The phenotypic consequences of genetic variation in the modulatory domain of the rat glucocorticoid receptor

by

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Thesis submitted to the faculty of science, University of Glasgow, for the degree of Doctor of Philosophy.

Division of Molecular Genetics

January 1998

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree

January 1998

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Abbreviations

APS	Ammonium persulphate
BSA	Bovine serum albumin
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediamintetraacetic acid
FCS	Foetal calf serum
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid
IPTG	Isopropylthio-β-D-galactoside
MOPS	(3-[N-morpholino] propane-sulphonic acid)
NBCS	Newborn calf serum
ONPG	o-Nitrophenyl-β-D-galactopyranoside
PBS	Phosphate buffered saline
PMSF	Phenyl methyl sulphonyl fluoride
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride-sodium citrate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TBS	Tris buffered saline
TEMED	N, N, N', N'-Tetramethylethylenediamine
X-gal	5-Bromo-4-chloro-3-indoyl-β-D galactopyranoside
MMLV	Molony murine leukaemia virus
DOTAP	N-[1-(2,3-Dioleoyloxy) propyl] -N, N, N-trimethyl-ammoniummethylsulphate
Taq	Thermus aquaticus DNA polymerase

Acknowledgements

My thanks to family and friends (if I still have any) for their help and support, especially my human spellcheckers (here's to the ones that got away!).

I thank my supervisors, Dr. C. J. Kenyon and Dr. R. G. Sutcliffe for their continued help, support and advice during the course of my studies.

My sincere gratitude to Dr. S. Rusconi for his expert advice in the handling of CV-1 cells and extreme generosity in providing plasmid clones, Dr. R. Leake for helpful advice and discussions and Professors Bianchi, vanZutphen and Groner for useful collaborations.

Summary

Previous investigations have reported differences in the affinity of hepatic glucocorticoid receptors (GR) for glucocorticoids between the Milan hypertensive (MHS) and normotensive (MNS) strains (Kenyon *et al.*, 1994), the Zucker lean and obese strains (White and Martin, 1990) and hypertensive SHR and normotensive WKY strains of rat (Panarelli *et al.*, 1995). To determine the possible causes of this phenotypic variation, a series of investigations were carried out into the molecular genetics of strain specific allelic variants of rat GR.

The original cloning of the rat GR cDNA (Miesfeld *et al.*, 1986) reported a polyglutamine encoding triplet $(CAG)_n$ repeat sequence in the amino terminal modulatory domain of the receptor. Given the highly polymorphic nature of simple sequence repeats, I investigated the repeats as potential mediators and as genetic linkage markers, of altered receptor function. CAG repeat length polymorphisms were identified. The GR alleles of the hypertensive strains, MHS and SHR, were found to have triplet repeat lengths shorter by one codon than in controls; MNS 21, MHS 20, WKY 20 and SHR 19-CAG repeats. GR triplet repeat lengths in Zucker rats did not differ between strains.

As part of a collaborative investigation using the Milan strains of rat, the GR triplet repeat polymorphism was used to screen for genetic linkage between *Grl* and glucocorticoid related phenotypes in F2 rats of a reciprocal MHS x MNS cross. A significant association ($p < 0.005$) was found between the MHS *Grl* allele and hypercalcuria in female rats. Homozygous MHS alleles were also found to be weakly associated ($P < 0.01$-0.02) with reduced systolic blood pressure in female and reduced body weight in male rats.

Optimised typing methods were used to characterise a further 61 inbred rat strains and substrains and 155 wild rats from England and central Scotland. A discontinuous distribution was found: no GR alleles with repeat lengths of

less than 7, 8-16 or greater than 23 CAG repeats were identified, suggesting that these alleles are either absent, or at least are significantly under-represented. In the 155 wild rats analysed, homozygotes were found for each allele, with the exception of Grl^{CAG17}, indicating that the observed alleles are compatible with significant viability. The presence of a gap in the allelic distribution of GR raised fundamental questions regarding the genetic origin of this discontinuity. The observed rGR alleles may have a selective advantage through their function as transcription factors or alternatively, may reflect a non-random mechanism by which triplet repeats expand and contract.

The reported differences in affinity for glucocorticoids of GR from hyper- and normotensive and of Zucker strains, indicated the possibility of a primary sequence difference between receptors. The coding sequence of GR was therefore determined in MHS, MNS, SHR, WKY and Zucker lean and obese rats. Each was found to differ from the published sequence. Re-sequencing of rat hepatoma cell line 6.10.2.-derived GR cDNA, which was used in determination of the original published sequence, indicated that the differences were probably due to mistakes in the original report. Apart from the polyglutamine tract, no intrastrain coding differences were found. Silent substitutions were detected at nucleotide positions 198, 531, and 711. All three were present in MHS and SHR strains, none were present in MNS and only that at position 711 was present in WKY. The GR sequences of Zucker lean and obese rats were identical.

The four sites of genetic variation identified in rat GR lie in close proximity (within about 500 bp), constituting six distinct haplotypes in which the rate of recombination is expected to be low. These haplotypes were useful in helping to confirm the genetic relationship of rat strains reported by others (Greenhouse *et al.*, 1990; Hedrich, 1990). The Milan rat GR haplotypes were distinct, suggesting the separation of GR alleles for thousands of generations before fixation in the Milan selection lines (Bianchi *et al.*, 1984).

To address the difference between MHS and MNS in GR ligand binding in liver cytosol preparations, I investigated the possible effect of differences in CAG repeat length. Accordingly, cDNA alleles, $Gr1^{CAG20}$ (MHS) and $Gr1^{CAG21}$ (MNS) were constructed and expressed in CV-1 cells, together with GR cDNAs with 7 and 18 CAG repeats. Western blotting and Scatchard analysis of steroid binding properties of the expressed GR proteins showed no differences, either in stability, or affinity (Kd) for dexamethasone and corticosterone.

Whether or not the gap in the distribution of rat GR CAG repeats reflected abnormal receptor properties, was addressed by the construction of full length GR cDNAs with 4, 8, 10, 20, and 80 CAG repeats and their expression in CV-1 cells. GR steroid binding properties were determined as for natural GR proteins. Receptor affinity and capacity (R1) was not different between alleles, implying that intra-allelic differences in the length of the polyglutamine tract are unlikely to affect receptor affinity. However, the binding affinity for dexamethasone and corticosterone was significantly higher for construct, compared with natural alleles ($p < 0.001$ for dex and B). This difference in affinity was *not* the result of inter-allelic differences in the length of the long homopolymeric repeats in the different alleles. The difference in sequence between the natural and construct GR alleles is shown in bold in the following figure for residues on the flanks of the homopolymer:

20Q (natural allele):

	66	67	68	69	70	71	72	73	74	75	76	77	78-96	97	98	99	100	101	102	103	104	105	106	107	
NH_2>	F	S	**K**	**G**	**S**	**T**	**S**	**N**	**V**	**Q**	**Q**	**R**	$(Q)_{18}$	**P**	**G**	**L**	**S**	**K**	**V**	**S**	**L**	**S**	M	G	<COOH

20Q (construct allele):

	66	67	68	69	70	71	72	73	74	75	76	77	78-98	99	100	101	102	103	104	105	
NH_2>	F	S	**T**	**L**	**A**	**C**	**G**	**S**	**L**	**E**	**E**	**D**	$(Q)_{20}$	**G**	**V**	**R**	**Y**	**G**	M	G	<COOH

Amino acid differences between natural and construct rat GR alleles.

Altered residues are shown in bold.

It is not known which residue differences contribute to the change in receptor affinity, but the phenomenon strongly implies that the structure of the modulatory domain can influence the function of the LBD, presumably through a proximity effect in the native molecule. This raises the more general question of whether the amino and carboxyl termini are able to affect each other's activity, either by contact, or through a mediator such as HSP90.

The transcriptional regulatory properties of the different GR alleles has been tested in two types of transactivation system:

1.) As activators of MMTV-lacZ

All of the GR variants (natural and construct) showed a similar ability to activate MMTV-lacZ through a GRE-containing promoter following hormonal induction. This implies that none of the structural differences between GR molecules had any measurable effect on their ability to bind a GRE and activate transcription.

2.) As modulators of STAT5

Because of the remaining possibility of tissue or promoter-specific effects involving interaction of GR with other transcriptional regulators, studies of the gene modulatory effects of GR have also been addressed. GR can interact synergistically with the signal transducer and activator of transcription, STAT5, to enhance β-casein gene transcription (Stocklin *et al.*, 1996). This work is currently in progress.

Chapter 1
Introduction

Part 1

Cellular and molecular aspects of glucocorticoid action

1.1.) Endocrine hormones

From the early 1900s, there has been an enormous increase in our understanding of endocrine organs and the diverse physiology that they coordinate. The hormones that these organs secrete have been shown to be profoundly important for controlling vertebrate development and physiology and maintaining homeostasis. Consequently, each has become a major focus of biological and clinical investigation.

In general, hormones can be described as substances produced by cells or tissues which, following secretion, have a defined physiological effect on their target tissue(s) or cell(s). Hormones which affect the cells which secrete them are termed autocrine, whilst those affecting other cells in their local environment are termed paracrine in function. In contrast, endocrine hormones are secreted by one tissue and exert their effect on another distant tissue, normally carried to their site of action by the circulation. Some hormones such as cortisol have auto, para and endocrine functions.

Endocrine hormones can be classified biochemically as peptides (e.g. insulin), amines (e.g. thyroxine) or steroids (e.g. cortisol). Their effects are mediated following the binding of the hormone to a specific receptor molecule, either on the surface, or in the cytosol or nucleus of the target cell. Resultant effects are either direct (e.g. on gene transcription) or indirect via an intracellular second messenger. Regulation of hormone action is possible at several levels: synthesis, secretion, transport in the circulation, distribution, clearance, tissue uptake and/or receptor interaction, receptor down regulation and signal transduction. The experiments described in this thesis concern glucocorticoid hormone

action. To put the results in context, this introduction will include information specifically about regulation involving the glucocorticoid receptor (GR).

1.2.) Steroids and the adrenal gland

1.2.1.) Adrenal zonation

Glucocorticoid hormones are secreted by the adrenal gland, which in mammals consists of two major parts of different embryonal origin, an inner medulla synthesising catecholamines (adrenaline, noradrenaline and their precursor, dopamine) and an outer cortex which produces a large number of steroids, some of which are hormones (McNicol, 1992). The cortex can be further subdivided into three histologically distinct regions called, from centre to periphery, the zona reticularis, zona fasciculata and zona glomerulosa.

There are three major categories of cortical steroids; the adrenal androgens, which are required for the expression of secondary male sexual characteristics, and the glucocorticoids and mineralocorticoids controlling intermediary metabolism and salt and water balance, respectively. Figure 1.1. shows the main steps in the pathways for adrenal corticosteroid biosynthesis. All share a common precursor cholesterol, the basic ring structure of which is maintained throughout enzymatic conversion.

1.2.2.) Steroid hormone biosynthesis

Adrenocortical steroidogenesis proceeds via a series of cytochrome P-450 hydroxylase and dehydrogenase enzymes. Enzymatic conversions are compartmentalised, depending on the subcellular localisation of the enzymes involved. Cholesterol from cholesterol esters, de-novo synthesis or from dietary uptake, is initially transported from the cytosol to the inner mitochondrial membrane where cytochrome $P450_{SCC}$ (side chain cleavage), also known as cholesterol desmolase, cleaves the side chain of cholesterol at C21 to form pregnenolone. This is the rate limiting step in steroidogenesis.

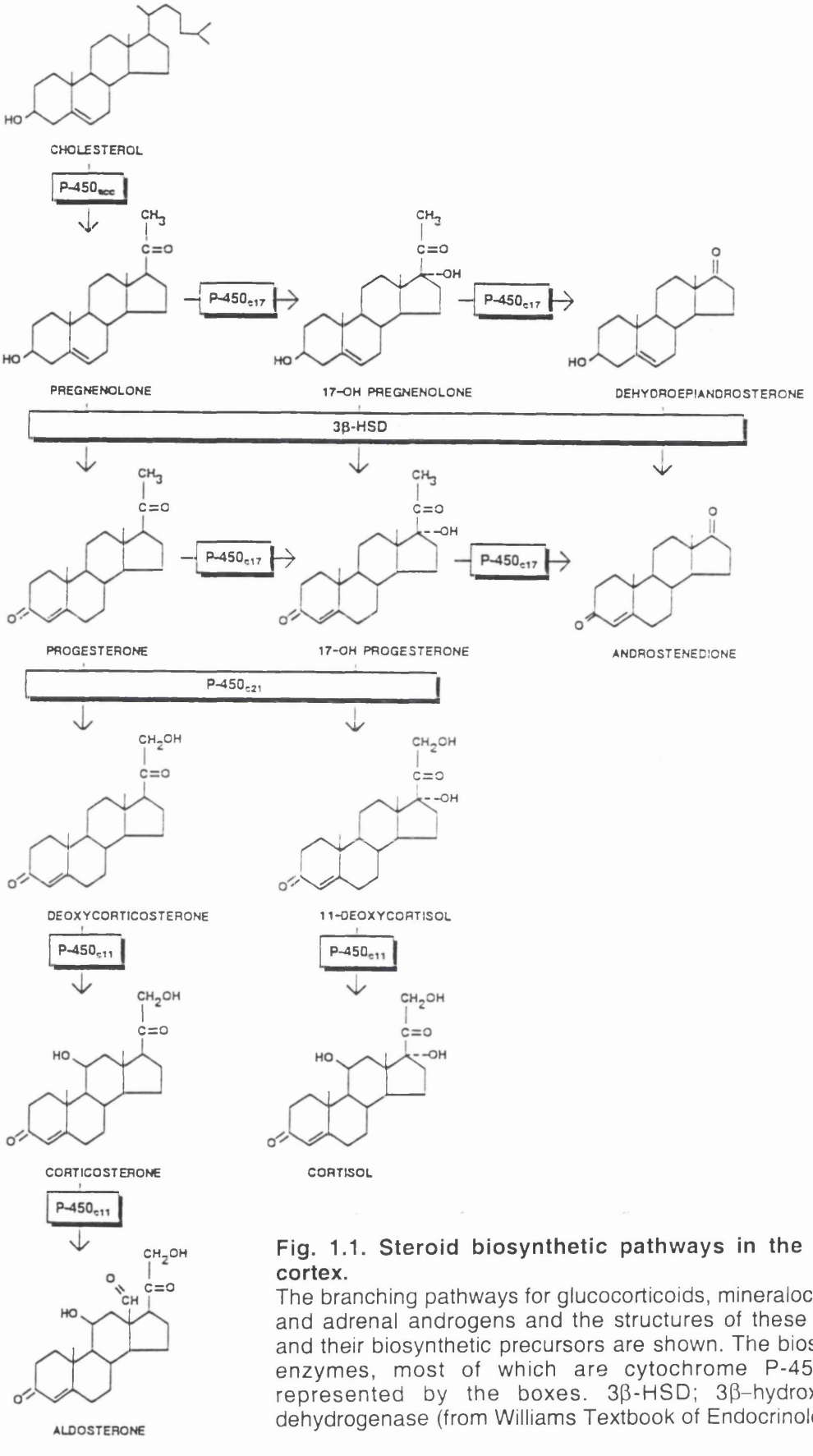

Fig. 1.1. Steroid biosynthetic pathways in the adrenal cortex.
The branching pathways for glucocorticoids, mineralocorticoids and adrenal androgens and the structures of these steroids and their biosynthetic precursors are shown. The biosynthetic enzymes, most of which are cytochrome P-450s, are represented by the boxes. 3β-HSD; 3β–hydroxysteroid dehydrogenase (from Williams Textbook of Endocrinology).

Mitochondrial 3β-hydroxysteroid dehydrogenase (3β-HSD) then converts a portion of the newly synthesised pregnenolone to progesterone. Both of these compounds act as intermediates, providing the substrates for two further enzymes associated with the endoplasmic reticulum, $P450_{c17}$ (17α-hydroxylase) and $P450_{c21}$ (21-hydroxylase). These enzymes catalyse hydroxylations at positions C17 and C21 of the steroid ring respectively, in separate biosynthetic pathways. Corticosterone and cortisol are formed from the respective 11β-hydroxylation of deoxycorticosterone and deoxycortisol by mitochondrial 11β-hydroxylase (cyp11b1). The end products of corticosteroid biosynthesis fall into two main catagories, the C21 steroids, which have a two carbon side chain at position C17 and the C19 steroids with either a hydroxyl or a keto group at position C17. Glucocorticoid and mineralocorticoid activities are attributed to the C21 steroids, which display only small structural differences (Fig. 1.1.). Aldosterone is derived from deoxycorticosterone in three steps by the actions of a single enzyme, aldosterone synthase (cyp11b2).

In man, C19 steroids with weak androgenic activity, dehydroepiandrosterone (DHEA) and its sulphate (DHEAS), are the most abundant products of the adrenal cortex. They are derived from 17α-hydroxypregnenolone and 17α-progesterone by the enzyme $P450_{c17}$ (Fig. 1.1.). The products of this reaction, DHEA and androstenedione are subsequently converted to low levels of testosterone in the periphery. DHEA is known to have anti-glucocorticoid properties (Svec *et al.*, 1995).

The adrenal cortex is functionally zonate. Aldosterone, the most important mineralocorticoid in rats and man, is synthesised only in the zona glomerulosa while the major glucocorticoids, cortisol in man and corticosterone in the rat and the adrenal androgens are synthesised in the inner cortical zones (Berne and Levy, 1993). The conversion of corticosterone to cortisol requires 17-hydroxylation, which is absent in the rat. Therefore, the synthesis of cortisol and adrenal androgens does not occur in this species.

1.3.) The control of adrenocortical secretions

The normal secretion pattern of mineralocorticoid and glucocorticoid hormones is maintained by different trophic stimuli; aldosterone secretion is regulated by angiotensin II and dietary potassium and is also affected by ACTH (Quinn and Williams, 1988). In the zona fasciculata, ACTH is the most important factor regulating the secretion of glucocorticoids (Waterman and Simpson, 1989; White *et al.*, 1994).

