- GR. This construct was determined to be localized to the cytoplasm prior to hormone treatment, in a similar manner to the NL1- receptor, but to import more like WT GR in response to hormone, although not quite as rapidly or as completely (see fig 20). This implied that the N-terminal NLS, contrary to expectations, was not exposed prior to hormone binding, but was capable of mediating the nuclear import of liganded GR.

In order to determine whether the addition of the N-terminal SV40 NLS had increased the nuclear retention of the liganded or ligand-withdrawn receptor, FRAP and hormone-withdrawal assays were performed as described above. In FRAP assays, the nSV40 mutant behaved similarly to the parent NL1- receptor, with the percentage of total
Figure 10: Addition of an N-terminal NLS does not fully restore retention to NL1-GR in the presence of agonist.
A) Diagrams of FL GFP-GR constructs used showing sequences of inserted NLS signals at N-terminus of NL1-GR. B) FRAP assays show that addition of the SV40 NLS does not affect the export rate of the NL1-GR, but addition of GR NL1 sequence gives partial restoration of nuclear retention.
A

GFP-GR (WT)

GFP-GR<sub>NL1</sub>- (NL1-)

GFP-SV40-GR<sub>NL1</sub>- (nSV40)

GFP-nNL1-GR<sub>NL1</sub>- (nNL1-)

B

% fluorescence, acceptor nucleus

0 5 10 15 20 25 30

0 1 2 3 4 5 6 7

time (min)

nSV40
NL1-
WT
nNL1
fluorescence in the bleached nucleus 1h post-FRAP of 25.1 +/- 1.9% for the nSV40 versus 21.0 +/- 3.8% for the NL1- (fig 10b). The slight increase in transfer rate may be due to more rapid re-import in the presence of the strong SV40 NLS. The hormone withdrawn nSV40 receptor also showed a strong lack of retention. While it was significantly more nuclear than the NL1- receptor directly after hormone withdrawal, it exported rapidly, showing 19% nuclear cells at 8 h post-withdrawal, compared to 8% nuclear cells for the NL1- and 78% for the WT receptor (fig. 11). Therefore, the N-terminal addition of the SV40 NLS to the NL1-GR did not increase the nuclear retention of the receptor.

To determine whether the lack of nuclear retention observed in the nSV40 construct was due to the position of the NLS at the N-terminus of the receptor or whether it was influenced by the sequence of the NLS, a sister construct to the nSV40 was produced. This one placed a GR NL1 fragment (RKTKKKIKG) at the N-terminus of the NL1- GR in place of the SV40 sequence in the nSV40 construct (fig 10a). This new mutant was named N-terminal NL1, or nNL1, and its localization and behaviour in FRAP and withdrawal assays were determined as for the nSV40. In the absence of hormone, the nNL1 had a mostly cytoplasmic localization, similar to the nSV40, but translocated to the nucleus somewhat more readily and completely than the nSV40, as determined by localization after 1 h treatment with different concentrations of hormone (see figure 20b). FRAP results showed that the liganded nNL1 construct possessed an intermediate degree of nuclear retention, having a recovery of 15.6 +/- 0.8% total fluorescence in the acceptor (bleached) nucleus at 1h post-FRAP, significantly different from both the 21.0 +/- 3.8% recovery for the NL1- and 10 +/- 6% for the WT (fig. 10b). This indicates that the sequence of the GR NL1 possesses intrinsic nuclear retention activity which is not present in the SV40 NLS sequence.
Figure 11: Addition of N-terminal NLS does not fully restore retention to NL1-GR in the agonist-withdrawn receptor.

Graph of the nuclear localization of GFP-tagged constructs described in figure 10 over time post agonist withdrawal shows that, similarly to FRAP assays, the addition of the SV40 NLS does not affect export rate of NL1-GR, but addition of GR NL1 sequence gives partial restoration of nuclear retention.
% cells with N+N>C localization

WT
NL1-
nSV40
nNL1

0 h 4 h 8 h 24 h
time post hormone-withdrawal, 4h 1uM cortisol
However, the NL1 sequence per se was not able to completely recapitulate the degree of retention seen in the WT receptor. An intermediate retention phenotype was observed for the ligand-withdrawn nNL1 receptor as well, as the localization of the receptor at 8h post-withdrawal was nuclear in approximately 49% of cells, significantly different from both the 19% of the cells with nSV40 nuclear fluorescence and 78% of those with WT GR nuclear fluorescence (fig. 11). Thus it seems that while the NL1 sequence of GR possesses retention activity, the location of the NLS within the protein may also affect the degree of nuclear retention of the receptor. It could therefore be hypothesized that the substitution of SV40 sequence for that of GR within the endogenous context of the hinge region should produce a receptor which is deficient in nuclear retention.

**Sequence surrounding K513-515 of GR is involved in nuclear retention:**

In order to test this idea, I made a series of three mutants within the hinge region of the WT GFP-GR, which gave the NL1 and surrounding sequence an increasingly SV40-like character. Both the GR and SV40 sequences contain a KKK_K sequence within the core NLS. This was not altered, and was used as the basis for aligning the sequences. The first mutant developed had a point mutation at I516, which changed the KKK_I of GR to the KKK_R of SV40. The second mutation was just N-terminal of the 3-lysine cluster, altering the GR sequence KT_KKK to SV40 sequence PP_KKK, in addition to the I516R mutation, to create the SV511-517 mutant. This completed the change of the GR NL1 to that of the SV40, and corresponded in extent to the fragments used in the nSV40 and RKT-AAA mutants previously described. As both of the last-named constructs showed decreased nuclear retention relative to their WT counterparts, it was likely that the SV511-517 would
show some effect on nuclear retention. The third and most extensive mutation altered all the residues in GR between 506 and 523 to the comparable SV40 sequence, to give SV506-523, with the aim of determining if residues within the hinge region but outside of the NL1 basic sequence played a significant role in nuclear retention of the FL GR (fig 12a). These three mutants were characterized with respect to their nuclear localization abilities, and their retention and export behaviour in FRAP and withdrawal assays, as had been done previously for the nSV40 and nNL1 mutants.

The I516R point mutant had a very minor effect on the behaviour of the GR receptor. The initial localization of the mutant receptor was very similar to that of the WT GR, and it translocated well to the nucleus, although not quite as efficiently as the WT receptor (Fig. 20). In FRAP assays, the liganded I516R did not show any loss in nuclear retention relative to WT GR. In fact, although the difference was not significant, it almost seemed that the I516R was slower to transfer between nuclei than the WT receptor, with a recovery of only 7.9% +/- 2.2% after 1h for the I516R as compared to 10% +/- 6% for the WT (fig. 13a). In hormone withdrawal assays, the ligand-withdrawn I516R did not export much faster than the WT receptor, although by 24 hours post-withdrawal, there is more of a difference between the two, with the WT having 68% of cells mostly nuclear, where the I516R was mostly localized to the nucleus in only 41% of cells (fig. 13b).

Localization assays of the SV511-517 mutant determined that it behaved in a manner similar to that of WT GR with respect to naïve distribution and its ability to transfer to the nucleus following hormone treatment (fig. 12b). FRAP assays, however, showed that the liganded SV511-517 exhibited significantly weaker nuclear retention than the WT GR, at a level similar to that of the nNL1 construct, but still stronger than the NL1- mutant. At 1h post-FRAP, the SV511-517 had a recovery of 15.4 +/- 1.5% fluorescence in the bleached
Figure 12: SV40 substitution mutants of GR respond rapidly to hormone treatment:
A) Modular diagrams of receptor mutants in order of increasing SV40 character at the NL1/hinge region. Expanded below is the aligned sequence of GR and SV40 NLSs, with the furthest extent of replacement highlighted in teal, and the lysines used to align sequence are boxed in red. B) Percentage of total cells transfected with SV40 substitution mutants having mostly nuclear localization before and after treatment with 1uM dexamethasone for 1h.
A

GFP-GR\textsuperscript{I516R} (I516R)

\begin{center}
\begin{tikzpicture}
  \node (A) {GFP};
  \node [right of=A, xshift=1cm] (B) {GR};
  \node [right of=B, xshift=1cm] (C) {795};
  \node [above of=A, yshift=-0.5cm] (D) {1};
  \node [above of=B, yshift=-0.5cm] (E) {1};
  \draw[->] (A) -- (B);
  \draw[->] (B) -- (C);
  \draw[->] (D) -- (E);
  \draw[->] (A) -- (B) node [midway, above] {I516R};
\end{tikzpicture}
\end{center}

GFP-GR\textsubscript{SV127-133} (SV511-517)

\begin{center}
\begin{tikzpicture}
  \node (A) {GFP};
  \node [right of=A, xshift=1cm] (B) {GR};
  \node [right of=B, xshift=1cm] (C) {795};
  \node [above of=A, yshift=-0.5cm] (D) {1};
  \node [above of=B, yshift=-0.5cm] (E) {1};
  \draw[->] (A) -- (B);
  \draw[->] (B) -- (C);
  \draw[->] (D) -- (E);
  \draw[->] (A) -- (B) node [midway, above] {I516R};
  \node [above of=B, yshift=0.5cm] {GR 511-517 -> SV40 127-133, NLS in-line};
\end{tikzpicture}
\end{center}

GFP-GR\textsubscript{SV506-523} (SV506-523)

\begin{center}
\begin{tikzpicture}
  \node (A) {GFP};
  \node [right of=A, xshift=1cm] (B) {GR};
  \node [right of=B, xshift=1cm] (C) {795};
  \node [above of=A, yshift=-0.5cm] (D) {1};
  \node [above of=B, yshift=-0.5cm] (E) {1};
  \draw[->] (A) -- (B);
  \draw[->] (B) -- (C);
  \draw[->] (D) -- (E);
  \draw[->] (A) -- (B) node [midway, above] {I516R};
  \node [above of=B, yshift=0.5cm] {GR 506-523 -> SV40 122-139, NLS in-line};
\end{tikzpicture}
\end{center}

WT GR 500 CLQAGMNLEARKTKKIKGIQQATAG 525

SV40 seq, in line CLQAGMR\textcolor{red}{AHSTPPKKK}RKVEDPKDAG

B

\begin{center}
\begin{tikzpicture}
  \draw[->] (0,0) -- (0,6);
  \draw[->] (0,0) -- (6,0);
  \node at (0,0) {0};
  \node at (0,2) {20};
  \node at (0,4) {40};
  \node at (0,6) {60};
  \node at (2,0) {80};
  \node at (4,0) {100};
  \node at (6,0) {120};
  \node at (0,6) {\% total cells N+C};
  \node at (6,0) {WT};
  \node at (3,0) {SV511-517};
  \node at (4.5,0) {SV506-523};
  \node at (6.5,0) {I516R};
  \draw[blue, fill=blue] (0,3) rectangle (1,4);
  \node at (0.5,3.5) {no H};
  \draw[red, fill=red] (1,3) rectangle (2,4);
  \node at (1.5,3.5) {1h 1uM dexam};
  \draw[blue, fill=blue] (2,3) rectangle (3,4);
  \node at (2.5,3.5) {no H};
  \draw[red, fill=red] (3,3) rectangle (4,4);
  \node at (3.5,3.5) {1h 1uM dexam};
\end{tikzpicture}
\end{center}
nucleus, compared to 21.0 +/- 3.8% for the NL1- and 10 +/- 6% for the WT GR (fig 13a, compare to fig 10b for nNL1). An intermediate phenotype was also observed for the SV511-517 mutant in hormone withdrawal assays. The SV511-517 mutant exported more rapidly than the WT GR, with 44% of the SV511-517-expressing cells exhibiting mostly nuclear receptor 24h post-withdrawal, versus 68% of WT-expressing cells (fig. 13b), a rate that was again similar to that of the nNL1 construct.

The presence of the NL1 at the N-terminus was not sufficient to induce a complete reconstitution of the retention activity lost in the NL1- mutation, and the SV511-517 substitution did not produce as significant a loss of retention as was observed in the presence of the same sequence (SV40 NLS) at the N-terminus of the GR NL1-protein. These observations suggested that sequence besides that altered in these constructs (GR 511-517) might play an auxiliary role in GR nuclear retention. To determine whether any further residues in the hinge region under study (GR 500-525) had an additional effect on GR nuclear retention, the SV506-523 mutant was created. Here, all residues between the GR DNA-binding region (up to residue 505) and the end of the original hinge fragment were substituted by SV40 sequence (fig. 12a). Interestingly, this construct was found to have a high degree of nuclear localization (72% of cells having mostly nuclear receptor) even in the absence of hormone, possibly due to an alteration in hinge conformation leading to a constant exposure of the NLS, or increased interaction with importin. The cytoplasmic fraction of the receptor did import rapidly and completely upon hormone treatment, to a degree equal to that of the WT GR (fig 12b). The constitutive nuclear localization of the majority of the receptor meant that hormone withdrawal assays were not capable of yielding much information about the export behaviour of this mutant, although an alteration in its localization from 97% to 88% of cells exhibiting mostly nuclear receptor occurred by 24h
Figure 13: Replacement of GR NL1 with SV40 NLS decreases nuclear retention of liganded or ligand-withdrawn GR.
A) Binucleate FRAP assays of SV40 substitution mutants, with WT and NL1- GR as controls for comparison. The two larger mutations show decreased retention relative to WT GR, but not as strongly as NL1-. B) Graph of the average nuclear localization of cells transfected with SV40 substitution mutants over time following agonist withdrawal, with WT and NL1- GR as controls. The SV506-523 mutant is largely nuclear in the absence of hormone.
post-withdrawal (fig 13b), suggesting that the nuclear export of the fraction which could export was enhanced over that of the WT GR.