ACTH is produced by the anterior pituitary gland as the derivative of a precursor peptide, proopiomelanocorticotrophin [POMC] (Eipper and Mains, 1980). Multiple hormonal factors regulate ACTH secretion (Antoni, 1986; Antoni, 1993), the most important being the peptide hormone CRF (corticotrophin releasing factor). Neural mechanisms stimulate the release of CRF from the hypothalamus. This causes a rise in pituitary ACTH, which in turn stimulates the zona fasciculata, increasing the secretion of glucocorticoids (Fraser, 1992). Elevated levels of glucocorticoid in the plasma inhibits further ACTH secretion (by feedback inhibition of POMC gene expression; Drouin *et al.*, 1989) and CRF gene transcription (Lundbland and Roberts, 1988) and consequently, further glucocorticoid secretion. The basis of this negative feedback inhibition process is outlined in Figure 1.2. The stimulation of ACTH by CRF is potentiated by vasopressin (AVP), which can also stimulate ACTH secretion directly. In addition, catecholamines, angiotensin II, serotonin, oxytocin, atrial natriuretic factor and several others have been implicated in the control of ACTH secretion (Rivier and Vale 1983; Antoni, 1993).

ACTH generally regulates glucocorticoid output through the hypothalamo-pituitary-adrenal (HPA) axis. Under normal circumstances in man, ACTH and therefore cortisol output is controlled in the long term in a circadian fashion (Kreiger *et al.*, 1971) and is increased by various types of physical and psychological stress (Weitzman *et al.*, 1971).

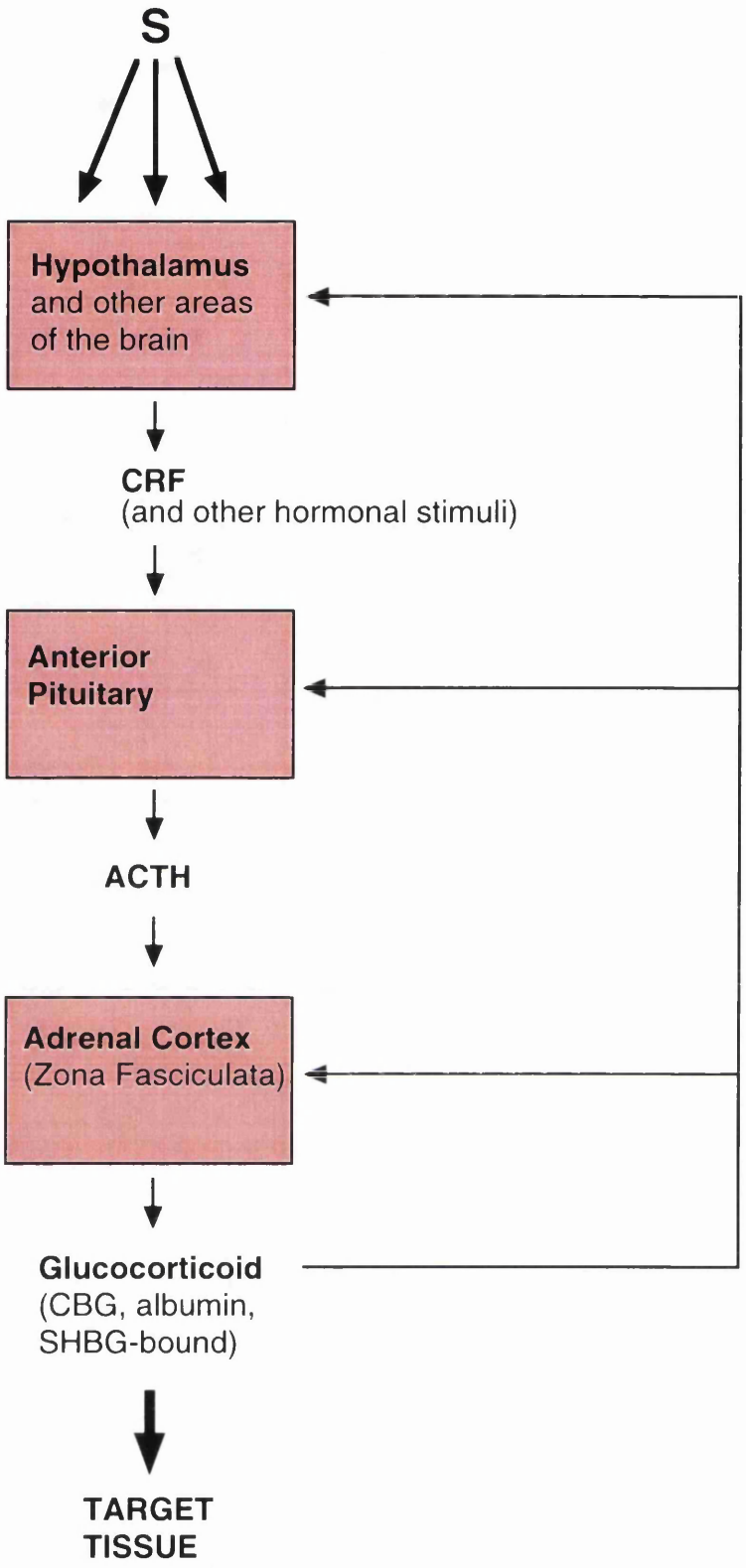

Fig. 1.2. Diagram showing the essential components of Glucocorticoid feedback inhibition of secretion from the adrenal cortex.CRF, Vasopressin and other hormonal factors stimulate ACTH secretion from the hypothalamus, which leads to the eventual secretion of glucocorticoids from the adrenal cortex. Most of the circulating glucocorticoid is associated with plasma binding proteins [such as; CBG, 80-90%; albumin and SHBG, the remainder] (Dunn *et al.*, 1981), which renders it biologically inactive. The active fraction which remains free in the circulation is normally in the nM range, which in man constitutes arround 3-4% of the total. Sites of feedback inhibition due to excessive glucocorticoid secretion are shown (see text for details). S: stimulus; environmental, physical or psychological, CRF: corticotrophin releasing factor, ACTH: adrenocorticotrophic hormone, CBG: corticosteroid binding globulin, SHBG: sex steroid binding globulin. Circled arrow relates to high endogenous level.

Since steroids are not stored to any great extent, biosynthesis rates must be increased on demand. ACTH stimulates steroidogenesis (Fig. 1.1.) through the classical receptor-adenylate cyclase system. Ligand binding to ACTH receptors in the cell membrane (Mountjoy *et al.*, 1992) increases adenylate cyclase activity, causing stimulation of a G-protein. This in turn leads to higher endogenous levels of cyclic AMP (cAMP). The resultant activation of a cytoplasmic protein kinase causes the phosphorylation of regulatory proteins which induce the process of steroidogenesis.

The secretion of aldosterone is also under tight physiological control. Both angiotensin II and potassium act as specific zona glomerulosa agonists which stimulate aldosterone synthesis. Angiotensin II is a pressor hormone generated in the circulation under the control of the enzyme renin from the kidney (Brown *et al.*, 1983). Figure 1.3. summarises the main components of the renin-angiotensin system. The inactive precursor angiotensin I is first formed by the splitting of the renin substrate angotensinogen, which is converted to angiotensin II by angiotensin I converting enzyme (ACE). In keeping with blood pressure control, the plasma concentration of angiotensin II, through the action of renin is directly linked to sodium levels. The normal physiological response to sodium depletion is the release of renin, which leads to an increased angiotensin II output followed by a respective increase in aldosterone secretion. The resultant effect is sodium and water retention, which inhibits further renin release and causes an expansion in plasma volume (Fraser *et al.*, 1981). Small increases in plasma potassium leads to aldosterone synthesis. In contrast to sodium, large increases in dietary potassium and hyperkalemia inhibit aldosterone synthesis through inhibition of renin release (Beretta-Picoli *et al.*, 1983).

Both potassium and angiotensin II regulate aldosterone synthesis by their effects on calcium metabolism (Radke *et al.*, 1989). Increases in potassium depolarises the cell membrane, opening voltage-dependent calcium channels; angiotensin II also opens calcium channels, as well as stimulating the release of stored Ca^{2+} within adrenocortical cells via a

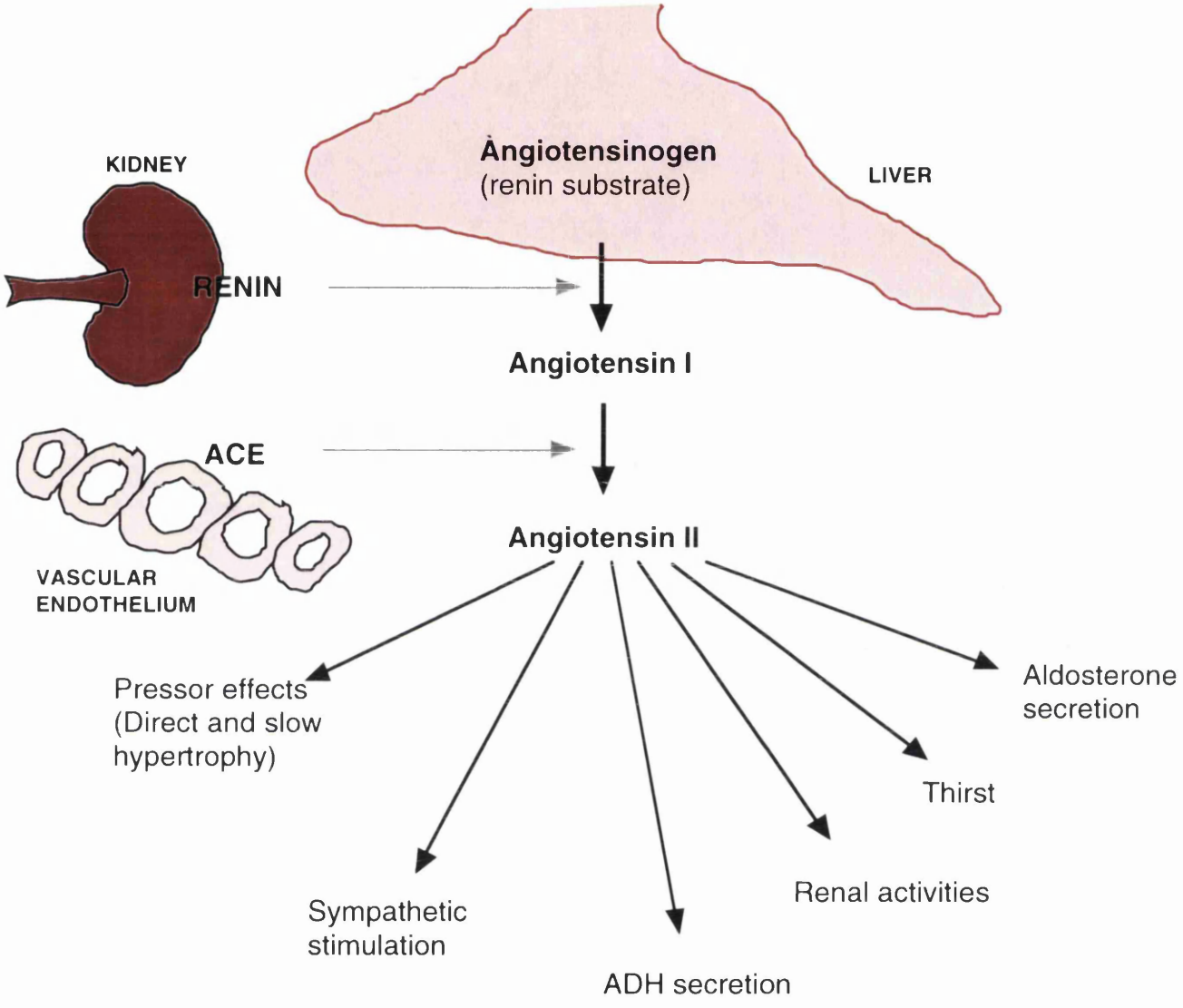

Fig. 1.3. The molecular components of the renin-angiotensin system.
The diverse range of physiological processes responsive to angiotensin II is shown. ACE: angiotensin converting enzyme, ADH: antidiuretic hormone.

cascade of events involving phospholipase C and the release of IP3. Ca^{2+} activates cholesterol side chain cleavage and the steroidogenic conversion of cholesterol to aldosterone (see Fig. 1.1.).

1.4.) The major physiological roles of adrenal corticosteroids

1.4.1.) Glucocorticoids

The glucocorticoids are of fundamental importance, exerting a multiplicity of actions in a wide range of tissues. They play important roles in the control of intermediary metabolism, aspects of cardiovascular regulation such as the regulation of vascular tone and in the function of skeletal muscle, lymphoid and connective tissue and the central nervous system. They perform a permissive function in many tissues by potentiating the actions of other cellular mediators such as catecholamines. Glucocorticoids have a well recognised anti-inflammatory effect and are known to be secreted in response to various forms of stress originating from internal or external environments. The activities of glucocorticoids are summarised in Figure 1.4.

Glucocorticoids generally have an anabolic effect on the liver. In their maintenance of hepatic glycogen stores, glucocorticoids both activate glycogen synthase (Hornbrook *et al.*, 1966) and inactivate the glycogen mobilising enzyme, glycogen phosphorylase (Stalmans and Laloux, 1979). Hepatic glucose production via gluconeogenesis is increased by promoting substrate availability and stimulating the release of glucogenic amino acids from peripheral tissues such a skeletal muscle. Glucocorticoids mediate this process by activating key gluconeogenic enzymes, such as glucose-6-phosphatase and phosphoenol pyruvate carboxykinase (PEPCK) (Exton, 1979)

A catabolic effect is elicited in most other tissues. Lipolysis is acutely activated by glucocorticoids in adipose tissue (Fain, 1979). The glycerol

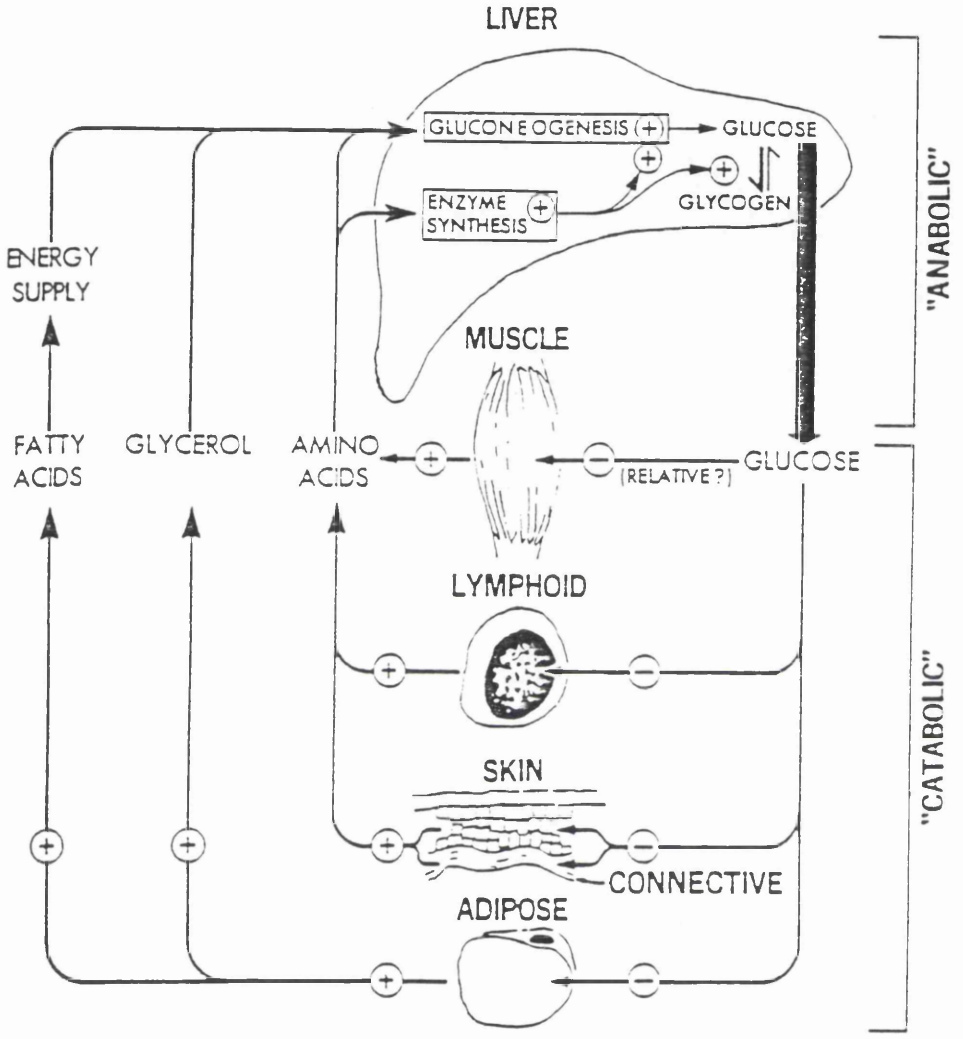

Fig. 1.4. The principal metabolic effects of glucocorticoids.
Hepatic glucose metabolism and peripheral tissue metabolism of protein and fats are all regulated by glucocorticoids. Stimulation is indicated by + signs and inhibition by - signs (from Williams Textbook of Endocrinology).

released during lipolysis provides a substrate for glucose production and the liberated fatty acids provide an energy source for the process. The breakdown of proteins (Fain, 1979) and reduction of glucose uptake from the circulation and its utilization by peripheral tissues (LeBoeuf *et al.*, 1962; Munck, 1962) are also mediated by glucocorticoids. The net effect is to keep plasma glucose and free fatty acid levels high. The catabolic effects on muscle protein provide an explanation for the profound myopathy seen in conditions of glucocorticoid excess.

Glucocorticoids exert a direct and indirect effect on bone metabolism. Indirectly, glucocorticoids have been shown to inhibit calcium absorption from the gut (Lukert and Adams, 1976) and enhance urinary calcium excretion by decreasing reabsorption in the kidney (Laake, 1960). Excessive exposure to glucocorticoids reduces or inhibits osteoblastic activity, leading to osteoporosis (Cheng *et al.*, 1994). This suggests the possibility of a direct steroid effect on bone cells.

Distribution and behaviour of cells of the immune system; T cells , (Gillis *et al.*, 1979), B cells (Cupps *et al.*, 1985), monocytes, neutrophils and granulocytes (Dale *et al.*, 1975) is affected by glucocorticoids, which may be manifest as immunodeficiency (Orth *et al.*, 1992). Endogenous glucocorticoid excess generally suppresses immune responses (Graham and Tucker, 1984). Glucocorticoids also modulate the activity of components which mediate local inflammatory responses. The anti-inflammatory and immunosuppressive effects of glucocorticoids are well established (Cato and Wade, 1996). The activities of histamine, a potent vasoactive agent (Fauci, 1979) and prostaglandins (Russo-Marie *et al.*, 1979) are both inhibited by these steroids (see Fig 1.4.).

Supraphysiological concentrations of glucocorticoids inhibit linear growth in children, possibly due to the direct inhibitory effects on bone and connective tissue (Ferraris and Pasqualini, 1993). In the lung, glucocorticoids stimulate the maturation and differentiation of many cell types (Snyder *et al.*, 1992). They are also responsible for regulation of

surfactant production by type II pneumocytes (Boggaram and Mendelson, 1988). Lower glucocorticoid levels are stimulatory, higher levels are inhibitory. In the nervous system, glucocorticoids regulate the development of neural crest epithelial cells, the precursors of more differentiated cell types including autonomic ganglion cells and adrenomedullary cells, into cromaffin cells. Under the influence of glucocorticoids, neural crest precursor cells that invade the embryonic adrenal gland cease to express 'neurone-specific' gene products such as neurofilaments and acquire the characteristic morphology of adrenomedullary chromaffin cells (Federoff *et al.*, 1988).

Evidence for glucocorticoid effects on blood pressure (reviewed more extensively in part 2 of this chapter) comes largely from patients with glucocorticoid excess or deficiency. Those with glucocorticoid excess usually develop hypertension, often without evidence of functional mineralocorticoid excess (Saruta *et al.*, 1986). Patients with glucocorticoid deficiency show defective water clearance with increased arginine vasopressin (AVP) concentrations (Raff, 1987). Glucocorticoid deficiency increases AVP mRNA synthesis in the paraventricular nucleus where it is synthesised and secreted (Sonnenblick *et al.*, 1979). Increased AVP levels may play a role in maintaining blood pressure in states of adrenal insufficiency, such as in Addison's disease. Glucocorticoids also have direct effects on ion transport in the colon (Sandle, and McGlone, 1987). The use of glucocorticoid analogues, specific for the glucocorticoid receptor produce a saturable sodium transport (Bastl, 1987), which is not diminished by specific mineralocorticoid receptor antagonists (Bastl, 1988). In the rat descending colon, Na-K-APTase α_1 and β-subunit gene expression is acutely regulated by dexamethasone, but not aldosterone (Fuller and Verity, 1990).

1.4.2.) Mineralocorticoids

The main physiological role of mineralocorticoids is to promote sodium retention and potassium excretion in transporting epithelia such as the

distal nephron of the kidney, salivary and sweat glands and the colon (Morris, 1981). In excess, mineralocorticoids cause hypertension and increases in body sodium. Aldosterone effects may also be seen in non-epithelial tissues. There is evidence for aldosterone induced activity in the brain (McEwen *et al.*, 1986). Intracerebroventricular (icv) infusions of low doses of aldosterone effects salt seeking behaviour and ingestion. These effects are not mimicked or blocked by equivalent or higher concentrations of corticosterone, but are effectively blocked by specific aldosterone antagonists (McEwen *et al.*, 1986). The effects of aldosterone in both mononuclear leukocytes and vascular tissues, mediated through mechanisms which do not require MR activation, will be discussed in the following section.

1.5.) Non-genomic responses to steroids

Although the majority of evidence suggests that steroid hormones induce genomic responses through specific nuclear receptor activation (Beato and Sanchez-Pacheco, 1996; Hagar *et al.*, 1996; Jenster *et al.*, 1997), there is also implication of direct steroid effects mediated via membrane receptors. Some steroid effects, particularly of an electrophysiogical and behavioural nature occur too rapidly to be explained in terms of a classical genomic response (McEwan, 1991), which are normally manifest after a period of hours, or even days (Steimer and Hutchison, 1981). The rapid induction of steroid effects which are not blocked by protein synthesis inhibitors (Nabekura *et al.*, 1986) and the modulation of binding characteristics of neurotransmitter receptors implies the possibility of steroid hormone binding to neuronal membranes (Schumacker *et al.*, 1990).

In female rats, rapid behavioural effects due to short-term progesterone administration have been documented (Kubli-Garfias and Whalen, 1977). Progesterone causes a rapid (40-60s) and transient increase in intracellular Ca^{2+} concentrations in amphibian oocytes, a necessary first step in the resumption of meiosis and eventual maturation (Wasserman *et*

al., 1980). Progesterone receptors have also been identified on the surface of human sperm and have been implicated in initiating rapid calcium influx, a prerequisite for acrosomal exocytosis (Tesarik *et al.*, 1993). Oestrogens have been shown to produce adenylate cyclase mediated increases in cyclic AMP (cAMP) in target breast cancer and uterine cells and intact uterus *in vivo* by a mechanism that does not involve the genomic action of this hormone (Aronica *et al.*, 1994). Corticosterone-specific receptors in the synaptic membranes of amphibian brains appear to influence male reproductive behaviour (Orchinik *et al.*, 1991). More recently, the rapid *in vitro* effects of aldosterone on intracellular electrolytes, cell volume, and the sodium-proton antiporter have been described in human mononuclear leukocytes and vascular smooth muscle cells, which respond with a sustained rise in free intracellular calcium levels within 1-5 mins following aldosterone administration (Schneider *et al.*, 1997). The full length cDNA sequence of a progesterone membrane-binding protein of porcine vascular smooth muscle cells has now been described (Falkenstein *et al.*, 1996).

The remainder of this thesis will focus on the classical mechanisms of steroid action, with particular emphasis on glucocorticoids.

1.6.) Steroid hormone receptors: an overview

Classically, steroid hormone receptors are viewed as the cellular mediators of steroid hormone responses at the level of the genome. Corticosteriods bind to cytosolic receptors, the resulting complex interacting with the steroid responsive elements of a wide variety of genes, altering messenger RNA (mRNA) synthesis. Subsequent alterations in cellular protein synthesis lead to changes in peptide and protein secretion, enzyme levels, the synthesis of components of cell-signalling systems, such as adenylate cyclase and finally, growth (Miesfeld, 1989). Perhaps the earliest evidence that steroid hormones regulate gene expression came from experiments showing that ecdysteroid-induction of metamorphosis in insects is

associated with changes in chromosome structure (Ashberner, 1980). It is now well established that all steroid hormones, as well as thyroid hormone and retinoic acid, act by binding to specific intracellular receptors, coordinating complex biological events by regulating the expression of genes or gene networks (Yamamoto, 1985). The discovery of receptors and receptor-related molecules for these hormones in a wide range of species suggests that the general mechanisms underlying morphogenesis and homeostasis may be ubiquitous.

1.7.) Nuclear receptor superfamily

The expression cloning of the human glucocorticoid receptor provided the first completed structure of a steroid hormone receptor and revealed a segment of extensive homology with the viral oncogene, *erb*A (Hollenberg *et al.*, 1985; Debuire *et al.*, 1984). The independent cloning of the human and rat receptors for androgen (AR), oestrogen (ER), progesterone (PR), and aldosterone (MR), over the following 2-3 years produced evidence for a family of related hormone binding molecules (O'Malley, 1990; Laudet *et al.*, 1992). Further to these advances, the identification of the *erb*A protooncogene product (c-erbA) as the thyroid hormone receptor (Weinberger *et al.*, 1986) gave a unifying element to steroid receptor structure and hormone action as well as suggesting a common receptor origin from a primordial ancestral regulatory gene.

Figure 1.5. Shows the major components of the steroid receptor superfamily. These molecules form a subgroup of a much larger multigene family of nuclear receptors (reviewed by O'Malley, 1990; Evans, 1988), including those which mediate responses to thyroid hormone, T_3R_α and T_3R_β; vitamin D3, VDR and retenoic acid, RARα, RARβ and RARγ (Laudet *et al.*, 1992). The molecules in Figure 1.5. are aligned on the basis of regions of maximum protein homology (Johnson and Doolittle, 1986). Each functions as a ligand-dependent transcription factor with diverse roles in growth, development and homeostasis (Hollenberg and Evans, 1988). Their primary

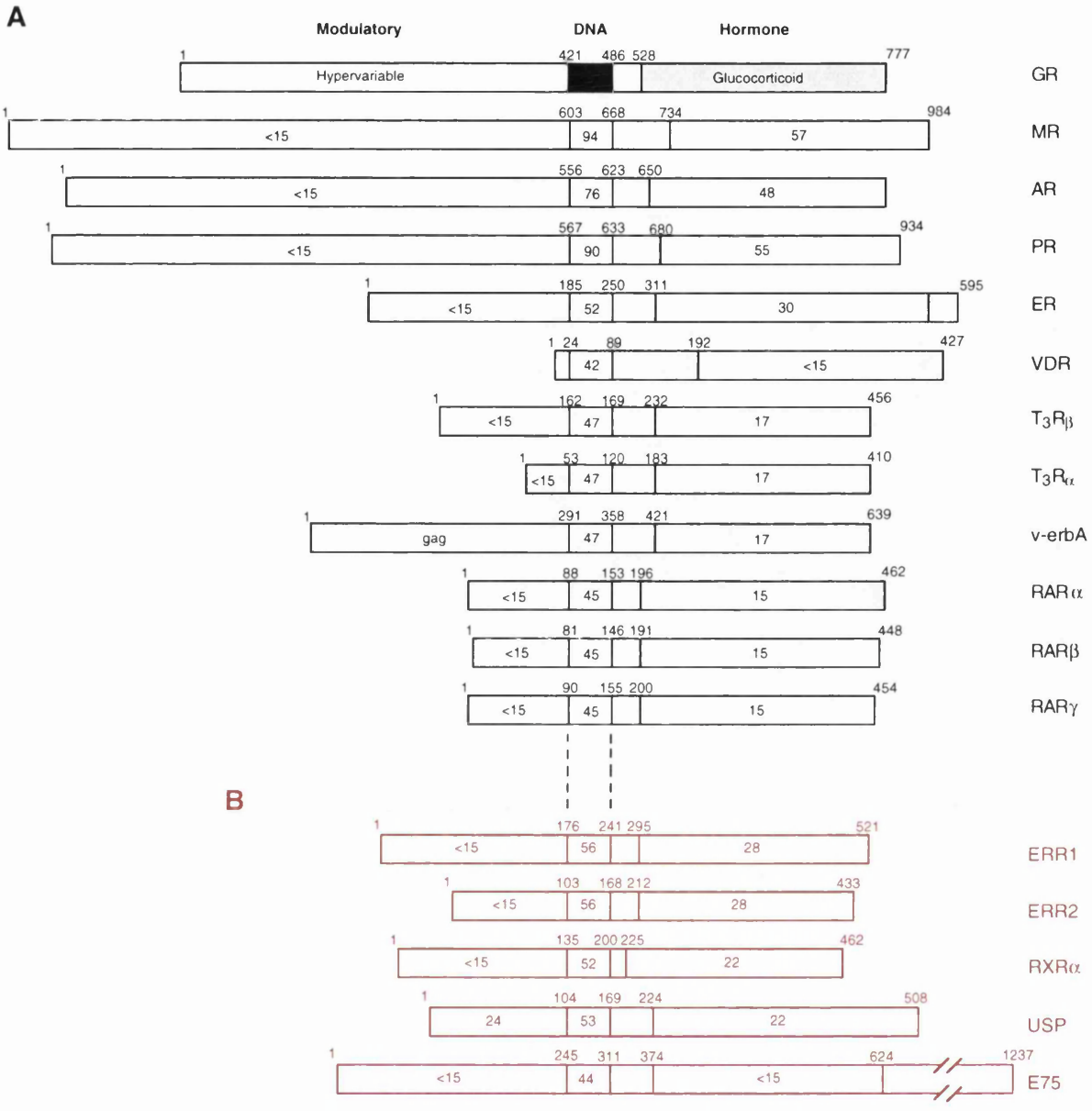

Fig. 1.5. Schematic amino acid comparison showing examples of members of the nuclear hormone receptor superfamily.
Structures are based on the results of molecular cloning studies (References in text). Primary amino acid sequences are aligned on the basis of regions of maximum homology (Johnson and Doolittle, 1986). Numbers indicate the percentage amino acid identity to hGR. Common domains shown: modulatory; N-terminal domain of variable sequence, required for maximum receptor activity, DNA; 66-68 amino acid DNA-binding core and hormone; the COOH-terminal hormone binding domain. The amino acid position of each domain boundary is shown. All receptors represent the human forms with the exception of v-erbA (viral oncogene), USP and E75 (from Drosophila). Receptors are grouped as A, the steroid/thyroid retinoic acid subfamily, for which an endogenous ligand activator has been identified: GR, glucocorticoid receptor; AR, androgen receptor; MR, mineralocorticoid receptor, PR, progesterone receptor; ER, oestrogen receptor; VDR, vitamin D3 receptor; T3Rβ and T3Rα, thyroid hormone receptors and RARα, β, γ, retinoic acid receptors and B, the orphan receptors: ERR1 and ERR2, estrogen receptor-related 1 and 2; RXRα, retinoid X receptor; USP, ultraspiracle and E75, for which endogenous ligands have not been identified.

sequences have since been characterised in detail (reviewed by Evans, 1988; O'Malley, 1990).

The observation that hormones, unrelated structurally or biosynthetically bind structurally conserved receptors, implies the existence of further unidentified genes, the products of which are also likely to be ligand-responsive transcription factors. The search for these initially hypothetical receptor genes was based on low stringency hybridisation techniques using cDNA sequences of already characterised receptor molecules (see Arriza *et al.*, 1987). Several new nuclear receptor coding genes have now been identified. By 1995, more than fifty members were reported, including at least thirty for which an endogenous ligand had not been recognised. Such receptors (exampled in Figure 1.5.) are referred to as 'orphans' (reviewed by Laudet *et al.*, 1992; Laudet and Adelmant, 1995). Some of the first examples of this class of molecule include the oestrogen receptor related proteins, ERR1 and ERR2, (Giguere *et al.*, 1988) and a homologue of the retinoic acid receptor subfamily, the retinoid X receptor (RXR) which is capable of mediating cellular responses to retinoic acid, but for which the true cellular ligand remains unknown (Kliewer *et al.*, 1992). The reasons for receptor multiplicity are debatable. They could be important in promoter-specific regulation, controlling the expression of overlapping gene networks, requiring tissue-specific patterns of expression (see section 1.13.). Alternatively, they may respond to different hormone metabolites. In contrast to positive gene regulatory roles, both COUP-TF (Wang *et al.*, 1989) and the closely related homologue EAR2 (Miyajima *et al.*, 1988) can specifically modulate the cellular retinoic acid response by interfering with RXR-mediated transactivation. This attenuation assigns a negative regulatory function to these orphan receptors (Kliewer *et al.*, 1992).

Several gene products with structural similarities to steroid receptors have also been identified in *Drosophila*. Examples include the gap segmentation gene, *knirps* (*kni*) (Nusslein *et al.*, 1980), *knirps-related* (*knrl*) (Ore *et al.*, 1988) and *ultraspiracle* (*usp*), required maternally and zygotically and the

products of the E75 locus, active in chromosome puffing (Segraves and Hogness, 1990). This inter-species homology between steroid receptor-like transcription factors suggests that similar chemical cues underlie morphogenic signalling in vertebrate and invertebrate systems and an evolutionary conservation of fundamentally important transcriptional regulatory motifs.

1.8.) The molecular mechanism of glucocorticoid activity

The first clue to the mechanism by which relatively simple steroid molecules could elicit diverse and complex physiological responses came from the identification of their cellular receptors through the use of radiolabelled ligands in the early 1960s (Jensen and DeSombre, 1972; Tata *et al.*, 1972). In each case, the hormone induced a change in the receptor which was then able to associate with high affinity binding sites in nuclear chromatin. This in turn led to the induction or repression of a limited number of genes (50-100 per cell) (Ivarie and O'Farrell 1978).

In addition to their influence over the rate of gene transcription, the stability and consequent turnover of mRNA may also be affected by steroids. These effects support observations that the general nuclear localisation of steroid hormone receptors is consistent with the site of action of their respective hormone ligands.

1.8.1.) Glucocorticoid signal transduction pathway

The overall molecular mechanism of the glucocorticoid signal transduction pathway and subsequent gene regulation is shown in Figure 1.6. (points 1-6). The first step (1) involves the passage of the steroid hormone across the cell membrane. The exact mechanism by which this is achieved is unclear, although processes of both passive diffusion, aided by the highly lipophilic nature of steroid hormones and specific uptake mechanisms involving

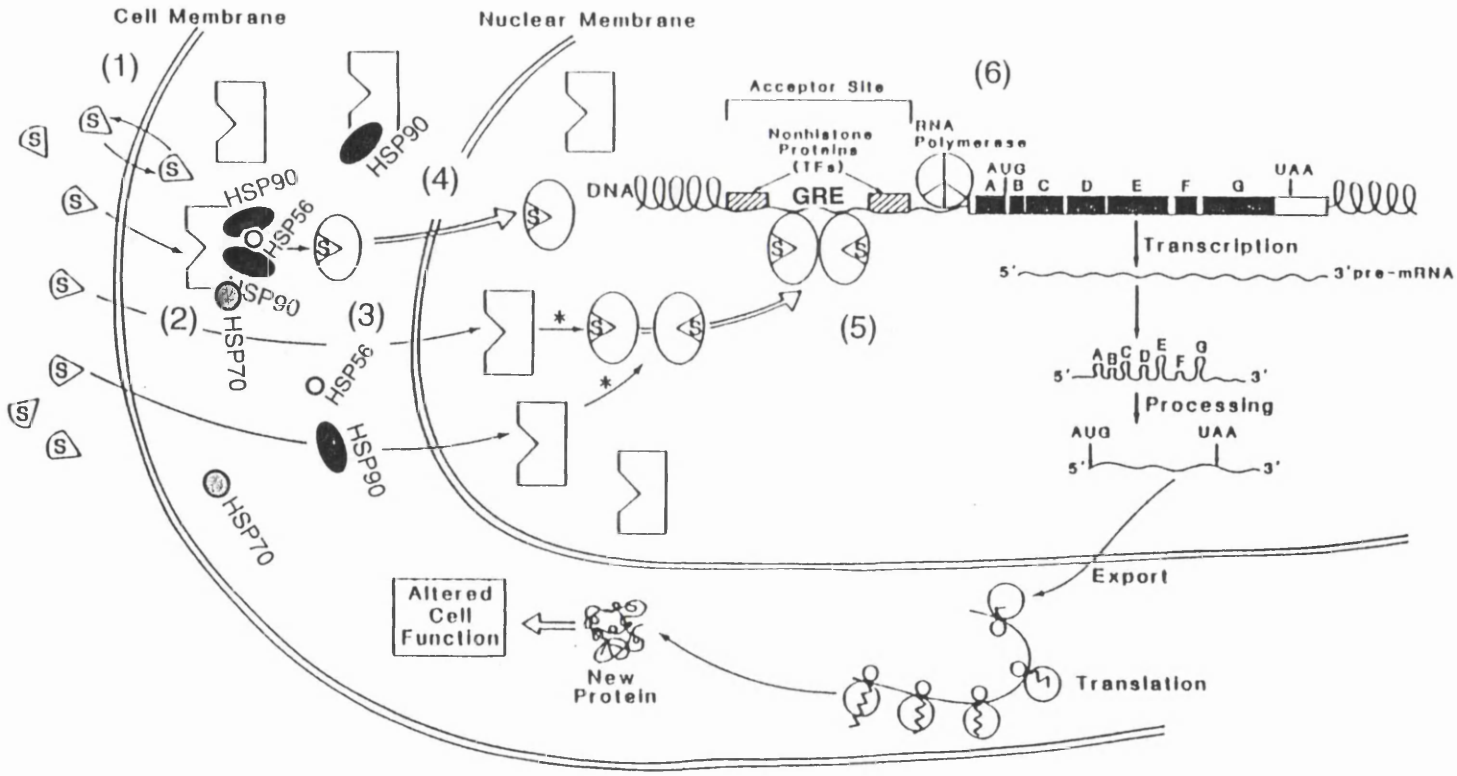

Fig. 1.6. Signal transduction pathway of glucocorticoid hormones and the molecular mechanism of GR.