FRAP assays yielded more useful results, showing that the SV506-523 mutant had a recovery level of 14.1 +/- 1.9% at 1 hour post-bleaching, which was not significantly different from that of the smaller SV511-517 mutation (fig. 13a). A slow shuttling movement is thought to underlie GR’s net localization (Walther 2003), and from these results it seems that this shuttling could explain why the constitutively nuclear mutant nevertheless exhibits some transfer between nuclei. The fact that the SV506-523 and SV511-517 show a similar level of recovery post-FRAP seems to suggest that the hinge residues outside of the 511-517 sequence do not play a significant role in GR nuclear retention.

Further, comparison of the FRAP results of the I516R, SV511-517, and SV506-523 mutants shows that, with the exception of the KKK513-515 previously identified, mutation of the KT511-512 residues seem to have the greatest influence on nuclear retention of the FL GR. There is still the question of what role the location of the NLS within the protein plays, and whether residues outside of the hinge contribute to retention and relate to these results.

To verify that the FRAP results were reflective of the rate of transfer between nuclei of the GFP-tagged GR mutants without undue influence from incomplete or inconsistent photobleaching, the initial slopes of the FRAP curves obtained (over the first 6 minutes post-bleach) were also measured. As expected, the WT and I516R had the lowest slopes, confirming their slow rate of transfer (0.38 and 0.32% average rate of recovery/min, respectively), while the NL1- (0.65 % per min.) and its derivative constructs nSV40 (0.90% per min.) and nNL1- (0.74% per min.) had slopes reflecting their more rapid transfer and lower degree of nuclear retention. Interestingly, the N-terminal NL1- construct, although it
Figure 14: Initial rates of return of full-length GR retention mutants in FRAP.
The average rate of return for each mutant was calculated for the first 6 min post-bleaching, based on the slopes of the FRAP curves presented in figure 13. WT GR is significantly different (p<0.05 using ANOVA) than all mutants, with the exception of SV506-523. There are no significant differences between NL1-, nSV40, and nNL1 and SV511-517.
WT NL1- nSV40 nNL1 I516R SV511-517 SV506-523

slope per minute

0-6 min
showed a decreased transfer in the FRAP assays relative to the nSV40 and NL1- constructs, had an initial slope which was not significantly different from the other NL1- constructs, and the SV511-517 construct (0.86% per min.), which also had an intermediate phenotype in the FRAP (figure 14). This discrepancy may suggest that the export rate of the intermediate nNL1 and SV511-517 constructs are similar to the nSV40 and NL1-, but a smaller proportion of the nNL1 and SV511-517 mutants may be competent for export due to increased interaction with nuclear factors.

**Antagonist treatment increases nuclear retention of receptor mutants:**

The nature of the ligand present has been shown to have significant effects on the activity of hormone receptors such as GR. For example, the alteration in the configuration of the ligand-binding domain caused by the binding of antagonist rather than agonist ligands results in the recruitment of corepressor rather than coactivator factors, which may exert their repressive effects through masking or alteration of signal sequences, competition for binding sites, or changes in modifications and configuration of receptor domains. Previous work in our lab had shown that treatment of GR or of the mineralocorticoid receptor (MR) with antagonist decreased the export of the receptor following ligand withdrawal, although subsequent treatment and withdrawal with agonist restored the receptor’s export ability (Walther 2005, Haché 1999). This suggested ligand-specific effects on receptor behaviour that persisted even after removal of hormone. To determine whether the effect of antagonist on export was related to or dependent on the nuclear retention activity, it was of interest to determine whether receptor mutants with decreased nuclear retention were also susceptible to antagonist-induced export blockage.
To this end, the effect of treatment with the antagonist RU486 rather than agonist on the post-withdrawal export of the receptor was tested for a subset of the GR mutants, and withdrawal assays were performed, as described earlier. The WT GR and SV506-523 mutant did not show an alteration in their ability to import to the nucleus, although the NL1- and nSV40 constructs did not localize as completely to the nucleus in the presence of antagonist as was observed with agonist. As the NL1- in the presence of antagonist did not import to a level of greater than N=C, both the N>C and N=C are shown for the NL1- in order to demonstrate that its export was also affected in the presence of RU486. Both the WT GR and the GR retention-deficient constructs tested showed similarly low levels of export after antagonist withdrawal, notwithstanding their varied export rates in the presence of agonist (fig. 15). This suggests that whatever mechanism is responsible for promoting GR nuclear retention in the presence of RU486, it is not affected by mutations that increase nuclear export of agonist-bound or –withdrawn GR.

**GR Retention Mutants Exhibit Decreased Activation at the MMTV Promoter:**

GR functions to directly or indirectly mediate the activity of genes involved in inflammation, appetite, mood, and stress response, among others, and in some cell types, over 20% of the genome may be influenced by GR (Derijk 2008, Stahn 2008, Chrousos 2007, Rose 2010, Nader 2009). It is therefore possible that alterations in GR retention, despite the net nuclear localization observed in the liganded retention mutants, could influence GR transcriptional activity through mechanisms such as cofactor binding and post-translational modification of the receptor. This makes an investigation of GR transcriptional
Figure 15: Comparison of localization of selected constructs after agonist versus antagonist withdrawal.
A) Localization over time after withdrawal of 1uM cortisol agonist. B) Localization over time after withdrawal of 1uM RU486 antagonist. For both graphs, as the NL1- control for retention deficiency does not become nuclear to the same extent as the other constructs, the percentage of cells in both the mostly nuclear (N+N>C) and N=C localizations are shown for comparative purposes.
activation a logical next step in determining what role retention might play with respect to GR function.

Historically, most studies looking at GR activity have utilized the mouse mammary tumor virus LTR promoter, a glucocorticoid-inducible promoter which contains a one whole and three half glucocorticoid/hormone response element sites for steroid receptor binding, as well as binding sites for the nuclear factor 1 (NF-1) and Octamer (Oct) transcription factors (fig. 16a). A high level (c. 100-fold) of transcriptional activation by GR can be achieved on this promoter under appropriate conditions. In conjunction with a luciferase reporter gene and a constitutively active CMV-renilla luciferase internal transfection control, this provided a useful model system for examining the ability of GR nuclear retention mutants to activate transcription. The system was tested and titrated to determine the optimal conditions for observing potential differences in transcription without saturating the system.

Initial experiments were performed with 50 ug of selected GR construct transfected in per sample, and treated with the physiological level of 1 nM dexamethasone for 16 h. While the I516R point mutant showed only a slight decrease in transactivation under these conditions relative to the WT GR, the SV506-523 substitution mutant and the nSV40 mutant showed a very low level of activation, similar to the NL1- mutation, with 10% of the level of the WT (figure 16b). This likely was in part due to the decreased ability of these mutants to translocate to the nucleus at low hormone concentrations (figure 20). When this experiment was repeated using the more pharmacological concentration of 1 uM of hormone, the differences observed between the WT and mutant GR were decreased, suggesting that the retention mutants may be generally less efficient at activating transcription than the WT GR, but that saturating conditions can partially overcome this defect (figure 16b). In order to
Figure 16: Retention-deficient mutants show a decrease in transactivational ability on the MMTV promoter.
A) Diagram of the elements in the promoter of the MMTV reporter. B) Comparative levels of transactivation seen from the MMTV promoter with WT and retention-mutant GR after 16 h treatment with 1 nM and 1 uM dexamethasone agonist, 50 ng transfected of GR constructs. C) Levels of transactivation seen from the MMTV promoter with WT and retention-mutant GR after 16 h treatment with 10 nM dexamethasone, 12.5 ng transfected of GR constructs. Transcription graphs are presented as the fold activation of hormone treated cells over untreated cells. * denotes p<0.05 from WT and # denotes p<0.005 from NL1-, using ANOVA.
**A**

![Diagram of the MMTV promoter with various elements such as GRE, NF1, Oct-1, and TATA boxes.](image)

**B**

- **1nM dex 16h**
  - Fold activation graph showing data for WT, NL1-, SV506-523, nSV40, and I516R.
  - Signatures: #, *, *.

- **1uM dex 16h**
  - Fold activation graph showing data for WT, NL1-, SV506-523, nSV40, and I516R.
  - Signatures: *, #.

**C**

- **12ng GR 16h 10nM dex**
  - Fold activation graph showing data for WT, NL1-, nSV40, nNL1-, SV506-523, SV511-517, and I516R.
  - Signatures: #, *, #.
achieve a level of transcription which would allow the retention mutant GR to activate
transcription while still observing the differences between constructs, an intermediate
concentration of 10 nM dexamethasone was selected, in conjunction with a decreased level
of 12.5 ng GR transfected into the cells. The ability of the retention mutants to activate
transcription from the MMTV promoter under these conditions showed a clear
division between those mutants having the K513-515N mutation (NL1-, nSV40, and nNL1)
and those based on in-line substitution of SV40 sequence (I516R, SV511-517, and SV506-
523). The K513-515N group of constructs showed the most significant decrease in ability to
activate transcription from the MMTV promoter, and although the original NL1- was
slightly worse at activating transcription than the mutants with an NLS added back (3.2 fold
activation for NL1- versus 4.5 fold for nSV40 and 4.3 fold for nNL1), there was no
significant difference between the three constructs. Similarly, although all three mutants
having SV40 NLS character to their sequence showed an intermediate ability to activate
transcription, there was no significant difference between the three (9.1 fold for SV506-523,
12.9 for SV511-517, and 13.2 for I516R). The results for the K513-515N mutant receptors
(p< 0.04) were significantly different from the wild-type GR (21.3 fold activation), while the
differences between WT GR and the I516R and SV511-517 and 506-523 mutants
approached significance (p<0.08) (fig. 16c). Under these conditions, therefore, the ability of
each of the retention-compromised mutant constructs to activate the MMTV promoter was
noticeably affected. Interestingly also, with the exception of the I516R mutant, the decrease
in the retention mutants’ transcriptional ability relative to the wild-type GR roughly
corresponded to the degrees of retention loss seen in FRAP assays. That is, the decrease in
transactivational ability observed in the mutants was more marked in the case of the NL1-
mutants, and was less severe in the mutants which were based on in-line substitution of the
SV40 NLS for GR sequence, with a few exceptions. It did appear that in the transcription assays, the presence or absence of the triple-lysine cluster within the hinge region was most significant in determining transcriptional activation, and that the sequence of the surrounding residues had less impact on transactivation than it had on the nuclear retention observed in FRAP. Notably, as the I516R mutant had no major effect on the localization or retention of the receptor, it was somewhat surprising that it should have an effect on the ability of the receptor to activate the MMTV promoter similar to that seen with the larger substitutions of SV40 sequence which did affect receptor nuclear retention. This suggests that this site may have a minor role in factor binding and transcriptional activation. As well, the nSV40 exhibited less nuclear retention than the nNL1 mutant, but these two mutants had essentially the same effect on transcriptional activation of the MMTV promoter. This disconnect also indicates the possibility that the effects on retention and transactivation observed do not stem from a single cause or signal, but from overlapping signals. Conversely, the correlation of the retention and transcription results, however, might indicate a common factor required for nuclear retention and transcriptional activation of the receptor.

Heterodimerization of Mutant GR with WT Receptor Produces a Mild Dominant-Negative Phenotype:

Because GR is known to act as a dimer (Hard 1990, Savory 2001), we questioned whether transcriptional activation in the presence of both WT and mutant GR would show an intermediate ability to activate transcription, or whether one phenotype would predominate. We tested this by co-transfection of the WT and SV506-523 mutant constructs into Cos7
Figure 17: Retention-mutant GR may exert dominant-negative activity over WT GR.

WT (wt) and SV506-523 (sv) receptors transfected alone or in combination. Numbers on x-axis indicate ng of receptor transfected. Transcriptional activity was measured after 16 h treatment with 10 nM dexamethasone. Significant differences (p-values as calculated using ANOVA) between the various combinations are shown in the grid below.
WT 6ng SV506-523 6ng WT 18ng 16h 10nM dex

<table>
<thead>
<tr>
<th>p-values</th>
<th>WT 6ng</th>
<th>SV 6ng</th>
<th>WT 18ng</th>
<th>WT 6ng SV12ng</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>0.024</td>
<td>0.16</td>
<td>0.52</td>
</tr>
<tr>
<td>SV 6ng</td>
<td>0.024</td>
<td></td>
<td>0.0014</td>
<td>0.0001</td>
</tr>
<tr>
<td>WT 18ng</td>
<td>0.16</td>
<td>0.0014</td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>WT 6ng SV12ng</td>
<td>0.52</td>
<td>0.0001</td>
<td>0.016</td>
<td></td>
</tr>
</tbody>
</table>
cells, using the same system as the previous transcription assays, and measured their ability to activate transcription from the MMTV promoter. Low levels of DNA were transfected in to ensure that transcriptional activation would not reach saturation and leading to skewing of the results. It was observed that transfection of 6 ng of WT GR per well gave 14-fold activation of the promoter, while the same amount of SV506-523 gave only a three-fold activation, and transfection of 18 ng of WT GR gave a 30-fold activation. Based on this, if the effect is additive, transfection of a mixture of 6 ng of GR and 12 ng SV506-523 would be expected to give approximately a 20-fold activation. However, this combination actually induced only a 13-fold activation, a little higher than what might have been expected if only SV506-523 were present (fig. 17). This showed that the heterodimers had an intermediate ability to activate transcription, suggesting that the effect of the mutations may be additive in the dimer, or alternatively, that the WT and mutant seem to act independently to activate transcription.