Steps, 1-6, outline the known mechanism of induction of gene transcription by glucocorticoid hormones. Abbreviations are as follows; S, steroid hormone; GR, glucocorticoid receptor monomer; HSP90, HSP70, and HSP56 heat shock proteins of 90, 70 and 56 Kd respectively; GRE, glucocorticoid response element (Adapted from Williams Textbook of Endocrinology).

transport proteins have been proposed (Brann, *et al.*, 1995, and references therein).

1.8.1a.) Hormone-receptor interaction and receptor activation

In Step 2 (Fig. 1.6.), the glucocorticoid hormone interacts with its cognate receptor. Inactive GR exists in the cytoplasm as a hetero-oligomeric complex (Pratt, 1993). The normal cellular location of other steroid receptor family members is in the nucleus (King, 1987). The complete GR molecule consists of one receptor polypeptide (the 4s monomeric form), one subunit each of HSP70, HSP56 and a smaller HSP of around 27 kd, an immunophillin of the FK506 class of cytosolic binding proteins and two molecules of HSP90 (Pratt, 1993; Hutchinson *et al.*, 1994). The HSP90 molecules perform two essential functions. By interacting with the GR ligand binding domain, they maintain the receptor in an inactive state in the absence of hormone (Dobson *et al.*, 1989; Picard *et al.*, 1988). HSP90 is also required for high affinity ligand binding, since in its absence, the receptor can still bind hormone but with a much lower affinity (Kauffman *et al.*, 1992). Binding of the hormone ligand causes molecular dissociation of the receptor heterocomplex and receptor dimerization (Eriksson *et al.*, 1991), liberating an activated receptor in a state competent for nuclear translocation (step 3) (Carson-Jurica *et al.*, 1990). Cellular HSP90 content can determine sensitivity to glucocorticoids, through its interaction with GR (Picard *et al.*, 1990).

1.8.1b.) Receptor dimerisation

Nuclear receptor family members can be sub-divided, depending on dimerisation properties. Classically, receptors such as GR are viewed to function as homodimers in gene activation mechanisms which involve DNA binding (Bamberger *et al.*, 1996). However, more recent evidence suggestes that heterodimers between steroid receptor family members, such as GR/MR (Trapp *et al.*, 1995) and GR/AR (Chen *et al.*, 1997) may be commonplace in the process of transcriptional modulation. TR, RAR and VDR can also bind DNA as homodimers, but this binding is relatively weak. DNA-binding is enhanced by a further member of the nuclear receptor

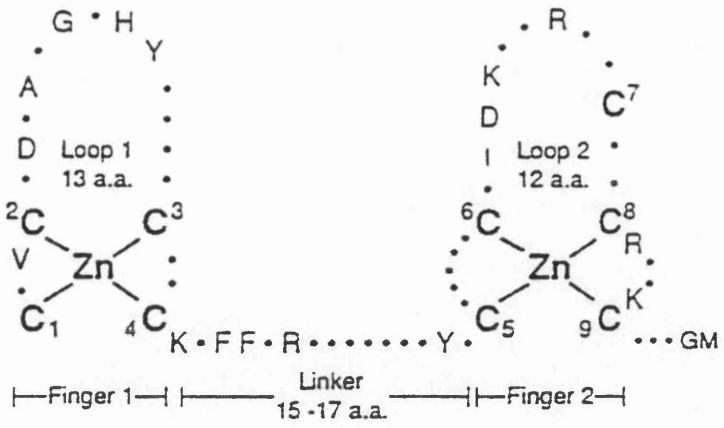

Fig. 1.7. Binding of the glucocorticoid receptor to DNA.
a.) Zinc fingers predicted from the conserved and variable amino acids in the DNA binding domains of hGR, hT3Rβ and hER. Amino acids are represented by the one letter code and dots respectively. Two zinc ions are coordinated between two clusters of four cysteines (C1-C4 and C5-C9), forming two zinc fingers (finger 1 and finger 2) separated by a spacer of 15-17 amino acids (taken from Beato, 1989).

b.) Model of the dimeric complex between the glucocorticoid receptor DNA-binding domain and the glucocorticoid rsponse element (GRE) (taken from Hard *et al.*, 1990).

superfamily, the retinoid X receptor, RXR (Yu *et al.*, 1991; Leid *et al.*, 1992). Both RXRα and RXRβ stimulate binding of TR and RAR subtypes, VDR COUP-TF and PRAP to their respective DNA response elements, suggesting that these receptors most likely function as heterodimers (Kleiwer *et al.*, 1992; Zhang *et al.*, 1992). Other nuclear receptors, such as NGFI-B and FTZ-F1 appear to function as monomers (Parker, 1993).

1.8.1c.) Translocation

Activated GR is transported into the nucleus as a homodimer (Wrange *et al.*, 1989), a process aided by nuclear localisation signals. One of these signals (NL1) is located towards the carboxyl terminal end of the DNA-binding domain, the second (NL2) is a composite part of the ligand binding domain (Picard and Yamamoto, 1987). Steps 5 and 6 depict the final stages of the signalling pathway, which involves specific binding of the receptor to nuclear chromatin, or interaction with other gene regulatory proteins and the subsequent regulation/modulation of gene transcription (Bamberger *et al.*, 1996).

1.8.1d.) Target gene recognition

The selective DNA binding of different steroid hormone receptors is achieved via two components, the receptor DNA binding domain, which forms a zinc finger binding motif (Green and Chambon, 1987; Severne *et al.*, 1988: see Fig. 1.7a.) and the hormone responsive elements (HREs). HREs (Table 1.1.) are discrete *cis*-acting nucleotide sequences which mediate the binding of nuclear receptors to the promoters of their regulated genes (Geisse *et al.*, 1982; Payver *et al.*, 1983). They function in a position and orientation-independent fashion, behaving like transcriptional enhancers (Benoist and Chambon 1981; Chandler *et al.*, 1983), but are dependent on the presence of hormone for activity (Scheiderit *et al.*, 1983; Ostrowski *et al.*, 1984). HREs all follow a consensus nucleotide sequence but maintain a specificity high enough to direct the binding of only the correct hormone receptor species (Beato, 1989). For example the glucocorticoid responsive element (GRE), can be shown to preferentially bind glucocorticoid receptors *in vitro* with high specificity (Willman and Beato, 1986).

Invert repeats		Binding receptors
1.) GRE (+)	GGTACAnnnTGTTCT	GR, PR, AR, MR
2.) ERE	AGGTCAnnnTGACCT	ER
3.) TRE/RRE	TCAGGTCAnnnTGACCTGA	TR, RAR
4.) GRE (-)	ATYACNnnnTGATCW	GR
Direct repeats		
5.) DR-1	AGGTCAn AGGTCA	RXR-RXR, RAR-RXR, COUP-TF-RXR
6.) DR-2	AGGTCAnn AGGTCA	RAR-RXR
7.) DR-3	AGGTCAnnn AGGTCA	VDR-RXR
8.) DR-4	AGGTCAnnnn AGGTCA	TR-RXR
9.) DR-5	AGGTCAnnnnn AGGTCA	RAR-RXR

Table.1.1. Consensus response elements for nuclear receptors.
GRE (+), positive glucocorticoid response element; GRE (-), negative glucocorticoid response element; ERE, oestrogen response element; TRE/RRE, thyroid/retinoic acid response element. The central core around the axis of symmetry for invert repeats shows a nucleotide conservation of 50% or more (Umesono and Evans, 1989). DR-(n); direct repeat with (n) number of nucleotides between direct repeats (Umesono *et al.*, 1991). N; any Nucleotide.

Gene transfer studies, particularly with the mouse mammary tumour virus (MMTV) promoter and the human metallothionein IIA promoter have shown that these elements can confer hormonal responsiveness (Robins *et al.*, 1982). Most HREs identified have a dyad symmetry, which suggests interaction with receptor dimers. It has been shown that selectivity of vitamin D3, thyroid hormone and retinoic acid receptors in binding to HRE direct repeat half sites is specified by the number of nucleotides which separates them (the 3-4-5 rule: Umesono *et al.*, 1991). Other steroid receptor-related molecules show different binding preferences which are detailed in Table 1.1.

1.8.1e.) DNA binding

The zinc fingers of steroid receptor DNA binding domains contain zinc atoms, essential for DNA binding. The first (N-terminal) finger helps distinguish between steroid response elements. Mutational analysis has identified three residues at the C-terminus of the first finger and in the interfinger region that specify GRE recognition. The second (C-terminal) finger is required for stabilisation of the binding reaction (Danielsen *et al.*,

1989). Nuclear magnetic resonance analysis (NMR) of the GR DNA-binding domain expressed in *E. coli* has shown that the region contacting the DNA is an alpha-helical structure located between the two zinc fingers (Hard *et al.*, 1990). This observation was substantiated by the solution structure of GR complexed with DNA (Fig. 1.7b). The crystallographic model of the hGR-GRE complex (Luisi *et al.*, 1991) revealed that amino acids in the two zinc fingers, important for target gene recognition, are found in a helical structure which makes direct contact with the major groove of the DNA. The residues in the second zinc finger are involved in dimerisation of the DNA binding domain.

DNA response elements bound by steroid receptors consist of only a limited number of sequence motifs, related to either AGAACT or AGGTCA. DNA-binding specificity seems to be determined by the orientation and relative spacing of these motifs. For example, GR, MR, AR and PR can all bind to a GRE inverted repeat of sequence AGAACA, separated by three nucleotides (see Table 1.1. and accompanying references). These recognition sequences for DNA-binding help determine hormonal specificity. However, additional factors in the DNA recognition process must operate to avoid unwanted cross-talk between hormonal responses.

1.8.2.) Transcriptional regulation by GR

Selectivity of gene regulation by steroid receptors is achieved, in part, by the restricted expression of receptors to specific cell or tissue types. A further level of selectivity may be achieved through variation in chromatin structure, which is uniquely organised as a result of cellular differentiation, leading to DNA condensation. This in turn is likely to influence the accessibility of the receptor to different subsets of genes. There is also increasing evidence that steroid receptors contain promoter-specific and cell-type specific transcriptional activation domains, leading to restricted transcriptional activity (Horwitz *et al.*, 1996; Guido *et al.*, 1996).

1.8.2a.) Activation and repression of gene transcription

Many glucocorticoid-responsive genes have been isolated and shown to be regulated at the transcriptional level, e.g. angiotensinogen, ACE, arginine synthase, POMC and β-casein (Parks *et al.*, 1974; Ringold *et al.*, 1975; Karin *et al.*, 1980; Evans *et al.*, 1982; Doppler *et al.*, 1989).

Various models have been proposed to explain the process of transcriptional activation by steroid receptors. Some of the first, developed originally for prokaryotic promoters (Ptashne, 1986) suggest that their gene regulatory effects are based on the interaction of the DNA-bound hormone-receptor complex with other proteinaceous components of the cellular transcription machinery (see Fig. 1.8. and section 1.8.2b for a current view). Under conditions of positive gene regulation, basal components of the promoter initiation complex such as TATA-binding protein (TBP), TAFs (TBP-associated factors) and RNA polymerase II, are thought to either 'initiate' or stabilise at the promoter.

Protein-protein interactions at the DNA level are mediated by specialised domains of GR, which for the process of gene activation are termed *trans*-activation domains (Webster *et al.*, 1988). The precise mechanisms by which steroid receptors affect gene transcription are still poorly understood. Another possibility is that of nucleosome displacement, allowing transcription factor recognition of previously inaccessible genes causing either gene induction or repression (Beato and Sanchez-Pacheco, 1996).

The mechanisms of gene repression by steroid hormone receptors are also dependent on specific regulatory surfaces, which may or may not require DNA binding by the receptor to promote their activity. For GR, novel repression mechanisms have been proposed (Drouin *et al.*, 1993). For example, the gene encoding POMC contains a negative GRE (nGRE), of sequence GGAAGGTCAGGTCCA, which binds three GR molecules. A GR homodimer binds to the GRE, followed by a GR monomer on the opposite side of the double helix. GR inhibition of transcription may be mediated by

interference with the activity of other transcription factors, including basal components.

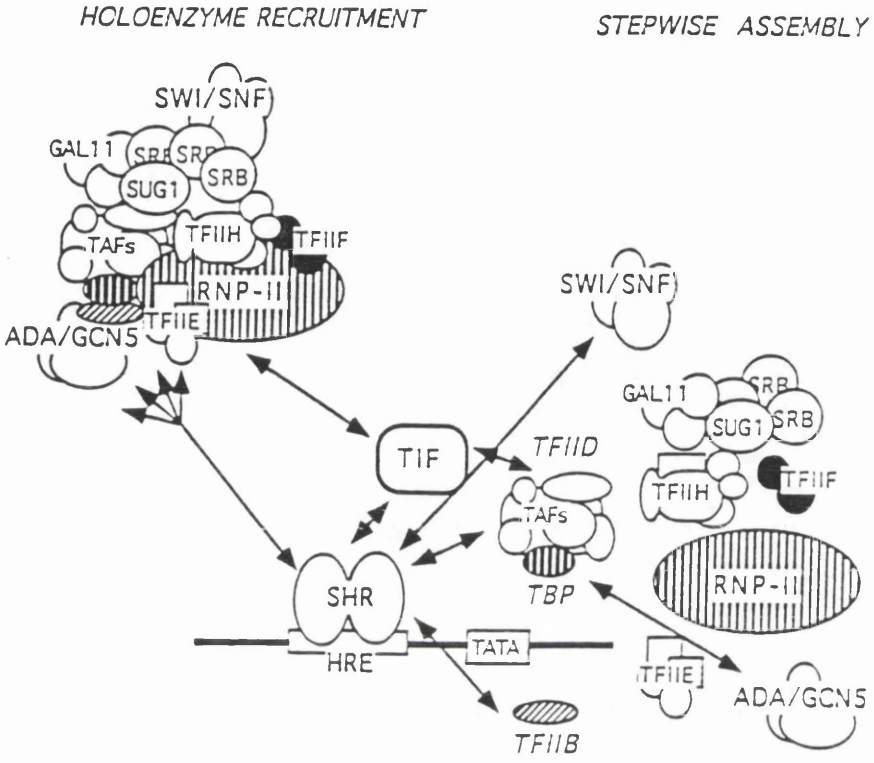

Fig 1.8. Assembly of the transcription initiation complex on a hormone-responsive minimal promoter.
Two possible alternate pathways are predicted. On the left, the recruitment of the complete RNA Pol-II holoenzyme; on the right, the stepwise assembly of the individual components. In both pathways, interaction with the receptors (shown as arrows) could be direct, or mediated by one or multiple coactivators (TIF).
(Taken from Beato and Sanchez-Pacheco, 1996).

Gel mobility shift experiments show that GR and the proto-oncogenenic transcription factor jun/AP1 can repress each others transcriptional activity in a reciprocal manner, through direct protein-protein interaction which is independent of DNA binding. Transfection analysis using transcription factor mutants shows that multiple domains of GR and the leucine zipper of jun/AP1 are involved in this process, either at the level of GR and jun/AP-1, or involving a common unidentified component of the transcriptional machinery (Schule *et al.*, 1990).

The interaction between steroid receptor superfamily and AP-1 family members in the regulation of gene transcription is well documented (Miner and Yamamoto, 1991; Miner and Yamamoto, 1992). It has been shown that the ability of GR to stimulate proliferin gene transcription is enhanced by c-jun homodimers, but repressed by c-jun/c-fos heterodimers. These effects are also mediated by an nGRE which is capable of binding both GR and AP-1 (see also Table 1.1.).

1.8.2b) Transcriptional modulation by GR

Signal transduction pathways enable many extracellular signals to activate latent transcription factors in the cytoplasm. Following common steps of dimerization, translocation to the nucleus and specific DNA or protein binding, these factors are able to regulate gene transcription. Different signalling pathways may even converge on the same promoter. The resultant transcriptional regulatory effects depend on the array of transcription factors present at the promoter and their molecular interactions.

Eukaryotic transcriptional activation is largely dependent on promoter context. Simple promoters containing a series of GREs in association with a minimal TATA box (e.g. the tyrosine aminotransferase promoter) depend solely on the functional properties of liganded GR for transcriptional activation (Jantzen *et al.*, 1987; Grange *et al.*, 1990). Composite promoters, on the other hand, are relatively more complex (e.g. the MMTV promoter) and contain additional binding sites for widely expressed and tissue-specific transcription factors, including OCT-1 (Truss *et al.*, 1995) and NF-1 (Granner *et al.*, 1996). NF-1 binding to the MMTV promoter is essential for activation by GR. The OCT-1 site modulates both basal and glucocorticoid-induced transcription (Granner *et al.*, 1996). Thus, protein-protein, as well as protein-DNA and protein-ligand interactions, which most likely take place through distinct polypeptide surfaces are all capable of influencing the activity of GR.

The efficiency with which GR activates transcription relies, in part, on the affinity of GR for its GRE, a property which depends on both the nucleotide sequence of the GRE and the receptor structure specified by the binding ligand (Delabre *et al.*, 1993; Lefstin *et al.*, 1995). For example, the glucocorticoid antagonist RU38486 can stimulate the translocation of GR to the nucleus and binding to a GRE, but the activated receptor is likely to remain transcriptionally inactive (Guido *et al.*, 1996). RU38486 can maintain a suppressed state of transcription, even in the presence of a constitutive GRE activator (C-terminally truncated GR). The effect of transcriptional repression elicited by an antagonist-activated receptor is thought not to result from the sequestering of an accessory transcription factor (the same effect can be reproduced *in vitro*), but from a receptor conformation, possibly influenced by the DNA binding sequence, which blocks the transfer of promoter activating signals from other factors. It is possible that different GREs induce different conformational changes in the bound GR in a manner analogous to different ligands, exposing unique receptor surfaces for activity with other cofactors. Different subsets of cofactors may in turn be required for different functional states of the promoter.

The idea that separate transcriptional regulatory sufaces of GR are required for promoter specific activity can be demonstrated using receptor mutants in which key residues are altered. For example, mutation of residue K442G in the DNA binding domain of hGR at one end of the DNA recognition helix affects function, not by inhibiting binding to DNA as might be expected, but by affecting the interaction of the receptor with other accessory proteins bound at the promoter (Montano *et al.*, 1995). Mutation of critical amino acid residues can genetically distinguish the promoter activity of GR, implying that different residues are important for interaction with different gene regulators. Consistent with this observation, hGR mutant K422G fails to repress AP-1 activity and activates promoters with AP-1 elements (Heck *et al.*, 1994; Starr *et al.*, 1996). Residue 442 is therefore probably involved in the transfer of information between the GRE and other parts of the GR protein and has implications regarding the flow of information between different domains of the receptor.

i.) Coregulators

Interactions between steroid receptors and basal transcription factors is necessary, but not sufficient for efficient transcriptional control. Recent evidence shows that apart from contacting the basal transcription machinery directly, nuclear receptors recruit an array of coactivators and corepressors, which are important in the mechanism of hormonally induced transcriptional regulation (Horwitz *et al.*, 1996). This third category of factors are thought to stabilise promoter specific interactions, providing transcriptional specificity (Goodrich *et al.*, 1993). A growing list of such molecules has been described. For example, the gene regulatory function of GR in yeast cells is dependent on the SWI/SNF family of transcription factors, which act to reverse the repressive effects of chromatin components (Chiba *et al.*, 1994). The human homologues of these factors, hSNFs also enhance the activity of GR as well as other steroid receptors (Muchardt *et al.*, 1993). The exact mechanism of action of these coactivators is still unclear, but for SWI/SNF factors, the currently favoured model is a stabilisation of the transactivator-PolII holoenzyme complex through the complimentary destabilisation of nucleosomes (Wilson *et al.*, 1996).

The binding of coactivators to steroid receptors is also likely to be a ligand regulated process. The nuclear proteins ERAP 160 (Halachmi *et al.*, 1994) and RIP 140 (Cavailles *et al.*, 1995) will only bind a conformationally active ER LBD in a reaction which is dependent on oestrogens and is destabilised by antioestrogens. These nuclear proteins are found in most tissues, which suggests they play a generic role in transcription. Several additional factors which interact with nuclear hormone receptors have also been described, which may form a large family of related proteins. Examples include SRC-1, which enhances the transcriptional activity of GR, ER TR and RXR *in vivo* (Onate *et al.*, 1995). It also interacts with agonist, but not antagonist occupied hPR (Onate *et al.*, 1995). GRIP-1, of the same family as SRC-1 is a coactivator of steroid receptor LBDs in yeast (Hong *et al.*, 1996). There are a whole host of other factors which bind either specifically (e.g. ARA_{70}: Yeh and Chang, 1996) or promiscuously (RAP_{46}: Zeiner and Gehring, 1995) to steroid hormone receptors. The CBP response element,

which is often found in association with GREs, is bound and coactivated by the cAMP response element binding protein, CBP. Fibroblasts microinjected with anti-CBP antibodies are unable to support GR regulated transcription, demonstrating the involvement of CBP in glucocorticoid signalling *in vivo* (Hanstein *et al.*, 1996). CBP could act as a cointegrator, coordinating the transcriptional effects of signals eminating both from cell membrane and nuclear receptors. The end result may be the integration of multiple, possibly conflicting signals which might impinge on a promoter.

This procession of recently discovered molecular interactions between steroid receptors and other nuclear proteins serves to illustrate the diverse molecular interactions which contribute to the transcriptional regulatory process. They also introduce a mechanistic complexity in which subtle differences in receptor steroid binding or protein sequence might be translated into different responses at the level of gene regulation. These additional regulatory layers might explain the heterogeneity of hormone responses seen in different tissues.

ii.) Synergism between the glucocorticoid and prolactin signalling pathways

The modulatory capacity of GR has already been used in an experimental approach to the assay of receptor function. In recent studies by Stocklin *et al.*, (1996) the glucocorticoid receptor has been shown to act as a coactivator for the transcription factor STAT5, enhancing STAT5-dependent transcription. STAT5 is a signal transducer and activator of transcription, one of a family of related transcription factors mediating the final stages of signal transduction through JAK/STAT pathways. These pathways are activated by cytokines, hormones and growth factors following binding to specific transmembrane receptors on the outside of the cell. The resultant activation of Janus protein tyrosine kinases (JAKs) leads to tyrosine phosphorylation of their associated STAT proteins and STAT dimerisation, conferring the ability to bind STAT-response elements in the DNA (Darnell *et al.*, 1994).

Although the glucocorticoid and JAK/STAT pathways are apparently unrelated, synergism between glucocorticoid and prolactin hormone action

in mammary epithelial cells is well documented (Doppler *et al.*, 1989). Prolactin is a lactogenic hormone (others include hydrocortisone and insulin) which, via the prolactin receptor, mediates prolactin-dependent induction of milk protein (β-casein) synthesis in terminally differentiated mammary epithelial cells through specific activation of STAT5. The coexpression of the prolactin receptor, STAT5 and GR in COS cells, in which only trace amounts of GR are normally expressed similarly results in synergistic activation of a cotransfected β-casein gene promoter (Stocklin *et al.*, 1996).

The direct relevance of the interaction between GR and STAT5 to the experimental focus of this thesis is discussed further in chapter 7.

Because cytokines can mediate inhibition of glucocorticoid-induced apoptosis of lymphocytes (LaVoie and Witorsch, 1995), it may be that STAT-GR mediated suppression of gene induction *in vivo* is responsible for this anti-apoptotic effect. It has also been proposed that the glucocorticoid receptor acts differently in its role as a negative transcriptional regulator in its interactions with AP-1 (Teurich and Angel, 1995) and NFkB (Cato and Wade, 1996). As anti-inflammatory agents, the transcriptional repression of several cytokines and adhesion molecules relevant to inflammatory processes are repressed by glucocortiocids (vanderBerg *et al.*, 1997). For example, the expression of intracellular adhesion molecule-1 (ICAM-1), an integrin which plays an essential role in the recruitment and migration of leukocytes to sites of inflammation, is repressed by opposing effects on the NFkB/RelA family member, RelA. This repression does not require GR binding to NFkB elements in the DNA, rather, GR interacts with the RelA protein. Of the family of steroid receptors, this effect is almost exclusive to GR, indicating a mechanism by which glucocortiocids specifically, function as anti-inflammatory agents. Few genes that are repressed by GR have GRE or nGRE sequences in their promoters, which suggests that DNA binding is probably not critical for all aspects of GR mediated gene repression (Truss and Beato, 1993).

1.9.) Detailed structure of the glucocorticoid receptor: localisation of functional domains

The glucocorticoid receptor (GR) was the first mammalian transcription factor to be isolated and studied in detail (Muller and Renkawitz, 1991). Cloning and sequencing of GR cDNAs from mouse, rat and man (Hollenberg *et al.*, 1985; Meisfield *et al.*, 1986; Muller and Renkawitz, 1991) has played a pivotal role in the structural and functional characterisation of this complex molecule.

The rat glucocorticoid receptor (rGR) at the molecular level displays properties characteristic of the steroid receptor superfamily of DNA-sequence specific transcription factors. The full length molecule comprises 795 amino acids (Miesfeld *et al.*, 1986) and divides into several autonomous domains showing cooperative molecular interactions. This interpretation is based on experimental evidence that discrete proteolytic fragments of GR possess some, but not all of the functional properties of the intact molecule (Vedeckis *et al.*, 1983). Residues 440-500 make contact with DNA through Zn-finger motifs, and present a nuclear localisation signal; the region from 595-614 interacts with heat shock protein 90 (HSP90), and the domain from 524-795 binds ligand and exposes a second nuclear localisation signal. The domains involved in transactivation of glucocorticoid-regulated genes are less well defined and several regions of GR show evidence of regulatory properties (McEwan *et al.*, 1997).

1.9.1.) Hormone binding domain (HBD)

Steroid hormone binding is the first step in a series of events which translate the structural information of the steroid into a biological response. Hydrophobic carboxyl-terminal residues in the HBD fold to accommodate the steroid binding pocket, which binds a single steroid molecule. Following hormone binding, steroid receptors undergo a structural (allosteric) alteration, or 'transformation', which for GR enables receptor dimerisation, translocation to the nucleus and finally, DNA binding (Garges and Adhya, 1985). The major (overlapping) sequences of

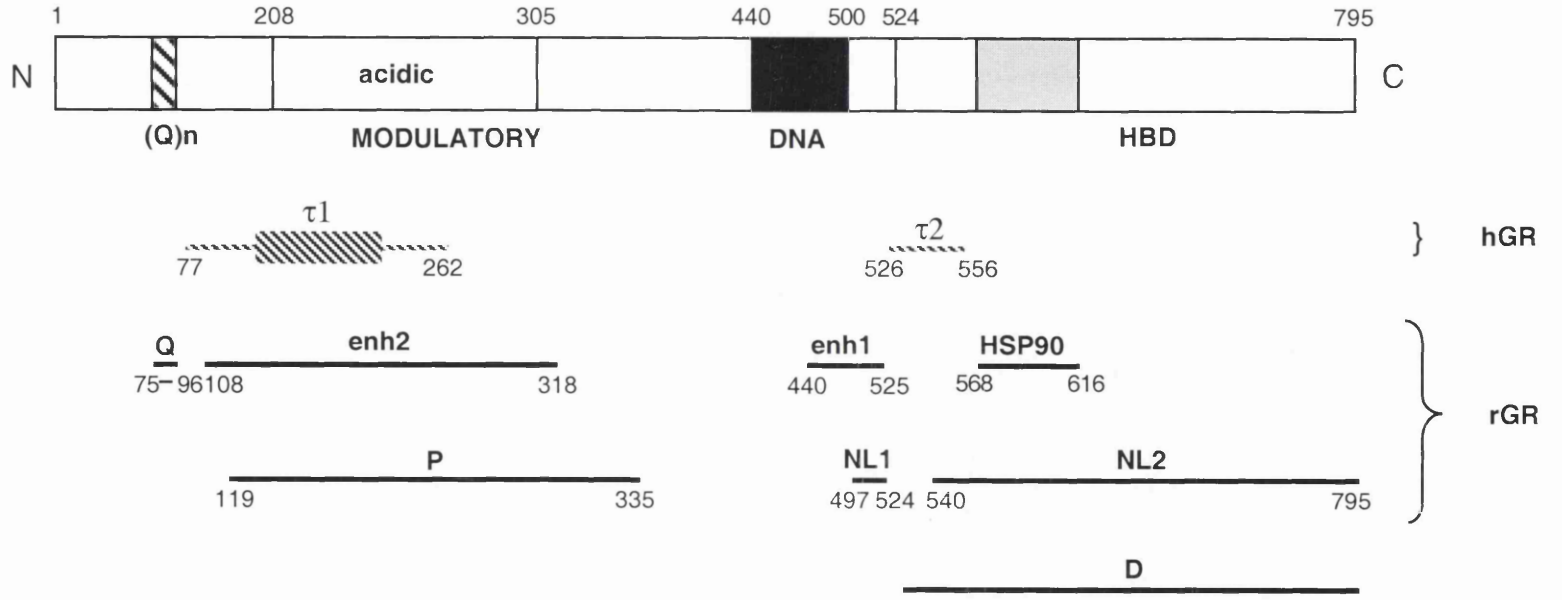

Fig. 1.9. Functional domains of the rat glucocorticoid receptor.
Estimated positions of domains are shown, with numbered residues for rat and human receptors, respectively (see text for references). The domains are: modulatory, modulatory domain; DNA, DNA binding domain; HBD, hormone binding domain; HSP90; HSP90 binding site; NL1 and NL2, nuclear localisation signals 1 and 2; P, phosphorylation domain; D, dimerisation domain; enh1 and enh2, transcriptional activation/enhancer domains 1 and 2; $(Q)_n$, polyglutamine tract (rGR residues 75-96; Miesfeld *et al.*, 1996). For comparison, the equivalent transactivation regions of human GR, τ1 (hGR, 77-262) and τ2 (hGR, 526-556) are shown as hatched lines. Hatched box in the τ1 domain localises the τ1-core polypeptide (hGR 187-244). Amino terminal transactivation domains of GR from different species overlap a highly acidic region required for maximum receptor activity. This region spans residues 208-305 (rat), 196-293 (mouse) and 187-285 (human) and is termed 'acidic'. The grey box marks the position (residues 595-614) of the HSP90 binding site, conserved in all members of the steroid receptor superfamily.

the HBD responsible for these events are shown in Figure 1.9. The entire C-terminal region of GR shows 96% amino-acid sequence identity between human, rat and mouse receptors. Homology of the same region between other members of the steroid receptor superfamily is less but still extensive, which probably reflects the structural differences between their hormone ligands (Evans, 1988). This homology may explain why virtually every steroid hormone appears to interact with more than one receptor subtype (Teustch *et al.*, 1988).

Many mutations directed to the steroid binding domain of GR cause a reduction or loss in steroid binding activity, measured by ability to induce an MMTV-CAT reporter gene (Rusconi and Yamamoto, 1987). In contrast, amino-terminal mutations reduce the ability of GR to transactivate. (Giguere *et al.*, 1986; Hollenberg and Evans, 1988, Dahlman-Wright and McEwan, 1996). Mutations occuring naturally in the steroid binding domain of GR (e.g., in familial glucocorticoid resistance; see section 1.13.) have a similar effect to directed mutations, in reducing steroid binding. There are exceptions; mutation C656G of rat GR results not only in increased affinity, but also in an enhanced specificity of steroid binding creating a 'super-receptor' (Chackraborti *et al.*, 1991). This supports the view that the hormone binding domain, at least in GR, contains a transcriptional inactivating function, possibly mediated through HSP90, which is known to bind to this region. The complete deletion of the carboxyl-terminus from human, rat and mouse GRs also produces constitutive (hormone independent) transcriptional activators (Danielsen *et al.*, 1987; Godowski *et al.*, 1987; Hollenberg *et al.*, 1987).

1.9.2.) DNA binding domain

The DNA binding domain of steroid receptors is comprised of a highly conserved 66-68 amino acid sequence, showing 42-94% homology between family members (Evans, 1988). It is the DNA binding domain which mediates specific recognition of the cognate steroid hormone response element (HRE). Each shows a clustering of basic residues, likely to interact with DNA (Umesono and Evans, 1989).

The critical DNA binding sequence contains nine conserved cysteines, eight of which are thought to form two zinc fingers. Each finger (both of around 25 amino acids: Giguere *et al.*, 1986) contains four cysteines coordinated with one atom of zinc (Evans and Hollenberg, 1987), forming motifs which interact with half turns of the DNA helix (Fig.1.7.). The amino-terminal finger makes sequence specific DNA contacts. Two amino acids located at the beginning of the alpha helix, which directly interacts with the DNA, are critical in determining binding specificity of GR and ER (Danielsen *et al.*, 1989). Mutational analysis of the second finger indicates that this region is also required for efficient DNA binding (Hollenberg *et al.*, 1987).

Proof of function of the central nucleic acid binding region from different receptors has been aided by 'finger swap' experiments, used to characterise receptor domains. For example, substituting the DNA binding domain from the human oestrogen receptor (hER) with that from hGR produced a hybrid molecule activated by oestrogen, but with the DNA binding specificity of hGR (Green and Chambon, 1987).

1.9.3.) Modulatory domain

The amino-terminal (modulatory) domain of steroid receptors is hypervariable in size and shows very little conservation between family members. However, this domain is still important for function. Deletions in this region of hGR can reduce transcriptional activity by 10- to 20-fold (Hollenberg *et al.*, 1987; Danielsen *et al.*, 1987). Additionally, NT^{i} (nuclear transfer increased) glucocorticoid receptor mutants with a truncated amino-terminus, can still bind hormone but are transcriptionally inactive (Dieken and Miesfeld, 1992). Mutants are retained in the cell nucleus more efficiently than wild type receptors, but fail to activate transcription. As well as a *trans*-activation function, receptors also appear to lose signals required for the efficient recirculation to the cytoplasm (Andreasen and Gehring, 1981; Westphal *et al.*, 1984; Dieken and Miesfeld, 1992). Similarly, an oestrogen receptor with an amino-terminal deletion can normally regulate the vitellogenin promoter, but is 10-fold less effective in

regulating the expression of the oestrogen responsive promoter, p52 (Kumar *et al.*, 1987). Structural differences in the amino-terminus may therefore influence the functional differences between receptors. The functional properties of this domain may involve interaction with general and promoter-specific transcription factors, modulation of DNA binding and responses to hormonal ligand by exerting an allosteric influence over the remainder of the activated receptor (Dellweg *et al.*, 1982).