The Effect of Retention Mutants on Transcription is Promoter-Specific:

The MMTV promoter which is commonly used as a reporter for GR transactivational ability requires interaction with the Oct and NF-1 cofactors to activate transcription. Previous studies have suggested that individual promoters, including the MMTV promoter, may have varying requirements for particular cofactors (Chen 2006, 2007, Trousson 2007). The possibility of promoter-specific interactions being involved was particularly of interest in light of the fact that our lab and others have found that GR can interact with Oct1/2 and other POU-domain proteins via their POU domain (Dong 2009, Kutoh 1992, Gonzalez 86)
2001), and our lab had identified the interaction as occurring within the hinge region of GR (Prefontaine 1998). We had considered the possibility that transcriptional activation and nuclear retention might both be influenced by cofactor interaction, and these things together suggested that examining the ability of the retention mutants to activate transcription from a promoter other than the MMTV reporter used might be pertinent, given the possibility that loss of retention and loss of promoter activation might both be due to alteration in GR interaction with a cofactor such as Oct. Therefore, I performed a side-by-side comparison of the abilities of the WT and retention-mutant GFP-GR constructs to activate transcription from both the MMTV promoter already described above and from a commercial GRE-luciferase reporter construct (Stratagene). The GRE-luciferase reporter contained 3 repeats of a consensus GRE, but no promoter sites for cofactors besides a TATA-like region (fig 18a). Under the same conditions as were used for the previous experiments with the MMTV promoter (12 ng GR plasmid per well, 10nM dex treatment for 16h), the correlation between retention and transcription previously seen was reiterated at the MMTV promoter, with the K513-515N group of mutants showing a low level of transactivational ability, and the SV40 substitution mutants an intermediate ability, with respect to the WT GR. By contrast, all of the retention mutant constructs activated transcription to similar levels from the consensus GRE reporter (fig 18b). Although the fold activation was at a lower level than that seen with the MMTV reporter, it was sufficient to show that there were no significant differences between the ability of the WT receptor and any of the retention mutant GR constructs to activate transcription from the consensus GRE promoter. As it had previously been noted that the assay conditions impacted the differences observed between constructs at the MMTV promoter as well as the maximum fold induction observed, these assays were also repeated using a higher transfection level of GR, and higher levels of hormone treatment
Figure 18: Retention mutants do not show decreased transactivation at the GRE promoter.
A) Diagram of the elements in the promoter of the GRE reporter. B) Levels of transactivation seen at the GRE promoter with WT and retention-mutant GR after 16h treatment with 10nM dexamethasone agonist, 12.5 ng transfected of GR construct. C) Transcriptional activation of the GRE reporter with 50 ng transfected GR constructs, 16h treatment with 10nM or 1uM dexamethasone. No significant differences between constructs was found in B or C using ANOVA.
A

Consensus GRE promoter

GRE1  GRE2  GRE3  TAL (TATA-like)

B

WT  NL1-  nSV40  nNL1-  SV506-523  SV511-517  I516R

fold activation

12ng, 16h 10nM dex

C

WT  NL1-  SV506-523  nSV40  I516R  SV511-517  nNL1-

fold activation

10nM dex 16h

1uM dex 16h

GRE, 50 ng GR transfected/well
(50 ng per well rather than 12 ng per well, and 10 nM vs 1 uM hormone treatment, fig 18c).

This showed that the lack of effect on transcription by retention mutant constructs of the GRE reporter construct was not a function of a particular set of conditions. These results tended to confirm the idea that a transcriptional cofactor of GR could be involved in retention, as well as being responsible for the promoter-specific effect on the ability of the mutants to activate transcription. Oct-1 and/or Oct-2 may be a candidate for this retention factor, based on their known interaction with the GR hinge, and the requirement for Oct in GR activation of the MMTV promoter.

Hormone sensitivity and response is altered in retention-mutant GR

Initial localization assays of the retention constructs had previously determined that all of the GR retention mutants were localized primarily to the nucleus one hour after treatment at pharmacological levels of 1 uM dexamethasone. In order to verify that the differences observed in transcriptional assays were not due to decreased expression of mutant receptors or, at lower concentrations of hormone, were not unduly influenced by incomplete localization of the mutant receptors to the nucleus, the expression levels and the distribution of receptors as a function of time and of hormone concentration were determined. Western blotting determined that all receptors were expressed at a comparable level (figure 19). It has previously been suggested that transcription is strongly activated initially, but is rapidly suppressed afterwards pending the next cycle of hormone binding, presumably as a method of allowing tighter control or connection between hormone concentration and transcriptional response (Qiu 2006, Freeman 2001). Thus, delayed entry and subsequent activation could lead to lower levels of cumulative activity (which is what is
Figure 19: Expression levels of WT and retention-mutant GFP-GR.
Western blots showing expression levels of the various GFP-GR constructs in transiently-transfected Cos7 cells, 40 hours post transfection. 50 ug of protein was loaded per well, and bands were visualized using the FiGR antibody, as described in Materials and Methods.
Figure 20: Hormone response characteristics of GR retention mutants.
A) Percentage of cells of each mutant with mostly nuclear localization in the presence of 1nM dexamethasone over a 24h period. B) Percentage of cells of each mutant with a mostly nuclear localization after 1h treatment with increasing concentrations of cortisol. Cells were transfected, fixed, blinded and counted as described in Materials and Methods.
measured by the luciferase reporter). In the presence of a physiological 1 nM concentration of hormone, the majority of the GR mutants appeared to translocate at a rate similar to that of the WT GR, and were primarily nuclear after 4 h of treatment (figure 20a). Of those which did not translocate as completely, the nSV40 and the NL1- also showed decreased nuclear localization previously at 1 h 1 uM dexamethasone, and so were expected to be delayed. The slight delay in the nuclear entry of the I516R, however, was not expected, and it is possible that this may be associated with the slight decrease in transcriptional activation noted with this construct at the MMTV promoter (figure 16b,c).

**An Oct-binding mutant of GR exhibits decreased nuclear retention after hormone withdrawal:**

As stated previously, it was known that Oct and NF-1 were required for the activation of the MMTV promoter but not the GRE reporter by GR, as well as that GR-Oct interaction takes place at the hinge region of GR (Prefontaine 1998). In conjunction with the observation that the retention mutant GR constructs showed a decreased ability to activate transcription from the MMTV but not the GRE promoter (fig. 16,18), these pieces of information suggested that Oct factors might be involved in GR retention. In order to test this theory, the C500Y GR mutant was employed. This mutant was originally described as a DNA-binding mutant, due in part to its inability to activate the MMTV promoter (Schena 1989), and the phenotype was subsequently found to be due to the Oct1/2 (POU domain)-binding loss of the mutant (Prefontaine 1998). In order to further examine the possibility that an interaction with Oct1/2 might be involved in GR nuclear retention, I examined the characteristics of the GR C500Y mutant (fig 21a) in transcriptional activation and hormone withdrawal assays. I first tested the ability of this mutant to activate transcription from both
the MMTV and GRE promoters, in comparison with wild-type and nSV40 retention mutant GR, the latter of which had strongly decreased retention and transcriptional activation, similar to the NL1- mutant. The C500Y mutant was not able to activate transcription from the MMTV promoter, showing a null result of 1 +/- 0.2 fold activation upon hormone treatment, versus 18 +/- 0.7 fold for nSV40 and 78 +/- 16 fold activation for WT GR under the same conditions (fig. 21b). The null activation of the C500Y is comparable to what was seen in the previously published results, and consonant with the phenotype of either an Oct-binding or a DNA-binding mutant. However, as had been observed with the retention mutants previously tested, there was no significant difference in the ability of the C500Y mutant and the WT GR to activate transcription from the consensus GRE promoter (9 +/- 5 fold versus 6 +/- 2 fold activation, respectively, with the nSV40 at 8 +/- 3 fold, fig 21b). This confirmed that the C500Y could not be a true DNA-binding mutant (Prefontaine 1998), and that the lack of transactivation seen at the MMTV promoter was more likely due to disruption of the ability of this mutant to interact with the necessary transcription factor, Oct, than to a defect in DNA binding.

If the decreased transcription from the MMTV promoter and the decrease in GR nuclear retention are indeed related, Oct factors could therefore be an obvious candidate as the GR nuclear retention factor. I therefore tested the ability of the C500Y Oct-binding mutant to export from the nucleus following hormone agonist withdrawal, hypothesizing that if an Oct factor was indeed involved in nuclear retention, the C500Y mutant should export more rapidly than the wild-type GR in this assay, and indeed, ought to export at least as rapidly as the nSV40 mutant, as the nSV40 mutant was still capable of stimulating some transactivation from the MMTV promoter, where the C500Y was not. The results showed that the C500Y mutant had an intermediate phenotype with respect to nuclear retention, with
Figure 21: Oct-binding GR mutant shows intermediate loss of nuclear retention only.
A) Sequence of the GR hinge region. Arrow and red letter indicates known Oct-binding mutation C500Y. B) Transcriptional activation under conditions as in figure 17 at the MMTV and GRE promoters, respectively, comparing the C500Y to WT GR and nSV40 GR retention mutant. ANOVA test for significance shows significant differences (p<0.02) between all constructs with MMTV promoter and no significant differences (p>0.05) with the GRE promoter. C) Proportion of mostly nuclear receptor over time post hormone-withdrawal for the same three GR constructs.
A  WT GR  500 YLQAGMNLEARKKKIKGIQQATAG 525

C500Y Identified as a Oct-binding and DNA-binding mutant. Oct site present on MMTV but not GRE promoter.

B

MMTV reporter

GRE reporter

C

% N+M>C

- H  + H  6h wd  24h wd
44 +/- 13% of cells showing mostly nuclear receptor 24 h after hormone withdrawal, similar to the level of export of the SV511-517 construct which was previously observed using this assay (fig 21c, compare to fig 13b). It did export more rapidly than the WT receptor (64 +/- 14 % nuclear after 24 h), but was still slow in comparison with the nSV40 mutant, which was known to export quite rapidly, and which was nuclear in only 3 +/- 1% of cells after 24 h hormone withdrawal. Thus, while mutation of the residues closer to the NL1 core of GR impacted both nuclear retention and promoter-specific transactivation to a similar degree, the Oct-binding mutant C500Y shows a separation between effects on retention and transactivation. This seems to imply that the hinge region may contain multiple overlapping signals, and that while Oct may indeed be involved in GR nuclear retention, it cannot be the sole factor involved, as loss of Oct binding was not capable of completely reproducing the phenotype observed of loss of nuclear retention resulting from mutation of the hinge region of GR.

Decrease of Transcriptional Activity by the SV506-523 Mutant on the MMTV Promoter is due to Failure to Recruit Necessary Cofactors:

Technical assistance for this experiment was provided by Dongmei Wu.

In order to investigate the basis behind the promoter-specific loss of transcription observed with GR nuclear retention mutants, the interaction of the GR and other factors with the MMTV promoter region was examined using chromatin immunoprecipitation (ChIP) assays. Cos 7 cells stably transfected with the MMTV promoter sequence were used, and were transiently transfected with appropriate GR constructs for experiments, as described in Materials and Methods. ChIP of GR interaction with MMTV DNA from cells transfected
with either the WT or the SV506-523 mutant GR showed that both constructs had a similarly increased interaction with the promoter after 1 h treatment with 10 nM dex. This confirmed that the DNA-binding function of the mutant appeared to be intact, and the loss of transactivation observed with the mutants was not due to loss of DNA interaction. However, in the presence of the wild-type GR there was a clear increase observed in the levels of histone 4 acetylation and recruitment of RNA polymerase II, which are classic markers for transcriptional activation, at the MMTV promoter after hormone treatment. In the presence of the retention mutant SV506-523, histone 4 acetylation was not observed, while the SV506-523 showed a higher basal level of Pol II than the WT GR, but did not show an increase in Pol II recruitment in the presence of hormone (fig.22). This suggested that the observed decrease in transcriptional activation by the retention-deficient GR mutants may be due to a failure of the receptor to recruit the necessary transcription factors and/or form effective complexes required for activation of the MMTV promoter. The currently accepted model for the majority of transcriptional activation involves changes in post-translational modification of histones, leading to a loosening of DNA and/or shifting of the histone octamer to facilitate interaction of necessary factors and activation of transcription. The lack of histone acetylation seen in the ChIP of the mutation in GR SV506-523 suggests that this mutation affects the recruitment of a crucial cofactor with histone acetyltransferase (HAT) activity to the MMTV promoter. This is not unlikely, as GR numbers several cofactors with HAT activity among its identified interacting proteins. In the absence of the HAT recruitment, the promoter would then remain in a closed conformation that does not favour the recruitment of the cofactors and Pol II necessary to activate transcription.
Figure 22: Decreased transcription on MMTV promoter by retention mutants may be due to decreased histone acetylation and polymerase recruitment.
Chromatin immunoprecipitation assay of binding to the MMTV promoter, comparing WT and retention mutant SV506-523 GR, before and after 1 h treatment with 10 nM dexamethasone. Both receptors interact with the promoter, but only the WT receptor shows acetylation of histone H4 and polymerase II recruitment upon hormone treatment.
**Discussion:**

Glucocorticoids and the glucocorticoid receptor have been studied for a long time. The use of glucocorticosteroids for therapeutic purposes has been established for over half a century, and the glucocorticoid receptor (GR) was the first member of the steroid hormone receptor family to be cloned. However, with respect to understanding its regulation *in vivo*, there is still much to be learned on the subject of GR function. An increased knowledge of GR regulation will hopefully aid both in developing improved therapeutic strategies which will enable the maximal benefit of these drugs to be utilized while avoiding the side effects associated with their use, and in better understanding the function and regulation of steroid hormone receptors in general.