1.9.4.) Trans-activation domains

Weinberger *et al.*, (1985) using insertional mutants, were able to demonstrate the existence of additional sequences, separate from DNA and steroid-binding domains with potent transcriptional activating properties. The *trans*-activation functions of the rat and human GRs have been localised to three distinct regions (McEwan *et al.*, 1997). The strongest transactivating region in human GR is τ1, located in the amino-terminal domain. The homologous region in rat GR is enh2, and is delimited by residues 108 and 318 (Godowski *et al.*, 1988). Evidence that these regions contain transcriptionally active surfaces comes from studies using chimeric proteins of both the human and rat GR amino-termini fused to unrelated DNA binding domains such as lexA (rat; Godowski *et al.*, 1988) or GAL4 (human; Hollenberg and Evans, 1988). Each is capable of promoting activation of a reporter gene (e.g. chloramphenicol acetyl transferase; CAT) containing the appropriate DNA binding elements. Domains τ1 and enh2, coincide with the major immunogenic domain of their respective GRs and therefore are likely to appear on the external surface of the molecule (Weinberger *et al.*, 1985). Weaker transactivating surfaces have been identified in the rGR protein: enh1 co-localises with the DNA binding domain (between residues 440-525), and is functionally equivalent to a similar region in hGR, τ2 (residues 526-556, human receptor sequence. See Fig. 1.9.). The τ2 domain of human GR (which, unlike τ1 is also present in smaller steroid hormone receptors largely devoid of a modulatory domain, such as ER) represents a *trans*-activator domain in its own right, functioning in a position and orientation-independent manner. Its activity, like that of τ1, is also increased when multimerised, or when

expressed in unison with τ1. When fused to the yeast transcriptional activators GAL4 or GCN4 as part of the ligand binding domain, τ2 generates a hormone-inducible activator (Hollenberg and Evans, 1988). The location of τ2, between DNA and hormone binding domains, suggests that it may act as a 'hinge' linking the two (Giguere *et al.*, 1986). Mutations in this region could therefore block the allosteric transformation necessary for receptor activation. McBroom *et al.*, (1995) have recently shown that a similar non-conserved 'hinge' region of the ROR receptor (a newly described orphan member of the steroid-thyroid-retinoid receptor superfamily) is required for maximal and efficient DNA bending. A third transcriptionally active region of GR locates to the C-terminus and is highly conserved between different steroid receptors (Danielian *et al.*, 1992).

i.) Detailed structure of the glucocorticoid receptor transactivation domains, enh2 and τ1

The experiments in this thesis are based largely on structure-function relationships of rat GR. An understanding of the mechanisms by which mutation of amino acid residues in the rGR amino terminus may alter molecular function depends on a detailed knowledge of the type and distribution of residues, their charge properties and likely contribution to specific molecular interactions.

The most detailed dissection of a transactivation domain of GR has been carried out in the amino-terminal τ1 region of hGR (Hollenberg and Evans, 1988). τ1 is the homologue of rat enh2 and contains a potent site of transcriptional activation in a core sequence located between residues 187-244. Recent studies have shown that the τ1 core sequence (Fig. 1.10.) is ordered into three α-helices (HRI, HRII and HRIII) with a defined loop structure separating HRI and HRII (Almlöf *et al.*, 1997). A sequence homologous to the τ1 core is also found in enh2 spanning residues 210-261. These core sequence polypeptides show 83% sequence homology, in which the residues key to receptor function are conserved (Almlöf *et al.*, 1997; Iñiguez-Lluhi *et al.*, 1997).

Charge properties, such as those resulting from acidity are known to influence the activity of many transcription factors (reviewed by Ptashne, 1988). However, the analysis of a series of τ1 mutants in hGR (TAs 1-8), each

of which encodes a small cluster of mutations affecting transcriptional activation (Almlof *et al.*, 1995), has indicated that the acidity of individual residues is not critical for function. All of the corresponding residues are conserved in the aligned rat GR sequence (see Fig. 1.10.). For example TA1, in closest proximity to the amino terminal end of τ1 (and only 16 residues C-terminal to a polyglutamine tract in the corresponding position in rat GR), defines the effects of mutation of acidic residues; Glu_{92}, Glu_{94} and Asp_{101}. The neutralisation of these residues produces a receptor protein with reduced transactivation activity (80% of wild type). Mutation of other acidic residues (predominantly glu, asp) throughout the majority of the τ1 domain also causes variable small changes in transcriptional activation function.

Three amino acid substitutions in the rat core peptide (E219 to K219, F220 to L220 and W234 to R234) selectively disrupt the activation, but not the repression function of the receptor amino-terminus. This supports the theory that specific residues of GR have defined functional roles (Iniguez-Lluhi *et al.*, 1997). Each of these residues occurs within a 16 amino acid sequence which aligns with the corresponding region of the τ1 core of human GR (Fig. 1.10.). Residues E219 and F220 (rGR) correspond to residues in helical region I (HRI) of the τ1 core sequence. Similarly, residue W234 of rat GR is homologous to residue W213, a critical residue of the τ1 core loop sequence. Mutation of W213 to G213 reduces hGR transactivating activity to 42% of wild type. It has been proposed (Almlöf *et al.*, 1997) that the structural components of the τ1 core are likely to present an array of discrete surfaces for interaction with other transcription factors.

The functional complexities of transactivating surfaces are currently being defined. The charge properties of different types of residues are important: the significance of acidic residues has already been discussed. Hydrophobic residues, as well as charged residues, also appear to provide important functional characteristics, contributing patches of hydrophobicity which may influence transactivation (Almlof *et al.*, 1995). The structural influences these residues induce in the τ1 core sequence suggest an activity mechanism which is more complex than that predicted for an acidic blob-like activator domain (Dahlman-Wright *et al.*, 1995) and has important implications for rGR function. Sequence alignments show that, as well as the 'core' sequence, a very high proportion of the acidic (Glu, Asp, phosphorylated Ser) and hydrophobic (Phe, Ile, Leu) residues are conserved between rat and human receptors. This homology extends

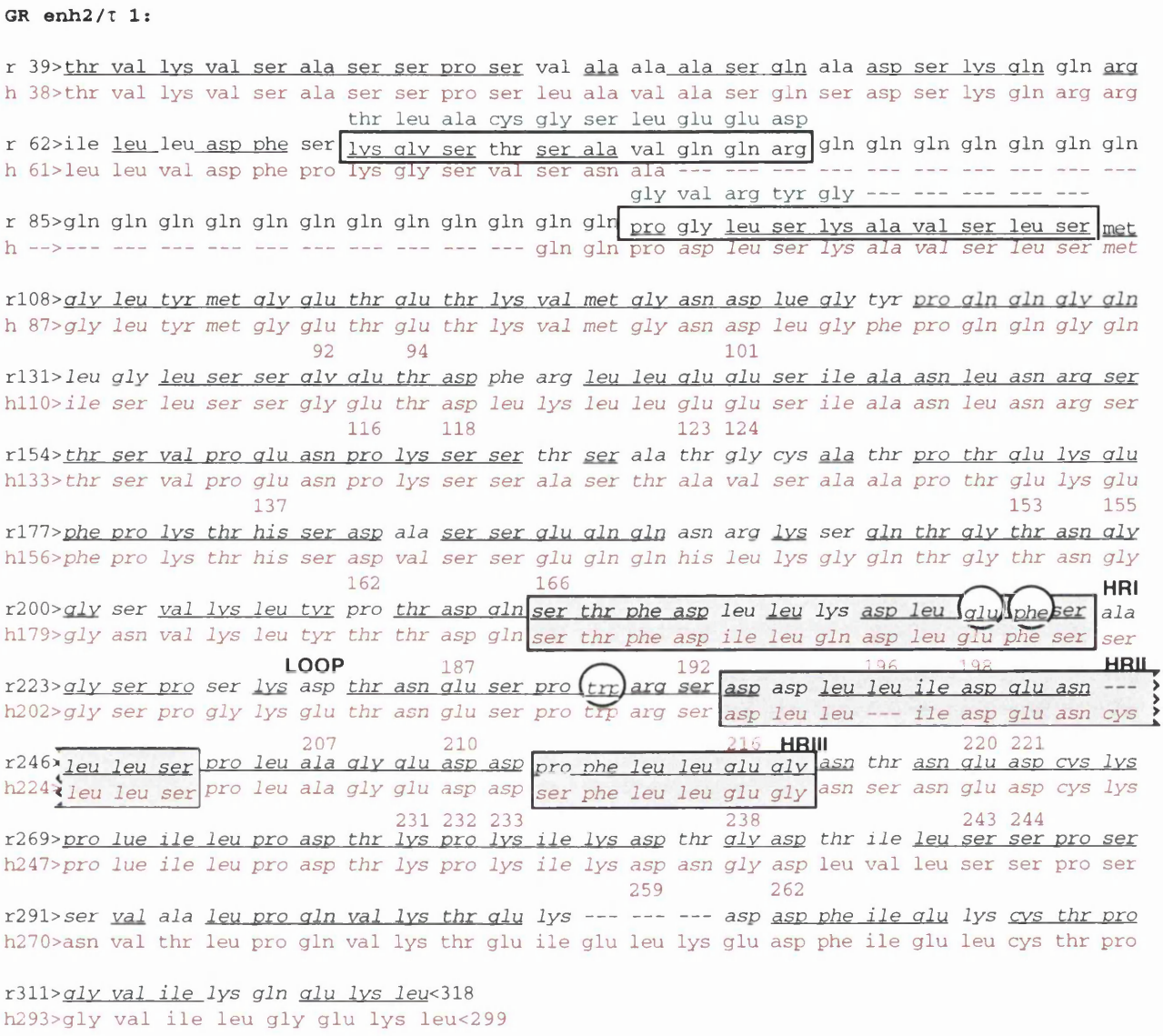

Fig. 1.10. Alignment of the major transactivation domains (τ1 and enh2) of the human and rat GRs. Open boxed residues in the amino terminus of the rat sequence (in black) denote those residues mutated in rat GRs constructed in this thesis. Residues above in green represent the corresponding amino acid mutations. Residues underlined represent sequences of homology between rat and human GR (shown in red). Amino acid coordinates within the human sequence mark acidic residues, which when mutated, bring about small alterations in receptor transactivation activity (Almlof *et al.*, 1995). Dashed lines locate residues missing in the corresponding sequence. Residues in italics mark the major amino-terminal transactivation domains; τ1 (human: residues 77-262) and enh1 (rat: residues 108-318). Shaded boxes localise those residues which make up the three helical regions of the hGR tau 1 core sequence (Almlof *et al.*, 1997). Homology in these regions between human and rat GRs are: 83% (HRI), 82% (HRII) and 83% (HRIII). Circles identify residues of the rat GR which are known to selectively disrupt transactivation function (see text for details).

beyond the perceived amino-terminal boundaries of enh2 and τ1 and into sequences which precede the polyglutamine tract of rat GR (see Figs. 1.9. and 1.10.).

Despite these advances in our understanding of the critical structural components of enh2 and τ1 the precise functional limits of these domains remain undefined. Most of the focus to date regarding GR amino terminal transactivation function has been centred around residues which comprise the 'core' polypeptide sequence. In addition, the identification of functionally important residues has been based largely on those which either significantly reduce, or completely abolish transcriptional activation activity of GR (Godowski *et al.*, 1987; Godowski *et al.*, 1988).

ii.) Polyglutamine tract

Protein sequences rich in basic residues such as glutamine and proline are also known to affect transcription. Many proteins have now been described which possess homopolymeric stretches of these amino acids (Ross *et al.*, 1993; Gerber *et al.*, 1994). Transactivation is shown to be affected when tracts of polyglutamine or polyproline are covalently inserted between the yeast GAL4 DNA-binding moeity and the transcriptional activation domain of herpes simplex-derived VP16 protein, indicating that these homopolymeric tracts could influence transcription (Gerber *et al.*, 1994). No such evidence exists for GR. Whilst in the rat, the length of the polyglutamine tract varies between 7 and 23 CAG repeats, the mouse and human receptors are constant, with 9 and 2 repeats respectively (Danielsen *et al.*, 1986, Hollenberg *et al.*, 1985). At the molecular level, no gross differences in transactivation function have been reported between human (2 repeats) and rat (21 repeats) GR cDNA clones (Miesfeld *et al.*, 1987). However, predisposition to prostatic cancer is positively associated with shorter CAG tracts in the human androgen receptor (hAR). Deletion of the CAG tract in rat or human AR is reported to enhance the activity of reporter elements, and long repeats reduce transcription to a degree (Mhatre *et al.*, 1993; Chamberlain *et al.*, 1994). In these reports it is difficult to exclude the possibility that two-fold differences in transactivation might be due, in part, to differences in protein stability. In a case of androgen insensitivity, a reduction of polyglutamine repeat length in hAR was

associated with increased lability of the receptor in tissue culture when associated with the mutation T761C in the steroid binding domain (McPhaul *et al.*, 1991).

The exact functional role of polyglutamine tracts is unclear, although by their very nature they are predicted to lie on the surface of proteins where, under non-pathological circumstances, could provide discrete surfaces which promote protein-protein interactions. Perutz and coworkers, (1994) have shown that synthetic polymers of glutamine residues are capable of forming regular beta-barrel structures, strongly held together by hydrogen bonds. These motifs may function as polar zippers, providing a means for the interaction of trans-acting factors even when bound to separate DNA segments. For example, the glutamine activation domains of Sp-1 (Courey and Tjian, 1988) and the POU (proline and glutamine-rich) domains of Oct-1 can specifically bind TBP *in vitro*. TBP mutants have now been identified which are defective in activated, but not basal transcription. A number of TAFs have been shown to bind specificaly to particular types of activation domains, e.g., dTAF110 which binds to the glutamine-rich domains of Sp-1 (see Triezenberg, 1995).

Interactions between polyglutamine tracts and other proteins has also been suggested as a molecular basis of the microsatellite expansion diseases such as Huntingtons disease (HD; Myers *et al.*, 1993) spinocerebellar ataxia type 1 (SCA 1; Orr *et al.*, 1993) spinal and bulbar muscular atrophy (SBMA; LaSpada *et al.*, 1992) and dentatorubral pallidolusian atrophy (DRPLA; Koide *et al.*, 1994). Of the gene mutations known to be involved in these diseases, only that causing SBMA is known to reside in a transcription factor (AR) and it is possible that altered transactivation function may lead to aberrent gene regulation. Alternatively, homopolymeric expansion may lead to progressive aggregation and co-precipitation of other cellular factors (Perutz *et al.*, 1994), although confirmatory evidence of cellular precipitates has not been reported.

Given the molecular complexities by which GR can modulate transcriptional activity; GR can even act as a corepressor through interaction with other transcription factors such as AP-1 (Pearce and Yamamoto, 1993; Diamond *et al.*, 1990), the potential for effects of polyglutamine sequences on interactions between GR and these other factors, or within GR homodimers becomes more apparent. Such interactions could have far reaching consequences metabolically or in a tissue or developmental stage specific fashion. GR has also been shown to form dimers with MR (Trapp *et al.*, 1995), which have an enhanced cooperativity in DNA binding and with AR (Chen *et al.*, 1997), which also carries an N-terminal polyglutamine tract in both rats and man. These interactions offer yet other ways of modulating glucocorticoid activity in tissues which express different combinations of these receptor types.

Part 2

Glucocorticoids and mechanisms of blood pressure control

1.10.) Corticosteroid receptor designation

Classically, corticosteroids are thought to exert their biological effects by differential binding to and activation of MR and GR. However, this distinction is possibly naive. There seems to be a clear lack of selectivity for natural mineralocorticoid and glucocorticoid hormones between these receptors. MR has a similar affinity (Kd, around 1 nM) for both aldosterone and glucocorticoids (cortisol/corticosterone) and is activated by both of these hormones *in vitro* (Krozowski and Funder, 1983). GR has only a slightly higher affinity for glucocorticoids (Kd, 10-50 nM) than for aldosterone (Kd, 50-100 nM) (Arriza *et al.*, 1987).

Given that total circulating levels of glucocorticoids are usually several orders of magnitude (100-1000-fold) higher than those of aldosterone, despite the fact that glucocorticoids are heavily bound to plasma proteins,

such as CBG (see section 1.3.) while aldosterone is mostly free, how is a mineralocorticoid response to aldosterone possible? Figure 1.11. outlines the basic mechanism. Primary sequence homology of hMR and hGR is high (DNA-binding domains, 94%; hormone binding domains, 57%. Respectively, 59% and 76 % in the rat). Thus, tissue-specific differences in aldosterone and glucocorticoid activation of mineralocorticoid (type I) receptors cannot be explained purely by receptor differences.

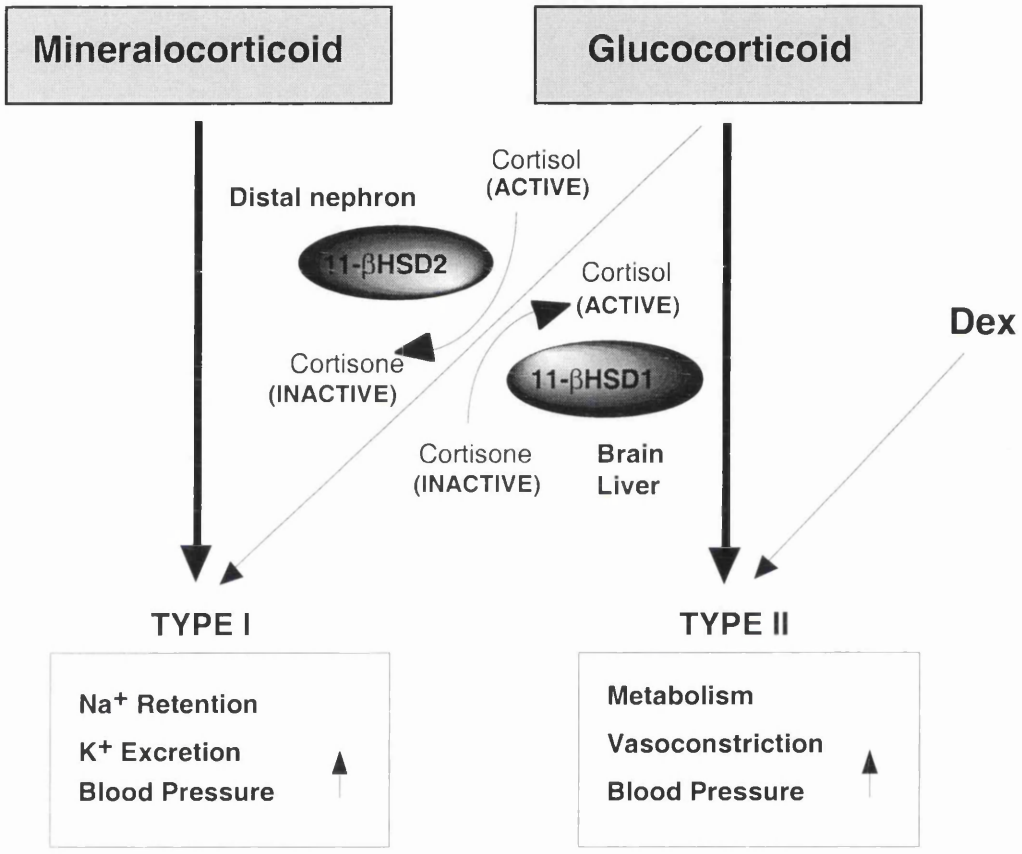

Fig. 1.11. The basic mechanism of aldosterone selectivity in target tissues. Glucocorticoids (cortisol/cortocosterone) have a roughly similar affinity for type I and type II corticosteroid receptors. In classical aldosterone responsive tissues, however, glucocorticoids are normally prevented from binding to mineralocorticoid (type I) receptors by 11β-hydroxysteroid dehydrogenase (11β-HSD) which rapidly metabolises these compounds. The glucocorticoid metabolites (cortisone and 11-deoxycortcosterone, respectively) have a much lower affinity for the mineralocorticoid (type I) receptor, thus allowing unhindered aldosterone binding. The reciprocal reactivation of cortisone and 11-deoxycortcosterone back to the active steroids occurs in other tissues, mainly the liver.