Previous work in the Haché lab found that a mutation of three core lysines in the GR NL1 sequence had the effect of not only decreasing the ability of GR to localize to the nucleus, but of dramatically increasing its export rate as well, suggesting that these residues were involved in nuclear retention as well as localization (Savory 1999). Although it was expected that mutation of the NL1 would decrease the import of the receptor, the receptor
binding to importins is lost upon import. Thus it was surprising that export should be affected as well as import, given that the mutation was mainly supposed to affect importin binding. The presence of a nuclear retention activity was confirmed when addition of the hinge region of GR, containing the NL1, was able to decrease the export rate of a rapidly-shuttling protein (Carrigan 2007).

In order to more closely study this retention, it was necessary to be able to separate it from the nuclear localization activity, both to confirm it was indeed a separate activity, and to ascertain its role in GR function. I was therefore interested in determining whether I could separate the nuclear localization and retention activities of GR, in order to more closely define the requirements for retention, and determine what role it might play in GR activity.

I found that in addition to the core lysine sequence initially identified at residues K513-515 of GR, mutation of the 511-512KT residues to sequence from the SV40 NLS region was able to decrease GR nuclear retention, but without significantly affecting the hormone-dependent nuclear localization of the receptor. These residues are key ones within the hinge, as further mutation failed to have any additional effect on nuclear retention of liganded GR. Retention also seems to be partly dependent on the position or context of the retention sequence within GR, as positioning an NLS sequence at the N-terminus of GR rather than in the hinge region decreases the nuclear retention of the receptor, with the degree of retention loss varying with the sequence of the NLS. The character of the ligand appears to influence retention as well, as antagonist-withdrawn mutants failed to show an increase in export rate relative to the WT GR. It was also of interest to explore the applicability of GR retention requirements and function to other proteins, to determine whether this might represent a general mechanism of regulation. Results from FRAP assays of different NLS sequences suggest that the KT sequence identified in GR, or more
specifically, the spacing of the basic residues, is important for nuclear retention in other NLS-linked sequences, and that proline may not have a fixed role in regulating nuclear retention.

In looking at the possible effects of retention, I found that mutants with decreased retention also showed decreased ability to activate transcription from an MMTV but not a 3xGRE reporter. The decrease in MMTV activation by an SV40-substituted GR mutant with decreased nuclear retention appears to stem from an inability to efficiently acetylate histones and recruit RNA polymerase II. It is formally possible, though, given that mutation of the NL1 lysine core has a much more dramatic effect than the SV40 substitutions on transcription from the MMTV promoter as well as on nuclear retention, that mutation at the NL1 lysine cluster may decrease transcriptional activation via a different mechanism. Although a GR mutant leading to loss of interaction with the POU-domain protein Oct, which is known to interact with the GR hinge, is sufficient to decrease promoter-specific transcription, this mutation led to a less dramatic effect in withdrawal assays than might be expected, suggesting Oct is not the primary retention factor for GR, but that binding site for Oct and the retention factor may overlap.

These results, taken together, suggest that there may be multiple overlapping signals in the hinge region near the NL1 of GR, which impact nuclear retention, cofactor interaction, and promoter-specific transactivation.

The GR Nuclear Retention Signal:

The localization of proteins within the cell frequently serves as a method of modulating their activity. By controlling localization, access to potential interacting partners
can be impacted, and consequently, functions such as transcription, post-translational
modification, signalling cascades, and protein turnover may be regulated. This provides a
mechanism for the cell to respond to an alteration in conditions and trigger a response.
Proteins such as GR, which can exchange between the nucleus and the cytoplasm, may
shuttle freely between compartments, or may be retained in either the nucleus or cytoplasm,
depending on the properties of the protein and the cellular conditions.

Several nucleocytoplasmic proteins are known to be retained in the nucleus, usually
upon activation. Smad proteins 2 and 4, for example, shuttle freely between the nucleus and
cytoplasm in the absence of TGF-β, but the presence of TGF-β leads to an accumulation of
Smads in the nucleus. These Smads are in a phosphorylated, complexed state, and show a
decreased intranuclear mobility as well as decreased export (Schmierer 2005). Similarly,
naïve mCAR shuttles freely between the nucleus and cytoplasm. Upon interaction with the
cofactor GRIP1/Src-2, a p160 protein with HAT activity, mCAR is activated, and
accumulates in the nucleus (Xia 2005). Interaction of p53 with E2F1 leads to nuclear
retention of the phospho-S315 form of p53 (Fogal 2005). Cdc7 also undergoes nuclear
retention, and this has been shown to occur via a basic 20-residue sequence near the C-
terminus, which appears to mediate interactions with chromatin. This sequence shows some
similarity to the GR hinge sequence, but does not contain NLS activity (Kim 2007). Thus,
the notion that a basic GR sequence might be implicated in nuclear retention of the protein
and be useful in controlling its activity is not unprecedented. It remains to be seen how
widespread this mechanism is among nuclear proteins, and how much variation may be had
in signal and mechanism within the potential group of proteins possessing active nuclear
retention.
The overlap of nuclear retention activity with NLS sequence in GR may exist in other proteins as well. Certainly other steroid hormone receptors (PR, AR, MR) have been reported to contain nuclear retention activity, and show sequence conservation with GR in the hinge region (Walther 2005, Man 2006, Poukka 2000, see appendix C for sequence alignment). Most of the residues identified as important to nuclear retention in this work are basic amino acids, commonly found in NLS sequences (Jans 1998, Macara 2001) – as well as in the cdc7 retention signal mentioned above (Kim 2007). It seems logical that retention and nuclear localization activities would be linked, or use overlapping residues, as both are concerned with proper localization of the protein, and could be interdependently controlling this function. This was taken as a working hypothesis in the selection and testing of NLS for retention activity in the latter part of this work. In GR, at least, this region is highly conserved between species (see appendix C for sequence alignments) and no natural mutants showing altered activity have been reported, implying that accurate sequence in this region may be required for proper protein function in vivo.

One of the observed consequences of mutations that decreased GR nuclear retention was a decrease in the ability of the mutants to import efficiently and rapidly into the nucleus. Although it seems likely that this might be at least partially due to the fact that many of the mutations made were close to or overlapping with the NL1 nuclear localization sequence, and could easily be interfering with importin binding and activity, it is perhaps noteworthy that the magnitude of the loss of nuclear import and nuclear retention closely parallel each other (compare figures 10 and 13 with 20). The presence of an NLS at the N-terminus of the NL1-GR is capable of restoring import ability, with the degree of restoration apparently influenced by the sequence of the NLS (fig 20, compare nNL1 and nSV40). A similar difference between these two constructs was seen with respect to their nuclear retention
properties (figures 10, 11) Although it seems unlikely, we cannot discount the possibility that perhaps a member of the import complex (not necessarily an importin, but possibly a heat shock protein or immunophilin) could be involved in both import and retention of GR, given the decreased efficiency of import observed with the retention mutants (figure 20). Alternatively, it is possible that the mutation of the hinge region could destabilize both importin and retention factor interactions, due to the presence of overlapping signals for retention and import. Something of this nature may occur in the related androgen receptor (AR), where mutation of a leucine residue near the NL1 (conserved in the GR) decreases the nuclear localization rate of the receptor, possibly by decreasing interaction with SNURF, a ring-finger protein which has been reported to increase import and nuclear matrix targeting and decreases export of AR (Poukka 2000). Mutation of this leucine in GR affected retention in the context of the hinge fragment but not the full-length receptor (figs. 7 and 9).

In the study of GR nuclear retention, it is likely that the properties of the closely related steroid receptors AR, PR, and MR are of interest in helping to elucidate the properties of GR. The hinge sequences of these receptors are well conserved until just past the NL1 of GR, particularly the positioning of the lysine residues within the NL1 (see appendix C). This includes the sequences noted here and previously which appear to have the most impact on GR nuclear retention. As mentioned above, there is evidence that all three receptors have some form of nuclear retention which may be similarly centred in the hinge region. Mutation of MR residues equivalent to the NL1- (K513-515N) of GR produces an increased export rate in MR (Walther 2005). Sumoylation of the equivalent lysines in PR destabilizes its nuclear retention (PR is normally constitutively nuclear) and leads to a decrease in transactivation (Man 2006). Interaction of AR hinge region with the RING-finger protein SNURF, increases AR import and net nuclear localization and decreases its
The cofactor interactions and behaviour seen in other steroid receptors thus may provide a broader perspective on effects on GR, and suggest a possible role for post-translational modification of GR hinge lysines in retention as well as receptor activity.

Acetylation of GR residues 511,513-515 (RKTKKKIKG, all of which have been found to have an impact on GR retention) has been reported, and deacetylation of this site by HDAC2 is required in GR control of NF-kB-mediated gene expression (Ito 2006, Nader 2009). Acetylation by p300 and deacetylation by HDAC have both been reported to be important for general transcriptional activation by GR (Glass 2000, Li 2002, Kinyamu 2004, Qiu 2006). Mutation of the analogous acetylation sites in AR delays ligand-dependent nuclear translocation, a phenotype also observed in GR retention mutants to a lesser extent (although this may be due to alteration of the NL1 rather than retention), as well as leading to AR misfolding and aggregation (Thomas 2004), while acetylation within the ER hinge by p300 enhances its DNA binding and transactivational ability (Kim 2006). PR undergoes SUMOylation by PIAS3 at its NL1 lysine site analogous to those in the GR and AR hinges, and this SUMOylation decreases the nuclear retention and transactivational ability of PR (Man 2006). ER SUMOylation within the hinge region has also been recorded, and appears to be involved in transcriptional regulation (Sentis 2005). GR SUMOylation has been reported in the N-terminal and C-terminal regions rather than in the hinge region, but the possibility of additional sites which may be used under specific conditions has not been ruled out (Le Drean 2002, Tian 2002). Thus, in other GR and other SHRs, the hinge lysines have been shown to undergo modification and to be involved in transcription and nuclear retention. Glutamine substitution for lysine has been shown to mimic constitutive acetylation in histone proteins and p53 (Luo et al, 2004). It might therefore be interesting to determine
what role acetylation might play in GR nuclear retention, by comparing the effect of glutamine versus alanine substitution of the key lysine residues identified. A second possibility would be arginine substitution, which would preserve the charge but block acetylation.

In the course of FRAP experiments to study the nuclear retention properties of various GR hinge mutants, it became clear that the degree of GR nuclear retention observed was affected by both the context of the hinge region within the receptor and the position of mutations within the hinge sequence. In the in-line SV40 substitution mutants of the full-length receptor, none of the mutations made were able to completely reproduce the loss of retention seen in the original NL1- (K513-515N) mutation. However, an analogous mutation, when positioned as an N-terminal NLS in the context of the full-length GR NL1-receptor or within the NES-GST-hinge-GFP-NLS construct, produced a much more dramatic loss in percentage of retention than was observed in the normal hinge context of the FL GR. For instance, compare the full-length receptor with the SV511-517 mutation (SV511-517 GR), the N-terminal SV40 NLS added to the full-length NL1- GR (nSV40 GR), and the hinge construct with the RKT510-512AAA mutation (NES-GFP-GR500-525mut RKT-AAA-GST-NLS). All three receptor mutants have similar basic alterations in the functional NLS region, with the 511-512 KT of the GR sequence mutated. For each of the three, a loss of retention was observed relative to the corresponding wild-type construct, but in the SV511-517, an intermediate retention phenotype is produced, not reaching the level of the NL1-, whereas in the other two constructs, the phenotype observed is much the same as that of the NL1- or a comparable ‘unretained’ control construct (compare in figures 13, 10, and 7). As the SV511-517 GR is the only one of the three constructs which has its primary
NLS in the original context of the FL GR receptor, this points to the possible co-operative involvement of sequence outside of the 26-residue hinge region in GR nuclear retention in interactions involved in maximal nuclear retention.

The above suggests the nuclear retention signal may have the aspect of a bipartite sequence, which has parallels in the existence of bipartite nuclear localization signals, such as that of GR (Ylikomi 1992, Tang 1997). Alternatively, it may reflect the different structural contexts of the mutations, and the possible role of a retention factor binding to the hinge region in creating or stabilizing a particular conformation. Thus, it is possible that in the full-length receptor, the binding of a retention factor to the hinge region of GR allows a stable configuration that favours cofactor binding, blocking of GR export signal sequence, and/or interaction of GR N-terminal/DBD and LBD regions, similar to what is observed in the androgen receptor (Van Royen 2007, Gonzalez 2001, Schaufele 2005). Interestingly, some direct evidence for GR hinge/LBD interdomain interaction comes from GR/MR chimeras, which showed that a receptor bearing both hinge regions was responsive to both GR and MR ligands, while chimeras at various breakpoints in the hinge/LBD region altered the specificity of receptor hormone response (Martinez 2005).

Interdomain interaction within GR might also occur indirectly via cofactors, as several reports note utilization of both the AF-1 and AF-2 by the same factor (Garside 2004, Chen 2006, Wang 2004, Kroe 2007). This would then allow for efficient function of GR. Mutations within the hinge/NL1 of GR would destabilize the binding of the retention factor, and lead to loss of retention, as observed. It can be imagined that in the context of the full-length GR, the central position of the hinge region within the protein might make it less likely that some mutations would destabilize the binding of factors sufficiently to cause a complete loss of retention or transcriptional activation, particularly if secondary contacts are
made outside the hinge region. Conversely, with the retention signal placed in the N-terminal region of GR or in the smaller hinge construct, there is likely to be less bulk nearby which might help keep interacting proteins bound to GR, and a lack of any other nearby sequence which might be implicated in retention in the WT context. This idea could be tested by determining what effect mutations of the AF-1 and/or AF-2 region have on GR nuclear retention. A less than ideal initial binding site for factor(s) involved in retention could also explain the more rapid transfer times seen with the WT GR hinge sequence in contexts other than the endogenous (i.e. in the nNL1 GR, or the 500-525 hinge construct). This could also provide an explanation for why the LE507-508AA mutation showed a strong lack of retention in the initial hinge construct experiments (fig 7) but failed to affect GR nuclear retention in the context of the full-length receptor (fig 9). An analogous leucine in the AR has been shown to be implicated in interaction with cofactor SNURF and affects nuclear localization of the AR (Poukka 2000).

Interestingly, the addition of an NLS sequence to the N-terminus of the FL NL1-GR, sandwiched between GFP and the receptor, produced a mutant with similar import characteristics to the WT receptor with respect to ligand-driven nuclear localization. The nSV40 and nNL1 receptors were more cytoplasmic than the WT GR in the absence of hormone, and slightly less efficient at being imported into the nucleus after treatment with hormone (requiring 0.1 to 1 uM dex to reach 80% nuclear localization in 1 h, while WT GR required 1nM, see fig 20b). However, they did make a complete transition to the nucleus, comparable to WT, while their parent NL1- GR had a slow or incomplete localization under the conditions studied– and unlike the SV506-523 receptor, which was constitutively nuclear. This implies that when the naïve GR mutants are in the chaperone complex, the N-terminal NLS sequences are likely not exposed in a manner which allows importin
interaction in the absence of hormone, whereas that of SV506-523 apparently is. This could be tested by examining binding/interaction of WT and mutant GR and importins in the presence or absence of hormone. Exposure of the N-terminal NLS allowing import subsequent to ligand-binding, however, would then suggest that perhaps interaction between the N-terminal and LBD domains of GR is occurring, similar to what has been reported for the related androgen receptor (Zhou 1994, Schaufele 2005, Van Royen 2007).

The use of FRAP and withdrawal assays to study the nuclear retention and export properties of various GR constructs allows real-time observation of effects in a minimally invasive system. These are not, of course, truly physiologically relevant systems, as they require substantial levels of expression of fluorescent tagged protein, treatment with fairly high doses of hormone, and observation techniques which entail exposure of the cell to high-intensity laser irradiation in FRAP, and fixing of the cell in paraformaldehyde in withdrawal assays. It is important to consider how the techniques employed may affect the behaviour of the receptor, and how this could impact the translation of results to an in vivo context. A previous case of this is shown in the finding that reports of calreticulin as a nuclear export partner of GR stemmed from the release of calreticulin into the cytosol during the assays used, when it would normally be sequestered away from GR in vivo (Walther 2003 and references therein). Thus while calreticulin may represent a valid export pathway in certain circumstances, it is not likely to function as an exportin under normal cellular conditions (Walther 2003).

While I have been looking mostly at differences between WT and mutant receptors, rather than absolute effects, such considerations may become relevant in attempting to translate the research presented here into a useful in vivo context. The overexpression of GR
constructs and the stabilization which can be conferred by the presence of the GFP tag might conceivably influence the balance of cofactor interactions and the transactivational activity of the receptor in the cell-based, essentially artificial system used. It might be expected that in vivo, a higher ratio of cofactor to receptor will be present, and that if cofactor interactions are indeed involved in GR nuclear retention, the transactivation, stability, and export characteristics of the receptor will be altered from those observed here. The high level of receptor relative to cofactor/retention factor probable in transfected cells might also account for the null result obtained in attempting to titrate the interaction causing nuclear retention of GR. During the course of my research, the notion was put forward that if retention was caused by interaction of the GR hinge with a nuclear component, then it should be possible to relieve retention of GR by titrating the interaction with a nuclear-targeted peptide containing the hinge sequence of GR. FRAP assays, however, did not show any alteration in the export rate of the WT GFP-GR in the presence versus the absence of the peptide. The need for a higher concentration of peptide to show any effect on retention is one explanation for this result. It has previously been shown that overexpression of GR can trigger a more nuclear localization of the receptor, even in the absence of hormone (Sanchez 1990), which might be due to the export capacity in the slow shuttling of the receptor being the rate-limiting factor for the exchange. I also noted in transcription assays that the maximal level of activity reached a plateau near transfection levels of 50 ng GR per well, using 250 ng of MMTV reporter DNA per well. Whether the limiting factor is the cofactor levels available or the MMTV reporter available is not known, but titration of the MMTV quantities could be used to determine this.
The Role of Nuclear Retention:

Experiments performed in various labs have established that nuclear retention such as that observed in GR is not a universal property of nuclear proteins, or even of nuclear receptors, leading to the question of what role retention might play in the function of those proteins which do possess this characteristic (Prufer and Barsony 2002, Bonaldi 2003). Increasing the residence time of a protein in the nucleus would be predicted to involve increased interaction with nuclear components, including DNA, nuclear matrix components, and other nuclear proteins. This would likely result in alteration in transactivational activity, alteration of signalling pathways and protein modifications, and alterations in the stability and nuclear mobility of the retained protein, either directly or indirectly, depending on whether these are governed by the binding of the protein to DNA or to the nuclear matrix. Increased residence time in the nucleus by particular nuclear proteins, or at certain points of the cell cycle, should therefore increase the efficiency of the retained proteins’ activity.

Some previous work on GR bears out this perspective: one paper found that nuclear retention but not the DBD of GR was necessary for gene expression (Cadepond 1992) and one noted that GR nuclear retention was lost in the G2 phase of the cell cycle, and that this corresponded with a loss of transactivational activity, and alterations in receptor phosphorylation (Hsu 1992).

Previous work by the Haché lab established that although hormone-withdrawn GR is rapidly recycled into hormone responsive complexes (1h), it is not re-exported until significantly later (12-24h) (Haché 1999). It has been suggested that this delayed export may allow for a faster response time in the event of a second hormone signal by eliminating the need for reimport into the nucleus. One of the few studies on the effects of GR nuclear
retention, apart from the work of the Haché lab, is a paper from the Defranco lab in which the addition of an exogenous NES to the WT GR was used to increase the export rate of GR. This was found to increase the turnover rate of the receptor, but did not lead to any alteration in transcriptional activation of the MMTV promoter (Liu 2000). By contrast, my findings showed that the GR mutants exhibiting decreased levels of nuclear retention had an impaired transactivation of the MMTV promoter (although not the synthetic GRE), but showed no consistent alteration in turnover rates.

These results are not necessarily contradictory, however, as differences in the constructs utilized by the two studies could easily explain the discrepancy. The Defranco group employed a construct containing an extra NES tagged onto the WT GR, but lacking a GFP tag, while the present work used GFP-tagged proteins throughout, but had constructs whose decreased retention was based on mutations within the GR sequence rather than addition of an NES signal sequence. GFP-tagged proteins were used throughout my work to enable easy, real-time visualization of proteins in live or fixed cells, as well as providing a convenient and consistent tag for immunodetection. Although it adds a 23 kD moiety, GFP is widely used as a tag. Most GFP-tagged proteins do not have their function significantly affected, and GFP-GR has been shown to be functionally competent to activate transcription (Htun 1996). One effect which has been noted of this tagging, though, can be the stabilization of the tagged protein, which can be of use in cases where a short-lived protein is to be studied (Haché lab, unpublished). In the case of a relatively long-lived protein such as GR, however, it makes it difficult to measure alterations in the turnover rate of the protein. Thus, although Liu et al observed that the addition of an exogeneous NES to GR increased both the export rate and rate of turnover of GR (Liu 2000), I was unable to see any consistent effect on the turnover rate of the GFP-GR nuclear retention mutants used, even though the
export rates of my mutants were significantly greater than that of the WT GR (unpublished observation). Increased turnover rates might have been expected, based on the findings of Liu et al. It is possible that in the absence of the GFP tag, an increase in the turnover rate of my mutants might be observed. The one caveat here would be based on the finding that following proteasome inhibition, unliganded GR binds to the nuclear matrix via residues in or near the hinge region (Schaaf 2003), leading to the possibility that the proteasome may interact with GR near the hinge/retention signal. In such a case, one might expect that mutation to the hinge might lead to a decrease in proteasomal degradation of GR.

Liu et al. also noted that the ability of the NES-GR to activate transcription from a transiently transfected MMTV reporter was not altered despite its increased rate of nuclear export, whereas I observed a decrease in transactivation roughly paralleling the decrease in nuclear retention in my mutants, using the same promoter. ChIP results at the MMTV promoter and the ability of mutants to activate transcription from the 3xGRE reporter indicate that the interaction of my retention mutants with the promoter is not likely to have been lost. This suggests that the decrease in transactivation observed in my mutants was not a direct result of the increased export rate or alteration in DNA binding, but rather due to an alteration in intranuclear interactions by the mutant GR. Again, the results are easily enough explained by the fact that the construct used by Liu et al was essentially a WT receptor, and the decrease in retention was produced by the addition of an NES. This would allow direct increase of export, where the increase in export of my mutants is more likely indirect. Multiple cofactor interactions are known to take place in the hinge region of GR, which might be predicted to be altered as a consequence of the mutations described in this thesis. This could consequently impact on transactivation, while the NES-GR of Liu should not show alterations in these interactions. This possibility is also consistent with my finding that
the connection between retention and transactivation was promoter-specific (fig. 16 and fig 18). Thus, it is possible that mutation leading to loss of GR nuclear retention likely involves loss or decrease of GR interaction with a (thus far unidentified) cofactor or cofactors which might otherwise interfere with the interaction of GR with the nuclear export machinery as well as being required for optimal transcription at specific GR-activated promoters. It remains also to be determined whether the same factor can also act in nuclear retention of proteins other that GR.

A further point is to be garnered from the work of the Defranco lab described above. Results from our NES-GST-GFP-NLS construct implied that the GR retention signal is capable of overriding an NES, while the Defranco results show that an NES can override the retention. These conclusions are not necessarily mutually exclusive. The different conclusions drawn may be a consequence of the positioning of the respective elements within the protein, or it may simply reflect an intermediate balance between export signal and retention signal which can be interpreted as a loss or gain of retention depending on what control construct the comparison is being made with (i.e. GR plus retention plus NES could be slower to export than constructs with NES only and faster than constructs with retention only).

In our system, the intranuclear mobility of GR mutants did not appear to be significantly altered with respect to the WT receptor (with the exception perhaps of the I516R mutation, which had a slightly reduced mobility based on direct observation). However, changes to intranuclear GR mobility are generally observed on a more rapid timescale (ms to s) than that achievable by the system used here (resolution of 20s post-bleach and up at 1s intervals). Ligand interactions have been shown to decrease the
intranuclear mobility of GR in a manner dependent on the type of ligand (Schaaf 2003). This is thought to result from altered interactions with cofactors, DNA, and scaffolding within the nucleus, and there appears to be an inverse correlation between receptor mobility and activity (Kino 2004, Schaaf 2005, Alarid 2006). By this theory, GR mutants lacking nuclear retention should have an increased intranuclear mobility and a decreased activity, such as was observed with transactivation from the MMTV promoter.

Despite the fact that FLIP assays showed that the entire population of GR within the nucleus appeared to be mobile, one anomaly in our binucleate FRAP assays has always been that the graph of fluorescence return to the bleached (acceptor) nucleus evinces a tendency to plateau prior to complete re-equilibration of fluorescence between nuclei (within the first hour of monitoring, or more dramatically within 30 min. in assays with the GR hinge – N2G2 context). Generally it has been easiest to look at the degree of re-equilibration between nuclei after a given time and interpret it as nuclear retention relative to positive and negative control constructs. The initial rate of return observed prior to fluorescence plateauing corresponds well to results obtained by measurement of the final ratio of fluorescence after recovery for 30 min to 2h, as well as to those obtained in hormone withdrawal assays, thus the plateau does not appear to alter the final conclusions made about the relative strength of nuclear retention in WT versus mutant GR constructs. However, the presence of the plateau suggests that a certain fraction of the receptor or shuttling protein, while mobile within the nucleus, does not undergo nuclear export at all. Normally one might speculate that this may be a population which is misfolded, but it seems unlikely, in light of the fact that the WT GR reaches a plateau at a lower level than most of the retention mutants. Consequently, this would imply that the WT GR has a larger proportion of misfolded proteins than the retention mutants. Proteasome activity has been shown to be implicated in
GR mobility and release from nuclear components, as well as transcriptional activity (Deroo 2002, Schaaf 2003, Stavreva 2004, Alarid 2006). Mutation in proteasome-interacting sites in GR might therefore explain alterations in intranuclear mobility and transactivational ability, but it is difficult to see how this could impact the nuclear retention of GR which is mobile within the nucleus.

The most likely explanation for the apparent block on export of part of the GR population might be interaction with a soluble factor within the nucleus. The correspondence of the plateau level with the initial rate of transfer in FRAP assays suggests that the retention of the general population and the block on export of a part of the population may be related. The same factor which is causing the nuclear retention under investigation may also be responsible for the apparent block in export, where just a slightly stronger interaction between GR and the retention factor in question could be all that is necessary to produce the stronger effect. It is accepted that receptors such as GR must form a heterogeneous population at the best of times, due to the multiple possibilities extant for post-translational modification and complex formation, and it is reasonable to postulate that some of these complexes are more stable than others, or less favourable to receptor export, resulting in a portion of the GR population which is retained more strongly in the nucleus. The MR, a close relative of GR with a very similar hinge sequence, and thought to interact with many of the same cofactors, appears to be retained even more strongly than GR in the nucleus after hormone treatment, to the extent that its export is negligible (Yang and Young 2009 and refs (incl. Ikonen 1997, Hultman 2005), Walther 2005).