Clearly, a protection function preventing unwanted glucocorticoid access to type I receptors is necessary in tissues which are required to respond almost exclusively to aldosterone. This is achieved by the enzyme 11-β hydroxysteroid dehydrogenase (11β-HSD) (Monder and White, 1993). This

enzyme has at least two isoforms (isozymes), the products of two distinct genes (Seckl, 1993). 11-βHSD2 (Albiston, *et al.*, 1994; Brown *et al.*, 1996), functioning as a reductase, converts cortisol and corticosterone to their respective inactive metabolites cortisone and 11-dehydrocorticosterone in aldosterone responsive tissues.

The other 11-βHSD isozyme, 11-βHSD1, is proposed to be an NADP(H) dependent enzyme (Agarwal *et al.*, 1989) which reactivates these glucocorticoid metabolites in glucocorticoid responsive tissues. In transport epithelia, particularly the distal nephron of the kidney, MR co-localises with the 11-βHSD2 isoform (Albiston, *et al.*, 1994; Brown *et al.*, 1996), which inactivates cortisol/corticosterone allowing aldosterone preferential access to the receptor. In brain and hippocampus, the 11β-HSD1 isoform predominates. In conclusion, the overall effects of corticosteroids on blood pressure are the composite result of activating more than one type of receptor.

1.11.) Glucocorticoid regulation of vascular tone: mechanisms of blood pressure control

Several components determine physiological blood pressure. These include cardiac output, resistance of the blood vessels, blood volume and viscosity. Total peripheral resistance is inversely proportional to vessel diameter, a function of vascular muscle tone and elasicity (Kenyon and Fraser, 1992). Each of these components is controlled by numerous neuronal and endocrine influences. The central nervous system control of cardiac function and total peripheral resistance is a critical factor in blood pressure regulation. Resultant effects are mediated through various neurotransmitters such as norepinephrine and acetycholine and their receptors (Axelrod and Reisine, 1984). Properties of resistance vessels, including structure, basal tone and sensitivity to various pressor agents also depends on short and long term influences of systemic and local tissue derived hormones (Benjamin and Vallance, 1991). The synthesis, action and

metabolism of many of these compounds are modulated by glucocorticoids and mineralocorticoids (reviewed by Walker and Williams, 1992).

All categories of adrenal steroids; glucocorticoids, mineralocorticoids, androgens and oestrogens are capable of influencing blood pressure but, glucocorticoids and mineralocorticoids are the major regulators (Sleight, 1986). The mechanisms by which steroids raise blood pressure differ. In the case of aldosterone, the exact nature and functions of all of the induced proteins have not been fully identified. In the kidney and in other transport epithelia, aldosterone-induced proteins (AIPs) are thought to increase cell permeability to sodium, increase the rate of ATP synthesis and stimulate the activity of the Na^+-K^+-ATPase (Horisberger and Rossier, 1992).

Despite their pronounced effect on blood pressure, mineralocorticoids and glucocorticoids do not effect smooth muscle contraction directly. Pressor effects of steroids can result from either the potentiation or stimulation of secretion of vasoconstrictors, or decrease in the effectiveness of vasodilators. The following two sections outline the principal modulatory effects of glucocorticoids on chosen examples of vasoactive molecules (see Table 1.2.).

1.11.1.) Vasoconstrictors

1.11.1a.) Catecholamines and adrenergic receptor interaction

The vasoconstrictor properties of glucocorticoids are based predominantly on their modulation of vascular smooth muscle sensitivity to noradrenaline (NA) (Yagil *et al.*, 1986; Frohlich, 1987; Darlington *et al.*, 1989; Sudhir *et al.*, 1989). Changes in vascular structure, second messenger events, adrenoceptor binding characteristics and the activity of enzymes which metabolise catecholamines are all known to be involved (Axelrod and Reisine, 1984).

Sites of modulation	Glucocorticoid effect	Reference
Vasoconstrictors		
α-adrenoceptors	In VSMCs of the rat, reduced high affinity α-adrenoceptor numbers are restored by dexamethasone	Haigh and Jones, (1990)
***ANP**	Glucocorticoids suppress ANP production *in vivo* and ANP activity in tissue culture	Kenyon *et al.*, (1990)
***ACE**	Glucocorticoids induce ACE in endothelial cells	Medelsohn *et al.*, (1982)
***Angiotensinogen**	Circulating levels of angiotensinogen are increased by glucocorticoids	Ben-Ari *et al.*, (1989)
***Renin**	In contrast to mineralocorticoids, glucocorticoids are reported to increase renin gene transcription	Morris *et al.*, (1984)
Vasodilators		
β-adrenoceptors	Rat aortic vascular smooth muscle cells show increased numbers of β-adrenergic receptors at physiological concentrations of dexamethasone	Jazayeri and Meyer (1988)
***Vasopressin**	Glucocorticoids secondarily down regulate vasopressin synthesis via ANP in response to vascular volume expansion	(Kenyon and Jardine, 1989)
***ANP**	Glucocorticoids increase ANP synthesis in response to surges in vascular volume	Kenyon *et al.*, (1990)
Phospholipase A2	Glucocorticoids reduce the production of vasorelaxant eicosinoids PGE1 and prostacyclin by down regulating phospholipase A2 gene transcription	Flower (1988); Axelrod, (1983)
***NO-synthase**	The inducible form of NO-synthase, identified in macrophages and VSMCs is inhibited by glucocorticoids	DiRosa *et al.*, (1990)

Table 1.2. Vasoactive molecules modulated by glucocorticoids. Abbreviations: VSMC, vascular smooth muscle cell; PGE1, prostaglandin E1; Gi, G-protein inhibitory subunit; Gs, G-protein stimulatory subunit; IP3, inositol triphosphate; DG, diacyl glycerol; NA, noradrenalin; ANP, atrial natriuretic peptide; ACE, angiotensin converting enzyme; NO, nitric oxide. Those molecules primarily involved in blood pressure control are marked in bold. Those molecules controlled at the genetic level by glucocorticoids are marked with an asterisk.

For example, dexamethasone has been shown to increase the number and affinity of aortic binding sites for the specific α1-adrenoceptor ligand, prazosin, in adrenalectomized rats (Haigh and Jones, 1990). This effect is not produced by aldosterone (Yagil and Krakoff, 1988). Similarly, increased

numbers of β-adrenergic receptors in various tissues, including human lymphocytes and granulocytes, rat liver and rat lung (Davies and Lefkowitz, 1980) have been observed.

Although the activity of adrenoceptors is modified in a post-translational manner by glucocorticoids (Haigh and Jones, 1990), there are a number of molecules with vasoconstrictor properties, which are regulated at the level of the genome. Examples include angiotensinogen and angiotensin converting enzyme (ACE) (Mendelsohn *et al.*, 1982), both important molecules of the renin-angiotensin system.

1.11.1b.) Renin-angiotensin aldosterone system

The influence of adrenocortical hormones over the renin-angiotensin system occurs at several levels. Renin, an aspartyl protease, important in the regulation of blood pressure and electrolyte balance is expressed in a variety of mammalian tissues, predominantly in the afferent arterioles of the kidney, but also in the adrenals and vascular smooth muscle cells (Makried *et al.*, 1988). The physiological effects of aldosterone suppress the renin-angiotensin system (section 1.3). Mineralocorticoid induced hypertension in rats (DOCA treatment, or high salt [1%] diet) causes suppression of renal renin gene expression, which is thought to be mediated by MR. Glucocorticoids oppose the effects of aldosterone and are reported to increase renin activity by activating renin gene transcription (Glorioso *et al.*, 1989). Possible GREs in the promoter of the renin gene (Dzau *et al.*, 1988) supports this proposal. Mineralocorticoid-induced increases in blood pressure are associated with increased vascular reactivity to angiotensin. Evidence of angiotensinogen gene regulationn by glucocorticoids is widely accepted and is likely to be important in conditions of glucocorticoid deficiency (Riftina *et al.*, 1995).

ACE is localised to the luminal surface of vascular endothelial cells, the major site of angiotensin I conversion *in vivo* (Caldwell *et al.*, 1976). Other prominent sites of ACE activity include the kidney, brain and small intestine. Cultured endothelial cells from bovine aorta and intact rat lung

in vivo show a net increase (6-7 fold) in ACE production in response to dexamethasone (100 nM). DOC and aldosterone are much less effective, stimulating only a 2-3 fold increase in ACE activity at 1 μM concentration (Mendelsohn *et al.*, 1982). Local levels of vasoactive pepetides, angiotensin II and bradykinin are likely to be influenced by glucocorticoids, with resultant effects on vascular tone. ACE normally inactivates bradykinin, a potent vasodilator (Erdos, 1977), but activates angiotensin II.

Recent studies have also demonstrated that type 1 angiotensin II receptors (AT1) are up-regulated by glucocorticoids in the rat hypothalamic paraventricular nucleus (PVN). AT1 receptor mRNA levels and All binding were reduced by approximately 20%, following adrenalectomy. This effect was prevented by corticosterone administration in the drinking water, or dexamethasone injection (100 mg, s.c., daily). Conversely, dexamethasone injection into intact rats caused a 20% increase in AT1 receptor mRNA. Again, this up-regulation would potentiate the vasoconstrictor effects of the renin-angiotensin system (Aguilera *et al.*, 1995).

1.11.2.) Vasodilators

Vascular tone is the balance between the action of vasoconstrictor and vasodilators mechanisms (Walker and Williams, 1992). Inhibition of vasodilation seems most relevant to glucocorticoid induced hypertension. The major vasodilators, atrial natriuretic peptide (ANP/ANH) (Kenyon *et al.*, 1990), prostaglandins (Bailey, 1991) and the endothelium derived relaxing factor, nitric oxide (DiRosa *et al.*, 1990) are all regulated by glucocorticoids.

1.11.2a.) ANP

ANP has direct vasodilatory properties, separate from its natriuretic effect. Physiological concentrations of glucocorticoids are required for the normal expression of ANP, but glucocorticoids may inhibit ANP synthesis and secretion, either by reducing plasma volume, or by regulating ANP

gene expression (Tonolo *et al.*, 1988). Glucocorticoids may also inhibit the responsiveness of vascular tissues to ANP by interfering with ANP signal response coupling. For example, renal vascular smooth muscle cells (VSMCs) treated with dexamethasone produce less cyclic GMP (cGMP) in response to ANP than untreated cells (Yasunari *et al.*, 1990). cGMP is the second messenger for a number of vasodilatory processes. Decreased ANP and cGMP levels will both tend to prevent vasodilation.

1.11.2b.) Nitric oxide (NO)

Synthesis of nitric oxide in endothelial tissues is controlled by two types of NO synthase, one constitutive and Ca^{2+}-dependent and the other inducible and Ca^{2+}-independent (Rees *et al.*, 1989). Glucocorticoids block the activity of the inducible form of NO synthase (Radomski *et al.*, 1990), limiting nitric oxide induced vasodilation. The effect of increase in blood pressure following inhibition of nitric oxide synthesis is well established (Haynes *et al.*, 1993). Similar effects of glucocorticoids have also been demonstrated in macrophages (DiRosa *et al.*, 1990) and have implications regarding the anti-inflammatory and immunosuppressive effects of glucocorticoids.

1.12.) Pathophysiological effects resulting from abnormal glucocorticoid signalling

Since corticosteroids exert powerful influences over intermediary metabolism, blood pressure control and electrolyte homeostasis, it follows that any deviation from their normal rates of production and metabolic clearance (Kaplan, 1983), will have profound consequences on receptor mediated responses. The effects of abnormal steroid production, and of abnormal receptor and catabolising enzyme activities are well documented.

The best known example of clinical glucocorticoid excess is seen in patients with Cushing's syndrome, in which primary hyperactivity of the adrenal cortex (low ACTH, high cortisol) or of the anterior pituitary (high ACTH, high cortisol) causes increased cortisol secretions (For reviews see: Fraser

et al., 1989; Connell and Fraser, 1991). A characteristic phenotype is observed; centralised body fat distribution, degeneration of muscle and connective tissue, growth impairment and osteoporosis. Patients may develop hypertension, sexual dysfunction and reduced resistance to infection. Synthetic glucocorticoids, or ACTH administered over a long period produces similar effects (Kenyon and Fraser, 1992). Excess aldosterone or 11-deoxycorticosterone (DOC) secretion causes mineralocorticoid-induced hypertension. Mineralocorticoid excess (resulting in hyperaldosteronism) typically arises from adrenocortical adenoma or carcinoma (Conn and Mich 1955) and is characterised biochemically by suppressed plasma renin and angiotensin II concentrations, together with Na^+ retention, K^+ loss and a metabolic alkalosis. The hypertension which ensues, is therefore an indirect consequence of changes in sodium levels and plasma volumes. As discussed in section 1.10., 11β-HSD type I and II regulate the access of cortisol to MR and GR. Suppressed 11β-HSD2 activity also leads to symptoms of hyperaldosteronism in the absence of aldosterone excess. Two clinical situations are known in which the activity of this enzyme is reduced; congenital apparent mineralocorticoid excess (AME), in which enzyme activity is deficient (Stewart *et al.*, 1988; Mune *et al.*, 1995) and acquired AME, which is caused by intoxication with inhibitors of 11β-HSD2 activity, such as glycerritinic acid, found in liquorice, or its derivative carbenoxalone (Stewart *et al.*, 1987).

Reduced adrenocortical activity (eg. in patients with Addisons disease, resulting from pathological destruction of adrenal tissue or ACTH deficiency) is normally associated with hypotension, hypoglycaemia, weight loss, fluid and electrolyte disturbances and abnormal development of secondary sexual characteristics (Brosnan and Gowing, 1996).

1.13.) Corticosteroid receptor mutations

The incidence of mutant corticosteroid receptors in humans is relatively rare. Studies of GR in patients with familial glucocorticoid resistance syndrome (FGR) has indicated that hypertension is a possible phenotypic consequence (Hurley *et al.*, 1991). Sequencing of human GR cDNAs from a kindred with FGR revealed a single point mutation in the hormone binding domain (V641D) which resulted in a 3-fold reduction in affinity for dexamethasone. The resultant hypertension was characteristic of mineralocorticoid excess. The mutant GR was unable to switch off ACTH synthesis from the anterior pituitary, resulting in elevated levels of plasma cortisol. These higher cortisol concentrations presumably overstep the capacity of 11-βHSD2 to protect MR from over-stimulation by glucocorticoids. A similar phenotype is encountered in patients with primary cortisol resistance, which is classified as a glucocorticoid receptor-mediated disease, resulting from a reduced affinity of the receptor for its hormone ligand (Bamberger *et al.*, 1996). Other forms of glucocorticoid insensitivity have also been described. Werner *et al.*, (1992) demonstrated instances of receptor thermolability in association with increased rates of GR gene transcription. A novel form of natural glucocorticoid resistance is found in the guinea pig GR, in which the ligand binding domain contains several novel amino acid substitutions (Keightley and Fuller, 1994). This altered GR structure directs constitutive transcriptional activity which is unsuppressed by RU38486 (1μM). Uncontrolled receptor activity in these cases may result from loss of interaction with HSP90. Mutant GR which do not bind HSP90 are transcriptionally active in the absence of hormone (Bronnegard *et al.*, 1991). Further abnormalities of GR function might be anticipated from mutations in the promoter region of the GR gene. As yet, no promoter effects on GR mRNA levels have been demonstrated. However, their identification may depend on promoter usage and the availability of tissue-specific transcription factors (Nobukuni *et al.*, 1995).

Glucocorticoid as well as progesterone, oestrogen and androgen receptors have been shown to be constitutively activated by loss of a portion of the ligand binding domain (e.g., Carson *et al.*, 1987; Godowski *et al.*, 1987; Brown *et al.*, 1988). Resultant proteins are still able to bind DNA, but are no longer regulated by steroids. hGRα and hGRβ are two isoforms of the human GR which result from alternate receptor mRNA splicing (Hollenberg *et al.*, 1985). hGRα is the biologically active isoform. The hGRβ isoform is carboxy-terminally truncated, has no reported intrinsic activity, but has been shown to interfere in hGRα steroid binding studies. The relative levels of these GR isoforms may affect GR function, either globally or in a tissue-specific fashion. Studies by Bamberger *et al.*, (1995), suggest that attenuation of transcriptional regulation by hGRα is brought about by competition by the truncated non-steroid responsive hGRβ for GRE binding sites in the DNA. In the rat, no obvious GR splice variants have been observed. However, lower molecular weight hepatic GR isoforms have been reported, which possibly result from alternate use of translation initiation codons in the GR mRNA (Miesfeld *et al.*, 1986).

Patients with depressive illness, characterised by excessive secretion of corticotropin releasing factor and cortoisol, due to hyperactivity of the HPA-axis also provide evidence of abnormal patterns of GR expression (Pepin and Barden, 1991). This observation derives from the action of anti-depressants which modulate the GR mRNA content of neuronal cells involved in the negative feedback control of HPA-axis function. Similar observations have been made by Lamberts *et al.*, (1994) who described female patients with reduced GR expression, overproduction of adrenal steroids and pathology resulting from excessive adrenal androgenic activity. In support of a consequential loss of GR activity, the directed knockout of type II glucocorticoid receptors in mice carrying an antisense GR RNA transgene (Pepin *et al.*, 1992) also generates increased activity of the HPA-axis, resulting in elevated corticosterone and ACTH levels, suggesting a general ineffectiveness of GR in feedback inhibition of glucocorticoid secretion.