The ChIP assays of the integrated MMTV promoter in the presence of WT and SV506-523 retention mutant GR led to two main conclusions. First, that the alteration in
receptor behaviour observed were likely not due to a loss of receptor DNA binding, as both the WT and mutant GR showed equal binding to the promoter. Second, the SV506-523 mutant failed to show the histone H4 acetylation (H4Ac) and RNA Polymerase II (PolII) recruitment which was observed with the WT receptor (fig 22), which offers an explanation for the decreased ability of the SV506-523 to activate transcription from this promoter, relative to the WT GR. This suggests that this mutant has a decreased ability to recruit one or more necessary cofactors to the promoter, likely including a histone acetyltransferase (HAT). This would lead to the loss of H4Ac observed, the promoter would stay in the ‘closed’, compacted and transcriptionally inactive form, and recruitment and activation of a functional transcription complex would be decreased (Shahbazian 2007, Kishimoto 2006).

GR is reported to interact with several HAT proteins, including p300, P/CAF, SRC-1 (although the latter has minimal HAT activity) (Glass 2000, Li 2002, Kinyamu 2004), and CLOCK (Nader 2009). Whether the decrease in GR nuclear retention and MMTV histone H4 acetylation are directly caused by the same factor is unknown, nor, if a specific HAT is involved in both effects, is it known whether the HAT activity or simply the protein interaction is significant for GR retention. This question might be answered by mutation or truncation of the candidate interacting proteins to remove their GR-interacting or HAT-activity domains. Mutants would be co-transfected into cells along with GR and alterations in retention or transcription relative to that seen in controls using GR and wild-type HAT could be measured. In the event that none of the candidate HAT proteins appears to be involved in GR retention, perhaps a broader approach could be tried. It is reasonable to assume that the decrease in GR nuclear retention seen in hinge mutants is due to decreased interaction with an unknown factor. An individual knockdown approach using an shRNA library might be used to determine which factors within a cell affect GR retention / export in
a hormone withdrawal assay. Alternatively, a comparison of proteins immunoprecipitated by WT and retention mutant GR might provide some candidate bands which could be sequenced using mass spectrometry.

MMTV promoter activation by GR also requires Oct 1/2 recruitment (Prefontaine 1998-99). Oct-1 and Oct-2, transcription factors which are members of a group of POU-domain proteins, have been shown to interact with PR, AR and GR via their POU-domains (Prefontaine 1999), and in AR it has been shown that Oct-1 binding increases the recruitment of SRC-1 to the MMTV promoter (Gonzalez 2001). Similarities in GR Oct-binding and retention deficient mutants in transcription and post-withdrawal localization assays (fig 21) suggest that while Oct1/2 is not likely to be the sole cause of GR nuclear retention, it may be implicated in both retention and promoter-specific transactivation aspects of GR function, and participation in a multiprotein complex would be consistent with my findings. Given that loss of GR-Oct interaction seems to produce only a partial retention phenotype, one of two possibilities seems likely. Oct (or more specifically, POU-domain proteins) may not be the sole, necessary and sufficient retention factor we had hoped to find, but it may be involved in a retention complex with GR and at least one other factor, such that the loss of Oct binding weakens but does not completely disrupt the complex and consequently GR nuclear retention. It is within possibility that such a complex might also contain other cofactors necessary for transcription on the MMTV promoter, which would explain the promoter specific decrease in transcription observed with loss of retention. Against this theory however, is the fact that unlike the other SHRs listed, MR does not interact with Oct or require it for activation of the MMTV promoter (Prefontaine 1999). This is particularly interesting, as MR and GR both respond to corticosteroids, and the MR hinge region has the closest sequence similarity of the SHRs to that of GR. The nuclear retention
of the WT MR appears to be as strong as that of GR, if not stronger (Walther 2005), and mutation of the MR NL1 lysine cluster, mimicking the GR NL1- construct, appears to decrease the nuclear retention of MR (Walther 2005), suggesting that MR and GR nuclear retention is likely to occur via a similar mechanism. Thus, although an Oct-binding mutant of GR shows a partial loss of nuclear retention (fig. 21), it seems probable that unless GR and MR nuclear retention use different mechanisms, any association of Oct interaction and nuclear retention observed with GR is due to an overlap of Oct-binding and nuclear retention sequences, or an indirect effect of Oct binding, rather than to any significant role of Oct in GR nuclear retention.

Although the particular HAT or HATs required for H4 acetylation of the MMTV promoter and this activation are not known, a possible role in retention for the p160 co-activator Src-1, even though it does not have a strong HAT activity, is a particularly interesting possibility. This protein is known to interact with GR, as well as other SHRs, in a cell- and promoter-specific manner, where it is thought to function as a platform for other interactions (Trousson 2007). One study found a connection between Oct-1, Src-1, and the AR and GR receptors, noting that the Oct-SHR interaction increased the recruitment of Src-1 to the promoter (Gonzalez 2001). Different reports have looked at the interaction of Oct proteins and GR in solution, and each has been suggested to recruit the other to the promoter, although their binding to each other and the promoter appears to be mutually exclusive (Prefontaine 1998, 1999). Perhaps a ternary complex of these two proteins along with Src-1 forms in the nucleus, and aids in efficiently activating relevant promoters. One current model, based on fluorescence imaging of live cells containing an MMTV array which allows real-time visualization of GR interaction at the promoter, suggests that GR and
its cofactors are all rapidly exchanging on and off the promoter site (McNally 2000, Hager 2004), and that transcriptional activation may be stochastic, an exercise in probability rather than the sedate, ordered event suggested by ChIP assays (Voss 2009). The prior interaction within the nucleus of multiple factors required for activating transcription could then be a method of increasing the probability that the correct factors will be recruited to the promoter in the right order for transcriptional activation to occur. The second possibility, not necessarily mutually exclusive with the first, is that the Oct binding and nuclear retention sequences in the GR hinge are actually separate but overlapping signals, much as the retention and NL1 sequences seem to be. Thus the retention mutants would weaken Oct interaction with GR, which would in turn affect MMTV promoter activation, but not completely disrupt it, whereas the C500Y mutant blocks Oct interaction and MMTV activation, but has only a small effect on retention, due to the overlap between Oct-binding and nuclear retention sites. This possibility would also agree with the theory that nuclear retention is partially context dependent, as exemplified by the differences in retention seen between the WT GR, the nNL1 construct with N-terminal GR NL1 and their respective SV40 substitution mutants, the nSV40 and the SV511-517.

A significant decrease was seen in the ability of the retention mutant GR constructs to activate transcription from the MMTV promoter relative to the WT GR, and this decrease was observed at varying levels of GR transfection, hormone concentration, and length of treatment (figure 16 and data not shown). These experiments were subsequently repeated with a synthetic 3xGRE promoter as well. While the MMTV promoter requires Oct and NF-1 binding for activation, the GRE requires neither of these, and has a TATA-like region rather than the TATA-box of the MMTV. The 3xGRE reporter was therefore chosen with the aim of determining whether effects of the mutations on the same cofactor interactions
might account for both the retention loss and decrease in transcription observed, and whether the decrease in transcription was due to the loss of retention or was a separate effect. Under the same conditions as used for the MMTV promoter, there was no significant difference observed in the ability of the WT and retention-mutant constructs to activate transcription from the 4xGRE promoter (fig 18). Although significant participation of the Oct cofactors in retention was later ruled out (figure 21), the equal ability of the WT and retention-mutant GR to activate the 4xGRE reporter does indicate that alterations in transcription at the MMTV promoter are not directly an effect of decreased nuclear retention.

The promoter-specific effect of nuclear retention mutants on transactivation suggests that nuclear retention of GR is important for the activation of a subset of endogenous genes, presumably those requiring a cofactor which is also involved in GR nuclear retention. This in turn raises two questions about the relationship between retention and GR regulation: first, is this subset of endogenous genes likely to be involved in a particular aspect of GR function such as differentiation or homeostasis? And second, could this subset of genes, if identified or defined via a microarray, ChIP-on-chip or similar genome-wide search, give us a clue as to the involvement of a cofactor or class of cofactors in nuclear retention and transcriptional activation of both GR and other proteins? It seems logical that there would be an organized, perhaps predictable system to co-factor interactions and promoter specificity, which would be of use in understanding and exploiting the regulation of GR and other proteins, but there is not enough evidence yet to say whether this is indeed the case. The recent results of Meijsing et al. seem to suggest that the promoter GRE sequence may be key for such regulation. Their findings indicate that small differences in the binding site sequence of the promoter can induce allosteric changes in GR conformation which in turn influence regulatory activity, presumably through alteration of cofactor affinity (Meijsing 2009).
Retention Mechanism:

Although I have not been able to identify a specific factor or factors that mediate GR nuclear retention, a number of clues have been amassed which should provide a good starting point from which future work on GR nuclear retention can proceed. Thus far, the evidence suggests that a coactivator protein is likely to be implicated, but it is possible that the solution may be more complex. Briefly, the following points arising from my research and others’ will have to be accounted for in any solution for the mechanism by which GR nuclear retention is accomplished: the mobility of GR within nucleus, the fact that retention of the mutants was lost in the presence of agonist but not with antagonist, the association between loss of retention and loss of transactivational activity on the MMTV promoter, and the promoter-specificity of the transcriptional effects, the loss of histone acetylation at the MMTV promoter with mutant GR, interaction of factor with GR hinge, and possible common interactions with other SHRs (MR, PR, AR) and other nuclear proteins.

Previously suggested mechanisms for nuclear retention in general as well as specifically of GR fall into two general categories – retention stemming from interaction with nuclear structures including the matrix or DNA, and retention stemming from interactions with mobile components (i.e. proteins) within the nucleus. For GR, a further sub-division may be made, of retention mediated directly by the hinge region, and mediated by sequence outside of the hinge region. My results have suggested that while the hinge region of GR is sufficient to impart retention on its own, other sequences may play a role as well.
Retention of proteins in a compartment has often been defined as, or assumed to include, binding to static components of the cell (Kim 2007, Jans 1998, Tang 1996, Faul 2005, Hoshino 2007). GR has been found to interact with these components, either transiently (Tang 1998) or more continuously (Tang 1996, Deroo 2002), and such interactions have been found to include basic sequence (Kim 2007) and GR sequence in or near the hinge region (Tang 1998, Schaaf 2003). It is difficult to envision, though, how transient interactions (necessarily transient, based on GR intranuclear mobility) would be capable of blocking GR export to as marked a degree as has been observed without some other factor being involved which could hinder export when GR is not interacting with static nuclear components such as the matrix or chromatin. Differences in mutant behaviour after agonist versus antagonist withdrawal likely have their root in altered receptor configuration, which could lead to changes in interaction with both cofactors and fixed nuclear components – indeed, differences in both have been indicated previously (Kroe 2007, Schaaf 2005, Htun 1996) – but promoter-specific differences in transcriptional activation between WT and mutant GR seem more likely to result from alterations in cofactor binding than matrix or DNA interaction.

The static components of the nucleus seem less likely than cofactors to be major mechanisms of GR nuclear retention or of the phenotypes observed in retention-mutant receptors. However, some involvement may be postulated. GR interacts with the matrix via its hinge/early LBD region (Schaaf 2003, Tang 1998). Interaction can be increased in the absence of ATP, and appears to require proteasomal activity to disrupt the interaction (Tang 1996, Deroo 2002). GR has also been shown to interact with matrix protein hnRNP U (Eggert 1997). Mutations producing a decrease of DNA-binding ability have been shown to increase the export rate of GR (Sackey 1996). As a segment of hinge region in an
exogenous protein was still capable of causing nuclear retention – indeed, partially
overriding the Rev NES also present in the protein – involvement of chromatin in GR
nuclear retention would infer that the hinge itself is capable of specific chromatin
interactions. Some minor support for this theory is given by the finding that part of the hinge
region of the peroxisome-proliferator activator receptor gamma (PPARg), another steroid
receptor, interacts with the minor groove of DNA in a crystal structure of a PPARg / RXR
(retinoid X receptor) dimer bound to DNA (Chandra V 2008). However, as WT and
retention-mutant GR are both capable of binding to DNA (figure 22), and can activate
transcription to the same extent on some promoters (figure 18), differences in retention are
likely not significantly regulated by DNA interaction of the GR.

There is some evidence for proteasome involvement in GR nuclear mobility, activity
and trafficking. Treatment with the proteasome inhibitor MG132 has been shown to
immobilize a fraction of the unliganded receptor via the hinge region (Schaaf 2003), and
inhibition of proteasomal activity has also been shown to decrease GR-mediated
transactivation, and intranuclear mobility, while increasing association with the nuclear
matrix (Deroo 2002). It is thought that perhaps proteasomal activity is required for
dissociating GR from nuclear structures involved in transactivation, as proteasome activity
has been shown to be required for the rapid exchange of GR on and off the promoter which
is observed in photobleaching experiments (Stavreva 2004). However, intranuclear mobility
and promoter residence time have also been shown to be positively correlated with
transcriptional activity, and influenced by ligand character (Schaaf 2003, Stavreva 2004,
Wang 2006), suggesting that mobility is the outcome of multiple factors which impact on
GR activity. If the proteasome is indeed involved in the decrease in transactivation and
increase in export noted for the GR retention mutants, it seems logical that intranuclear
mobility will also be impacted, based on the papers cited above. As well, if mutation of the hinge region impacts proteasomal effects on the GR, then proteasomal inhibition should affect transactivation and mobility of the WT but not the retention-mutant GR. I would hypothesize that the retention-mutant GR will ordinarily have a higher intranuclear mobility and exchange rate on and off the promoter than the WT GR, and that under conditions of proteasomal inhibition, the retention mutants will maintain a higher level of intranuclear mobility than the WT. The direction of the effect of proteasome inhibition on transcriptional activation is more difficult to predict, given that the previous studies had contradictory results, but could be determined using the system described in this work for measuring transcriptional activation from a reporter, and comparing results with or without treatment with proteasome inhibitor MG132. Intranuclear mobility is generally measured using photobleaching or photoactivating techniques on live cells, and requires a microscope capable of measuring in a scale of milliseconds, beyond the technical capability of the equipment used in this work, so this could not be tested here.

Based on my experimental evidence, it seems likely that interaction of GR with a soluble cofactor could account for both the fundamental mechanism of GR nuclear retention, and either directly or indirectly, the effects accompanying loss of retention in the mutant receptors. Of the cofactor proteins reported to interact with the hinge region of GR, some are classed as co-activator proteins, while a larger group are defined as co-repressor proteins. A co-repressor as retention factor would make it difficult to explain the promoter-specific decrease in transactivation seen in my retention mutants by the theory that the retention mutations might be decreasing GR interaction with a specific co-factor needed for activation of the MMTV promoter. Mutation within the hinge region produced a receptor which was
less competent than WT or similar to WT GR for activating transcription, depending on the
promoter examined – a result which seems more likely to reflect a loss of coactivator than an
increase of corepressor binding. The fact that in hormone-withdrawal assays, antagonist
treatment increased retention or decreased export of the mutants suggests that the receptor
configuration or cofactor interactions produced by antagonist binding produces a similar
effect to the nuclear retention seen with the agonist, but via a different mechanism, as it does
not appear to be influenced as strongly by mutation of the hinge region. Antagonist binding
recruits a different range of co-factors than agonist, and produces a different configuration in
the LBD (Kroe 2007, Garside 2004, Wang 2004). Thus, the retention seen in the mutant
constructs in the presence of antagonist could be due to a block on NES accessibility or
export machinery binding by a corepressor, or by changes in configuration of the GR leading
to altered interaction with chaperone proteins or coactivators, either blocking export
machinery directly, or by altering post-translational modifications which may be necessary
for export. A related indirect interference with retention and export through interdomain
communication, stemming from altered configuration of GR and leading to allosteric effects
on interaction with export or retention factors, could then be the explanation for effects on
GR retention and export observed with mutations to DBD or LBD, or Hsp90 inhibition

The MMTV promoter is certainly the best known system used for testing GR
activity, but GR is involved with such a range of promoters and cofactors that it is certainly
possible that the use of another promoter for reporter assays might give different results to
what was previously observed, as was exemplified with the transcription results using the
3xGRE promoter. As well, certain GR cofactors have been shown to exhibit both activating
and repressive activity, depending on promoter and conditions. For instance, HDACs are
generally supposed to act as repressors, yet their inhibition can decrease GR’s ability to activate transcription (Ito 2006, Qiu 2006). BAG-1/RAP46 cofactors can repress GR, but are also thought to have a positive role (Schneikert J 1999, Kullman 1998, Cato 2001). However, corepressor interaction with the hinge region might be predicted to have an effect on the distribution of the receptor within the cell, given that the hinge houses a strong nuclear import signal and has been found to contain nuclear retention activity as well. As well, both binding of factors to the hinge/DBD region and altered cellular distribution might be predicted to have an effect on transcriptional activity of GR.

The overlap of nuclear localization and nuclear retention activities in the hinge region, and the observation that even with the addition of an N-terminal NLS to the NL1-GR, the retention-deficient mutant receptors of this type were less efficient at importing into the nucleus suggested that perhaps a member of the chaperone/importin complex plays a role in GR nuclear retention. Hsp90 is the most obvious candidate here for several reasons. While Hsp90 was originally thought to interact with GR mainly in the cytoplasm, later reports give it a nuclear role as well. Hsp90 has been reported to co-localize with GR in the nucleus, to be required for GR nuclear mobility (Elbi 2004), and to have a potential role in retention, as Hsp90 inhibitors can increase the export rate observed for GR (Tago 2004). Further, Hsp90 appears to have a role in stabilizing GR interaction with the promoter and increasing its transcriptional activation (Stavreva 2004), thus it is involved in several activities which were observed to be altered in retention-deficient GR mutants. Opposing this notion, Hsp90 would have to be functioning indirectly as a retention factor, by hindering interaction of GR with the export machinery. Hsp90 is known to interact with the LBD of GR (Fang 2006, Ricketson 2007), and although it has been postulated to interfere with NL1 exposure (Scherrer LC 1993), Hsp90 has not been reported to interact with the hinge region of GR,
thus could not be directly impacting either the nuclear localization or the nuclear retention activity identified there, but would need to be acting via steric hindrance or stabilization of the retained complex.

Another candidate for a nuclear retention factor besides Hsp90, based on previous studies, could be a PIAS protein. Perhaps relevant is the finding that the progesterone receptor, a close GR relative, undergoes sumoylation by PIAS3 on hinge lysines analogous to those of GR. This is reported to decrease PR nuclear retention, DNA-binding, and transactivational ability (Man 2006). This may not be fully applicable to GR, of course, given that recruitment of PIAS3 to PR and its promoters is reported to be hormone dependent (Man 2006), and that sumoylation remains to be demonstrated in the hinge region of GR (Tian 2002). However, PIAS proteins are known to interact with GR, with PIASxb and y functioning as co-activators and PIAS1 as a co-repressor on the MMTV promoter (Tirard 2004), and it is possible that these interactions may be promoter-specific, given that other cofactors such as the p160/Src proteins and Mediator proteins are known to show promoter and cell-type specific recruitment (Trousson 2007, Chen 2006).

As shown earlier in the discussion, work on the octamer transcription factors (OTF or Oct) suggested that these might be linked to nuclear retention of GR due to interaction with the GR hinge region and Oct requirement on the MMTV promoter (Prefontaine 1998, 1999), but abrogation of Oct-GR interaction had only a small effect on GR export rate post hormone-withdrawal, eliminating Oct from consideration as a major mechanism of GR nuclear retention (fig. 20). Further, the mineralocorticoid receptor (MR), which exhibits a strong nuclear retention, does not require Oct for MMTV promoter activation (Prefontaine 1999), making a role for Oct in retention of SHRs unlikely.
Mutation of GR abrogating its nuclear retention appears to work largely through increasing the export rate of the receptor, given that liganded retention mutants show increased transfer between nuclei in FRAP assays, but maintain a predominantly nuclear localization, and ligand-withdrawn mutants show an increased return to the cytoplasm over time. The precise mechanistic reason for this increase has not been defined, however. It is thought that GR export is mediated in part via the classical CRM1 pathway (Liu 2000, Savory 1999), and it has been shown that CRM1-driven export is capable of exporting cargo much more rapidly than the normal rate observed for GR, as exemplified in the rates observed in the NES-GFP-GST-NLS construct used in our lab. Coupled with the apparently normal intranuclear mobility of both WT and retention mutant GR, this suggests that under normal conditions, GR may interact with one or more factors such that the export signal is masked or inaccessible by the export machinery, and that these interactions are altered in the retention mutants to allow greater interaction of the receptor with the export machinery. As export of GR has been reported to involve the DBD (Black 2001, Guiochon-Mantel 1994), it is also possible that intramolecular interactions, or interactions with cofactors, involving both the hinge and DBD of GR, normally block export. This would provide an explanation for why mutation of either the hinge or DBD has been reported to increase nuclear export of GR (Guiochon-Mantel 1994, Black 2001). These results were gathered using techniques such as cell fusion, however, which might be predicted to disturb cell compartmentalization and/or activate the calreticulin export pathway, rather than having export occur under conditions closer to those found in vivo. Work in our lab places the GR export signal in the ligand-binding domain, and the presence of both a putative export motif and co-factor binding sites in the same domain seems reasonable, as the two functions are likely to be linked in terms of receptor regulation.
In the course of my experiments, I found that the GR mutants with decreased nuclear retention showed this phenotype both when liganded (FRAP assays) and after ligand withdrawal (withdrawal assays) of hormone agonist, while withdrawal assays using antagonist resulted in nuclear retention of both WT and retention-mutant GR. This seems to indicate that for agonist at least, the key interactions involved in nuclear retention must be at least partially independent of the actual liganded status of the receptor, and may indicate that retention is mediated by or requires an alteration in the receptor configuration that is induced by ligand binding and remains after withdrawal of ligand. This would provide an explanation for the receptor mutants treated with the antagonist RU486, which do not appear to undergo export to the same extent as their agonist-treated counterparts post hormone withdrawal. Antagonist treatment is known to induce a different configuration of the hormone-binding domain of the receptor than agonist treatment does, and encourages recruitment of co-repressor proteins rather than co-activators (Garside 2004, Kroe 2007, Wang 2004). Work within the Haché lab (unpublished) as well as others (Qi M 1989) supports the notion that ligand-withdrawn receptor does not revert to its naïve configuration, and that this configuration is different for agonist and antagonist treatment. Given that the receptor retention mutants showed a loss of retention in both the presence and absence of hormone, but had their export curtailed after treatment with antagonist, it is possible that the RU486-induced configuration blocks interactions with export machinery, either directly or as a result of co-repressor recruitment, while the decision of retention vs export in the agonist-induced configuration involves competition between cofactors and export machinery, with the decreased cofactor interaction in the retention mutants leading to increased export. This may account for a previous observation made in our lab. FRAP assays were done on a series of
mutations within a possible nuclear export signal (NES) in the LBD, in order to determine whether these mutations affected GR nuclear export. Rather than the expected decrease in export rate, an increase in export was observed. As it was unlikely that the mutations had increased NES activity, this suggested that these mutations might be interfering with ligand or co-factor interactions, which both take place in the LBD, through either direct binding site alteration or configurational change. Decreased interaction with other intranuclear factors could then conceivably lead to increased export machinery interaction. (unpublished, Haché lab).

Most of my experiments utilized either dexamethasone or cortisol, both GR agonists which produced very similar results. However, a wide range of corticosteroid ligands - agonists, antagonists, mixed antagonists, and new ligands (SEGRAs) which attempt to discriminate between the target genes activated in order to refine control of receptor function – exists (Wang 2006, McMaster 2007, Stahn 2007). It might be interesting, in view of the results obtained with antagonist, to determine whether different ligands impact the phenotype of retention mutants, and whether there is any connection between nuclear retention and the subset of genes activated by the receptor via the cofactors recruited. This might ultimately prove to be useful in determining which types of glucocorticoids will be most useful in treating particular symptoms or patients. Indeed, support for this notion comes from a recent study showing that treatment of diabetic obese mice with RU486, although it did not affect weight or food intake, was able to improve blood glucose and insulin responsiveness, as well as decreasing transcription from 11b-HSD, PEP-CK, glucose-6-phosphatase, and LPL genes (Taylor 2009). It is known that the configuration of the ligand leads to allosteric configuration changes in the LBD of steroid receptors which can impact cofactor recruitment (Wang 2006, Alvarez 2008). Different ligands have also
been shown to alter the intranuclear distribution of GR, (the degree of speckling or foci observed) (Schaaf 2005), and this is hypothesized to have some connection with the interactions and activity of the receptor, although no definite conclusions have been reached regarding the role of these nuclear speckles (Hager 2000, Feige 2005).

Mutations leading to a decrease in nuclear retention of GR show a promoter-specific decrease in transactivation, and can be postulated to have defects in cofactor recruitment, possibly through altered post-translational modification or configuration of the hinge region. Further work on the question of GR nuclear retention might profitably address the question of what effect these mutations might have at the level of the whole organism. GR is expressed in many cell types, and it is thought that cell-specific and gene-specific effects may depend at least partly on the availability of particular cofactors, and promoter-specific requirements for particular complexes (Rogatsky 2003, Adams 2003, Luecke 2005, Chen 2006, Szapary 1999, Obradovic 2004, Trousson 2007). Thus, it could be predicted that one would also observe cell-type- and gene-specific effects as a result of a decrease in nuclear retention. Although several natural mutations of GR have been recorded which are associated with partial GC resistance and HPA axis alterations, none of these overlap with residues thought to be involved in retention (Bray 2003), which could suggest a critical role for retention in GR function. Natural GR mutations in humans have been previously found to affect stress response, mood disorders, and obesity (Charmandari 2004, Bray 2003), and studies with rodents have linked decreased levels of hippocampal GR with decreased anxiety and stress response and increased learning/cognition and exploration (Tronche 1999, Brinks 2007).

Conclusions:
The existence of GR nuclear retention was first recorded well over a decade ago (Cadepond 1992, Lacasse 1995). It has been described as playing a role in transactivation (Cadepond 1992), and in protein stability, and has been suggested to improve efficiency of response to a secondary hormone challenge (Liu 2000). Studies in various labs have noted that mutation of DNA- and ligand-binding domains of GR, or disruption of Hsp90 interaction, could decrease GR nuclear retention (Sackey 1996, Tago 2004, unpublished, Haché lab). The Haché lab identified a 26 residue segment of the GR NL1/hinge region which was capable of conferring retention on an exogenous protein, and showed that three core lysines of the NL1 were involved in nuclear retention of GR (Savory 1999, Haché 1999, Carrigan 2007). My work has attempted to expand on these results, both to improve our knowledge of the mechanism and function of GR nuclear retention, and examine the possibility that my findings on nuclear retention may be more generally applicable to nuclear proteins besides GR. This will hopefully aid future researchers in understanding and exploiting nuclear retention as a general method of protein regulation.

Within the 26-residue hinge region containing retention and NL1 activity, I have identified a KT sequence near the initially identified NL1 lysines as being involved in nuclear retention. A survey of other NLS sequences suggests that the KT sequence or spacing of the lysines may be associated with nuclear retention in other proteins as well. I have also noted that the context of the hinge/NL1 residues is significant in determining the degree of nuclear retention observed, as shown in the LE-AA, nNL1, and nSV40 constructs. The nuclear retention activity centered in the hinge region appears to overlap with other signals, including co-factor interaction and nuclear localization. This concentration of functions in a small area suggests that they will be tightly regulated and at least partly interdependent,
providing a rationale for the lack of natural mutations occurring in the hinge region.

Mutations in the hinge which compromise retention also have a promoter-specific effect on transcriptional activation by the GR, which may have a basis in alteration of promoter acetylation, based on the presence of acetyl-lysine sites within the residues found to have an effect on retention (Nader 2009, Ito 2006), and ChIP results showing decreased MMTV promoter acetylation with mutant GR. Although this topic has only lightly been explored in the experiments presented here, the results suggest that retention is mediated by one or more cofactors. Altered interaction of the GR with cofactors may be responsible for promoter-specific transcriptional effects, and directly or indirectly for the decrease in acetylation seen at the MMTV promoter in the presence of retention mutant GR. Much remains to be determined, however. The cofactors involved in retention need to be identified, and the basis for the retention mutants’ promoter-specific effect on transcription determined. As well, the possibility of residues involved in GR retention functioning as a broader retention signal sequence for a range of proteins requires further verification. Further work with different, endogenous promoters, and other steroid hormone receptors and NLS sequences would aid in understanding the general and specific implications of nuclear retention as a form of regulation.
Appendix A: Retention as a Property of NLS and the Role of Proline:

Results:

Although the GR retention mutants made by in-line substitution of SV40 NLS sequence for GR sequence indicated that the 511-512KT residues of GR are important in GR nuclear retention activity, a second possibility can also be envisioned. The exchange of SV40 sequence for that of GR in mutant receptors meant that the 511-512KT was substituted for by a PP sequence from SV40, which alters charge and size of the sequence significantly. Proline is a bulky amino acid, and has been referred to as a helix-breaker due to the fact that it is the only amino acid whose side chain loops around to interact with the amino group of the molecule, which introduces a kink in the peptide chain. This suggested that the results observed could actually stem from either of two possibilities: one, that it is indeed the KT sequence that is important for retention, and the proline is merely a coincidence, or two, that nuclear retention is acting as a default mechanism, so that a basic NLS, lacking proline residues, should exhibit nuclear retention regardless of its exact sequence. In order to distinguish between these two possibilities, as well as to determine whether my findings on nuclear retention were applicable to other proteins, I decided to test a selection of basic NLS sequences for nuclear retention. A list of known basic NLS sequences was compiled from publications, and 5 monopartite sequences of these were selected (Rac1, NUCKS, LXR, IFNγR, and VDR), along with the SV40 NLS and the GR NLS as controls (fig 23). It is perhaps noteworthy that of a total of 50 monopartite and functionally monopartite sequences surveyed, 15 contained at least one proline residue while only 6 contained a KT combination (and 2 had an RT combination). Furthermore, proline is included in the definitions of types
Figure 23: Sequences of NLS selected for determination of nuclear retention properties of KT and PP sequences.
Top section: previously used NLS from SV40 and GR, with prolines highlighted in red, and KT in blue, core basic sequences roughly aligned. Middle section: NLS sequences containing proline residues. Bottom section: Sequences containing KT but no proline, and neither proline nor KT sequences.
<table>
<thead>
<tr>
<th>NLS source</th>
<th>NLS sequence</th>
<th>Anti Export Activity (Nuclear Retention)</th>
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<tbody>
<tr>
<td>Previously used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV40 NLS GR NL1</td>
<td>PPKKKRKV RKKKKIK</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Proline-containing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rac1 NUCKS LXR</td>
<td>PPPVKKRKRK PPTKIRSS PERKRKKGPAP</td>
<td>?</td>
</tr>
<tr>
<td>KT or neither</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ VDR</td>
<td>AKTGRKRK FRRSMKRKA</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td></td>
<td>?</td>
</tr>
</tbody>
</table>
Figure 24: Preliminary FRAP results showing retention activity of selected NLS. FRAP assays were performed using the same conditions and the same backbone and control constructs described for the hinge mutants in figures 6 and 7.
of basic NLS sequence (Macara 2001). This combination of circumstances encouraged the
notion that the proline may be a determining factor in the nuclear retention characteristics of
a given NLS. Three of the NLS sequences selected contained proline residues in various
positions (Rac1 with 3 and NUCKS with 2 at the beginning of the NLS, and LXR with 1 at
the beginning and 2 at the end), one (IFNγR) had the KT sequence in approximately the
same location as GR, and one (VDR) had neither prolines nor a KT sequence. These NLS,
along with sufficient of their surrounding sequence to produce a fragment which
corresponded in both length and position of the NLS to the GR sequences previously used in
this context, were cloned into the NES-GST-GFP-NLS construct that was initially used to
investigate nuclear retention (see fig 6a for diagram). The behaviour of these constructs was
then investigated via FRAP assays. Preliminary results obtained suggest that the presence of
the KT sequence is indeed important for retention, while proline may play a varied role with
respect to its effect on nuclear localization and retention of proteins (fig 24). Specifically, the
IFNγR sequence appears to be transferring between nuclei in FRAP experiments with similar
or slower kinetics than the WT GR, whereas the LXR and Rac1 sequences transfer in a
similar manner to the control construct containing a random oligonucleotide.

Discussion:
In order to determine whether residues identified by my work were indicative of a
more general system with respect to linkage between NLS and nuclear retention, several
basic NLS sequences were selected for testing in FRAP assays. These were all inserted into
the same backbone previously used (pNLS-GST-GFP-NES, fig 6), and produced in the same
way, then verified by sequencing. Unfortunately, not all of the NLS selected to look for a
possible general criterion for retention in NLS sequences were expressed in transfected cells. Preliminary results from FRAP assays for the purpose of determining nuclear retention properties of the interferon gamma receptor (IFNgR), Rac1, and liver X receptor alpha (LXR) nuclear localization sequence regions did suggest that IFNgR showed nuclear retention, while LXR, and to a lesser degree, Rac1, lacked retention. The KT sequence is present in both GR and IFNgR, and the IFNgR NLS region appears to exhibit nuclear retention similar to that of GR in FRAP assays (Appendix A, figure 24), indicating that this may be a more general requirement for retention. However, as several of the GR-related hormone receptors (MR, AR, PR) which are thought to have some nuclear retention activity (Walther 2005, Man 2006, Poukka 2000) contain a KK rather than a KT sequence, it is possible that the position of the lysine relative to the core NLS rather than specifically the KT sequence is significant, and may play a role in stabilizing receptor interaction with a factor or factors involved in nuclear retention.

Rac1 and LXR both lack the KT sequence, but contain proline residues around the NLS core. A search turned up no prior information on Rac1 retention, but a previous paper on LXR showed NLS mutation to lead to decreased retention/increased export in digitonin permeabilization assays and altered levels of basal transcription at a reporter gene. They also showed that retention was dependent on agonist/antagonist type (with approximately the opposite results as would be obtained from GR (fig 15), having highest retention in the absence of hormone and lowest in the presence of antagonist), and was likely mediated by cofactors (Prufer 2007). Although the VDR construct, whose NLS contains neither proline nor a KT sequence, could not be tested in our system, some previous work suggests that the WT VDR protein lacks nuclear retention (Prufer 2002). The second construct which was selected but did not express contained the NLS from NUCKS. Although its nuclear retention
has not been studied, it has been found to exhibit cell-cycle dependent localization, and to require the proline in its NLS for efficient nuclear localization (Grundt 2007). The combination of my FRAP data and published information indicates that it is possible that the presence of proline may play a variable role, or be irrelevant, in nuclear retention. Depending on the number and context of the proline residues present, either an increase or a decrease in nuclear localization and retention may be indicated. A wider test group of NLS sequences would be necessary to draw any firm conclusions about a possible impact of proline on nuclear retention.
### Appendix B: Oligonucleotide sequences used in cloning:

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<th>Primer Sequence</th>
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<td></td>
<td>rev RKT-OX</td>
<td>TC GAC TCC TGC AGT GGC TTG CTG AAT CCC Pst I TTT GAT TTT TTT CTT AGC GGC CGC TGC TTC AAG GTT CAT TCC AGC CTG AAG ACA C</td>
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<tr>
<td>IKG516-518AAA</td>
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| GR FL LE-AA | LEA-FWD | CGG AAA TGT CTT CAG GCT GGA ATG AAC  
R K C L Q A G M N  
GCC GCG GCT CGA AAA ACA 
A A A R K T  
Sac II  |
| LEA-REV | TGT TTT TCG AGC CGC GGC GTT CAT TCC  
Sac II  
AGC CTG AAG ACA TTT CCG |
| GR FL SV506-523 | NL/RA FWD | (MNL to MRA, intro BssHII site)  
GT CTT CAG GCT GGA ATG CGC GCT GAA GCA  
L Q A G M R A E A  
CGA AAA ACA AAG  
R K T K  |
| NL/RA REV | CTT TGT TTT TCG TGC TTC AGC GCG CAT TCC  
Sac II  
AGC CTG AAG AC |
| SV-KD FWD | CGC GCG CAC AGC ACG CCT CCG AAA AAG AAG  
R A H S T P P K K K  
AGG AAA GTG GAG GAC CCG TTC CC  
R K V E D P F |
| SV-KD REV | TGC AGG GAA CGG GTC CTC CAC TTT CCT CTT  
CTT TTT CGG AGG CGT GCT GTG CG |
| RA/NL FWD | (Change RA back to NL)  
GT CTT CAG GCT GGA ATG AAC CTG CAC AGC  
L Q A G M N L H S  
ACG CCT CCG  
T P P |
| RA/NL REV | CGG AGG CGT GCT GTG CAG GTT CAT TCC AGC  
CTG AAG AC |
| GR FL I516R | I516Rins FWD | GG AAA TGT CTT CAG GCT GGA ATG AAC CTT  
K C L Q A G M N L  
GAA GCA CGA AAA ACA AAG AAA AAG CGG AAA  
E A R K T K K K K  
GGG ATT CAG CAA GCC ACT GCA  
G I Q Q A T A |
| I516Rins REV | GT GGC TTG CTG AAT CCC TTT CCG CTT TTT CT  
TGT TTT TCG TGC TTC AAG GTT CAT TCC AGC  
CTG AAG ACA T |
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<td>IFN gamma IFNG R</td>
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<td>LXRRalpha</td>
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<td></td>
<td>CCG AAG CCT AAG ATG CTC GCC AAC G P K P K M L G N</td>
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<td>MMTV promoter</td>
<td>MMTV -270</td>
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<td>(forward)</td>
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<td>MMTV +84</td>
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### Appendix C: Reagents and suppliers:

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<td>Bio-Rad Bradford Protein Assay Reagent</td>
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<tr>
<td>Cortisol</td>
<td>Steraloids Newport, RI, USA</td>
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<tr>
<td>Dexamethasone</td>
<td>Steraloids Newport, RI, USA</td>
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<td>FBS</td>
<td>Wisent, St. Bruno. QC</td>
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<td>FiGR primary antibody</td>
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<tr>
<td>Fugene 6</td>
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</tr>
<tr>
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<td>Clontech, Missisauga, ON</td>
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<td>GR M-20 primary antibody</td>
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<tr>
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<td>PVDF membrane</td>
<td>BioRad, Missisauga, ON</td>
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<tr>
<td>RU-486</td>
<td>Roussel-Uclaf, France</td>
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<td>Sheep anti-rabbit HRP-conjugated secondary antibody</td>
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<tr>
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<td>-------------------</td>
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<tr>
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<tr>
<td>Western Lightning Chemiluminescence Reagent</td>
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Appendix D: GR hinge region sequence conservation:

Conserved between species:

CLQAGMNLEARKtKK–kIKGiQq–ttg

Trout GR     CLQAGMNLEARKNZKLIRLKGQQTTMEP
Frog GR      CLQAGMNLEARKT–KK–KIKGIQQSTTA
Rabbit GR    CLQAGMNLEARK–KK–KIKGIQQTSTG
Human GR     CLQAGMNLEARKT–KK–KIKGIQQATTG

Rat GR 500 CLQAGMNLEARKT–KK–KIKGIQQATAG 525

Rat MR     CLQAGMNLEARKSKKLGKLGLHEEQPQ
Rat AR      CYEAGMTLGARKLKLGLNLK–LQEEGEN
Rat PR      CCQAGMVLGGRKFKFNKVR–VMRALDG

ClqAGMNLeRK–KKgLklgLqee–g

Conserved between receptors:

Red capital letters – conserved in all sequences listed in group.
Small black letters – conserved or semiconserved in at least half the sequences listed.
Bibliography:


through the nuclear pore complex is mediated by its interaction with Nup62 and importin beta. Mol. Cell. Biol. 29, 4788-4797.


Galigniana, M.D., Radanyi, C., Renoir, J.M., Housley, P.R., and Pratt, W.B. (2001). Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. J. Biol. Chem. 276, 14884-14889.