The effectivity of the glucocorticoid signal may be reduced by means other than interference of steroid binding to GR. Accessibility of activated GR to DNA, a vital component of the glucocorticoid signal pathway may also be affected. In steroid resistant (SR) asthmatics, (Barnes and Adcock, 1995) this condition seems to result from increased binding of GR to AP-1, presumably sequestering GR away from other modulatory roles concerning the anti-inflammatory actions of glucocorticoids .

1.14.) Essential hypertension in man

Although in man, specific abnormalities of glucocorticoid function have been shown to result in cardiovascular disease, in most cases, the cause of hypertension is not known. Essential hypertension, of unknown origin, represents a major public health issue due to its common occurrence (affecting 20-30% of the population) and its long-term complications. As a quantitative trait, blood pressure varies continuously in the whole population. Its regulation is controlled by a variety of mechanisms involving, probably several genetic loci and environmental and other factors such as diet, body weight, stress and physical exercise (Camussi and Bianchi, 1988; Corvol *et al.*, 1990; Guyton, 1991).

1.14a.) A role for glucocorticoids?

Several studies have implicated abnormal steroid hormone activity as a contributory factor in the aetiology of cardiovascular disease. For example, a well defined subgroup of patients have been described with low-renin hypertension (Gormez-Sanchez *et al.*, 1985). Since this effect is typical of excessive mineralocorticoid activity, but in the absence of a measurable mineralocorticoid excess, suggestions of novel steroids with mineralocorticoid properteis, reduced 11β-HSD2 activity, or abnormal GR function have been proposed.

Data from several groups support the conclusion that 11β-HSD is impaired in some hypertensive patients (Walker, 1993; Soro *et al.*, 1995). In certain

sample populations, however, neither mineralocorticoid or glucocorticoid levels are found to be sufficiently elevated to explain the rise in blood pressure. Abnormally high sensitivity to glucocorticoids might provide an explanation; firstly, dexamethasone treatment produces a paradoxical fall in blood pressure in a subgroup of hypertensives, possibly implicating the involvement of cortisol or another ACTH-dependent steroid (Whitworth *et al.*, 1989). In essential hypertension, in which cortisol levels remain normal, sensitivity to this steroid, as judged by dermal blanching following topical administration, is increased (Walker *et al.*, 1995). Again, the authors propose the possibility of defective cortisol inactivation by 11β-HSD, or abnormality of glucocorticoid receptor action.

1.14b.) Genetic evidence for the involvement of GR

Studies from a number of families have shown that essential hypertension is inherited as a polygenic trait (Higgins *et al.*, 1980). However, only 30% of the blood pressure elevation has a genetic basis. The search for the causative genes in humans has encountered several complex problems including; adequate phenotype definition, the availability of informative families for genetic studies, the apparent heterogeneity of the disease (Lindpaintner *et al.*, 1992a) and the confounding effects of environmental factors (listed above). Environmental factors can act over extremely long timespans affecting the genotype/phenotype relationship individually, within a family and regionally (Watt *et al.*, 1992).

Two main approaches have been used to identify the genes involved in hypertension. One approach, which is largely restricted to animal models, is to map a series of polymorphic genetic markers (simple sequence repeats; SSRs) distributed throughout the genome to establish a link between increased blood pressure and specific gene loci (Jacob *et al.*, 1991; Hilbert *et al.*, 1991; Jacob *et al.*, 1995; Pravenec *et al.*, 1995). The other approach is to study candidate genes that are known to participate in blood pressure regulation, such as those which encode hormonal factors (catecholamines, vasopressin and components of the renin-angiotensin system, inlcuding renin: Barley *et al.*, 1991 and ACE; Jeunmaitre *et al.*, 1992),

mediators of hormonal responses in target cells of heart and blood vessels (corticosteroid, catecholamine and other receptors, such as GR: Heeley *et al.*, 1996b; angiotensin II receptor, Koike *et al.*, 1995) and membrane components of the distal nephron of the kidney, which critically control sodium balance (Na^+ transporter: Husted *et al.*, 1997; Na-K-Cl cotransporter: AlvarezGuerra, *et al.*, 1997).

Neither of these approaches is without problems. For example, linkage mapping may be useful in identifying gene loci showing a significant linkage with blood pressure, but the precise identity of the genes involved remains unknown. There are also differences of opinion in what are considered to be candidate genes. The result is that a great many 'candidates' get studied, only some of which may be completely relevant to the disease phenotype. Candidate genes can also be shown to segregates with blood pressure. This could be due to either genetic variation within the gene of interest or in a closely linked gene with very different properties.

On the basis of differences in glucocorticoid-dependent physiological variables, epidemiological studies by Watt *et al.*, (1992) have suggested the association of *Grl* with high blood pressure in humans. The phenotypic and genotypic correlates of a predisposition to hypertension were investigated by comparing offspring with and without a family history of this disorder. Four groups were chosen on the basis of extremes of personal blood pressure and of their parents blood pressure. For those individuals selected, linkages between high blood pressure and a large number of other biochemical and physiological variables and various candidate genes were sought. Genetic linkage analysis was restricted to the extreme phenotypic groups (i.e. those with high blood pressure and those with low blood pressure) where an underlying genetic component would most likely be found. Only a few of the chosen variables showed a genetic link. Plasma concentrations of cortisol, 18-hydroxycorticosterone, and angiotensin II, were higher in those with a genetic predisposition to developing high

blood pressure. Most importantly, plasma levels of the renin substrate angiotensinogen were also raised.

RFLP analysis revealed two hGR haplotypes, A and a, which were identified following *Bc1*1 digestion of genomic DNA and probing with a full length hGR cDNA probe. Homozygosity for the AA genotype occured in 50% of those with high blood pressure whose parents had raised blood pressure, as compared with 36% of those whose personal and parental blood pressures were low. Comparisons of individuals homozygous for either AA or aa genotypes showed a significantly higher association of the AA genotype with higher blood pressure scores. Quite possibly, the raised plasma cortisol concentrations, increased renin-angiotensin activity and the genotypic differences in the glucocorticoid receptor could represent the genetic basis for at least a proportion of the glucocorticoid associated hypertension. In contrast, markers associated with several other blood pressure-relevant genes, including ANP, renin and ACE were uninformative.

Although these data relate to blood pressure distribution within a young normotensive population, it is possible that the alleles of *Grl* which associate with high blood pressure contribute to the development of hypertension in later life. The more recent studies of Walker *et al.*, (1995), in using the same population also identified abnormal sensitivity to glucocorticoids, which predicted predisposition to hypertension.

Of particular interest from these studies, is that elevated adrenal corticosteroid levels and a trend towards increased receptor affinity for dexamethasone binding (also see Panarelli and Fraser, 1994) were found associated with a specific *Grl* genotype (AA). It has been suggested that functionally unaccountable mutations of GR might be of general importance in contributing to the more common forms of hypertension (Lamberts *et al.*, 1994; Werner *et al.*, 1992).

Essential hypertension in man is complex because several genes are involved, as in cardiovascular control mechanisms (Kreutz *et al.*, 1992).

Analyses are complicated still further by heterogeneity of individual genetic background and differences in environmental exposure. Epidemiological considerations, a vital aspect of human studies, requires a large populus in order to provide meaningful data. Difficulties with population size becomes more apparent when relatively rare disease loci are to be isolated and studied in detail. Finally, the availability of human biopsy material is also very limited and the ease of investigations in hospitalised patients are hampered by ethical considerations. Well defined experimental animal model systems which provide more reliable genetic homogeneity are therefore a very useful alternative.

1.15.) Rat genetic models of hypertension and abnormalities of steroid metabolism

Several rat genetic models of hypertension have been developed over recent years, with phenotypes that include abnormal steroid action, as well as elevated blood pressure. These model strains include: the spontaneously hypertensive rat (SHR), its stroke-prone derivative SHRSP and their normotensive control strain, Wistar Kyoto (WKY); the Dahl salt-sensitive and salt-resistant; the Milan hypertensive (MHS) and normotensive (MNS); the Lyon strains (LH, LN, LL) and the New Zealand strains. Inbred strains displaying a particular phenotype are obtained by selective breeding. Once the trait of interest has become sufficiently well established, brother-sister matings are performed to achieve genetic homogeneity by theoretically fixing all genes of a given strain in the homozygous state. This determines a predisposition to the selected phenotype.

The following list summarises, for chosen rat strains, phenotypic characteristics which are thought to result from abnormalities of steroid metabolism. Many of these strains provide evidence of abnormal glucocorticoid activity. Included are those strains which are of specific relevance to the work of this thesis; SHR, Zucker obese and MHS.

i.) SHR

Of the available rat models of genetic hypertension, the SHR strain has been the most intensely studied. Several abnormalities in the HPA-axis have been reported, including elevated plasma corticosterone levels (reviewed by Panarelli, 1994). The adrenal cortex shows hypertrophy compared with WKY and adrenalectomy prevents blood pressure increase, reversible by the administration of aldosterone (Kenyon *et al.*, 1984). The development of hypertension is therefore dependent on an intact adrenal. Recent indication of slower rates of GR steroid binding depletion in SHR compared with WKY at elevated temperatures (Panarelli *et al.*, 1995) provides evidence of a thermostable GR heterocomplex. Receptor stability in SHR is concomitant with an increased affinity for dexamethasone (Panarelli, 1994).

ii.) Dahl salt-sensitive and salt-resistant

These strains are characterised by abnormal steriod 11- and 18-hydroxylase activity. Part of the salt-dependent increase in blood pressure (SS-strain) is due to altered 11 β-hydroxylase activity, producing an increased ratio of 18-hydroxy-DOC:corticosterone *in vivo* and *in vitro*. Breeding studies have shown that the preferential conversion of DOC to 18-hydroxy-DOC rather than corticosterone, which characterises the salt-dependent rats, accounts for at least 16% of the increase in blood pressure (Rapp and Dahl, 1972). The salt resistant (SR) and salt sensitive (SS) rats possess specific homozygous alleles of the 11 β-hydroxylase gene, which differ by five amino acid substitutions. The salt-resistant allele, by coding for an enzyme which synthesises proportionally less 18-hydroxy-DOC, is thought to protect the SR strain from salt-induced hypertension (Matsukawa *et al.*, 1993).

iii.) Lyon strains

Development of the hypertensive phenotype in Lyon hypertensive (LH), compared with the normotensive (LN) and low (LL) blood pressure strains, is characterised by elevated deoxycorticosterone (DOC) and reduced corticosterone levels (suggesting a lowering of 11β-hydroxylase activity). Levels of 18-hydroxy-DOC and aldosterone are unchanged. These

observations were made in young rats 5-weeks old (Vincent *et al.*, 1989). DOC levels normalise on reaching maturity (at 20-weeks). However, this normalisation is accompanied by a reciprocal increase in corticosterone and a lowering of 18-hydroxy-DOC. Whether these changes represent altered steroid secretion or metabolism is uncertain.

iv.) Zucker obese

Although this strain represents a genetic model of obesity, it shares many interesting abnormalities of steroid metabolism common to hypertensive strains, including mild hypertension and is presented here for comparison.

Zucker obese rats, compared with lean controls, like SHR and MHS display adrenal hypertrophy with elevated plasma ACTH and corticosterone concentrations. In addition, hepatic GR from this strain have a lower affinity for glucocorticoids (White and Martin, 1990). Body weight gain is normalised following adrenalectomy.

v.) MHS

Details of the MHS phenotype are given in chapter 5, and will only be considered briefly at the end of this section in relation to hypertension gene candidates.

Varying degrees of progress towards defining the genes responsible for genetic hypertension has been made with the above rat models. Recent studies involving numerous microsatellite markers have been carried out using the stroke-prone SHR derivative (SHR-SP), crossed with its normotensive control strain, WKY. These large scale breeding studies have identified several loci showing linkage with the hypertensive phenotype in SHR-SP, although the precise identity of the genes involved remains unclear (Hilbert *et al.*, 1991; Jacob *et al.*, 1991). From the mapping study of Jacob *et al.*, (1991), two 'interesting' loci were identified, one of which contained the growth hormone gene, GH. Whether the rat GH gene, or another closely linked gene was responsible for the linkage with blood pressure was not determined in this study. Another gene in close linkage

with GH in the rat and perhaps of more relevance to blood pressure control, is the gene encoding angiotensin converting enzyme (ACE). The possibility of a direct association between ACE gene polymorphisms and increased blood pressure is still unclear. Segregating markers have identified other loci on chromosome 18 and on the X chromosome as being significantly associated with blood pressure. In the rat, GR is also localised to chromosome 18.

The highest rate of successful genotype-phenotype associations in hypertension have been attributed to the candidate gene approach (Lindpaintner, 1992b). For example, the significant contribution of abnormal steriod 11β- and 18-hydroxylase activity to the development of hypertension in the Dahl salt-sensitive rat has been known since 1972 (Rapp and Dahl, 1972). In 1993, Matsukawa *et al.*, reported variation in the gene encoding the 11β-hydroxylase enzyme between salt resistant and salt sensitive strains. In the same year, linkage of 11β-hydroxylase polymorphisms with altered steroid biosynthesis and blood pressure was confirmed (Cicila *et al.*, 1993).

In addition, comparison of the salt-resistant and salt-sensitive variants of Dahl rats has revealed differences in renin gene structure. The renin gene locus (i.e. the renin gene, or another closely linked gene) has been found to be linked to hypertension in the Dahl strain of sodium-sensitive rat (Rapp *et al.*, 1989). In the Okamoto SHR strain, breeding studies suggest that between four and six independent loci contribute to high blood pressure (Jacob *et al.*, 1991; Koike *et al.*, 1995). Allelic variation has also been reported in the renin gene of SHR when compared to WKY, although inter-strain genetic variation in controls makes realistic interpretation difficult (Samani *et al.*, 1989).

Of particular interest and relevance during the course of this thesis has been the excitement regarding possible association between adducin genes and hypertension, both in humans and the Milan hypertensive strain of rat, MHS. Adducin is a heterodimeric membrane cytoskeletal protein, consisting of separately encoded α- and β- subunits. Adducin interacts with

other membrane skeleton proteins affecting ion transport accross the cell membrane. Initial genetic studies have demonstrated that a point mutation within the α- subunit of adducin in MHS increases Na-K-ATPase activity when transfected into rat renal epithelial cells (Tripodi *et al.*, 1996). *In vivo*, this mutation affects blood pressure, accounting for 40-50% of the blood pressure difference between MHS and MNS, when interacting with a mutated β-adducin subunit (Bianchi *et al.*, 1994). The effect of adducin on blood pressure is thought to be mediated by changes in cell signal transduction mechanisms, through effects on the polymerisation of actin filaments. Recent studies in independent sample populations have now confirmed significant linkage of the α-adducin locus with hypertension in humans (Cusi *et al.*, 1997).

The possibility that an abnormality of GR function could account for part of the remaining difference in blood pressure and/or provide the basis for other phenotypic differences between MHS and MNS is the subject of further investigation presented in this thesis.

Chapter 2
Materials and Methods

2.1.) Materials

2.1.1.) Reagents

All reagents were of analytical or molecular biology grade, purchased from either: Sigma Chemical Company Ltd. (Pool, Dorset, U.K.) or the BDH (Merck) Chemical Company (Lutterworth, Leicestershire, U.K.) unless otherwise stated.

2.1.2.) Animals

Rats of strains MHS and MNS were obtained from the Field Laboratories, University of Sheffield, U.K.. SHR, WKY and Zucker lean and obese rats were obtained from Harlan Olac (Bicester, Oxfordshire, U.K.).

2.1.3.) Tissue and DNA samples

English wild rat liver samples were obtained from Central Science Laboratory, Ministry of Agriculture Fisheries and Food, Slough, Berkshire, U.K.. Scottish wild rat carcasses were donated by regional pest control departments of Scottish District Councils (Stirling, Glasgow and Fife). Carcasses were stored at -20^{o}C prior to dissection and removal of the liver.

Inbred rat liver samples from strains SHR-SP$_{Gla}$ and WKY$_{Gla}$ (Glasgow strains) and SD were obtained from The M.R.C. Blood Pressure Unit, Western Infirmary, Glasgow.

Inbred strain and substrain liver genomic DNA samples were obtained from The Department of Laboratory Animal Science, Faculty of Veterinary Medicine, University of Utrecht, The Netherlands.

The following sub-headings list specific materials, followed by the supplier for:

2.1.4.) Genomic DNA extraction

Proteinase K (Sigma).

Phenol (TE saturated): Obtained pre-equilibrated with TE (pH 8.0) (Fisons, Loughborough, U.K.).

2.1.5.) Polymerase chain reaction (PCR)

PCR primer oligonucleotides (as well as single stranded complementary oligonucleotides and oligonucleotide probes) were obtained from Oswel (Oswel DNA service lab 5005, University of Southampton, Southampton, U.K.).

General PCR components, such as *Taq* DNA polymerase (from *Thermus Aquaticus*, compatible with reaction buffer B), 10x reaction buffer B, dNTPs, magnesium chloride (25 mM) acetylated BSA (10 mg/ml) (Promega, Southampton, U.K.). Mineral oil (light white: Sigma).
Vent$_R$ DNA polymerase (from *Thermococcus literalis*), 10x Vent polymerase buffer, magnesium sulphate (100 mM) and purified BSA (non-acetylated) (New England Biolabs, Hitchin, Hertfordshire, U.K.).

2.1.6.) Gel electrophoresis

Ethidium bromide (Sigma).

Agarose (GIBCO BRL [Life Technologies], Paisley, Scotland, U.K.).

Metaphor agarose and low gelling temperature (LMP) agarose (FMC Bioproducts, Rockland, U.S.A.).

1 Kb DNA ladder (GIBCO-BRL).

2.1.7.) Southern blotting

Sonicated salmon sperm DNA solution (10 mg/ml) (Pharmacia Biotech, Herts, U.K.).

Hybond-N^+ nylon membrane (Amersham International plc, Buckinghamshire, U.K.).

Autoradiographic film (Hybond-MP) and intensifying screens (Amersham).

2.1.8.) ^{32}P-labelling of DNA

T4 polynucleotide kinase (PNK) and 10x PNK reaction buffer (Promega).

Sephadex G50 (Sigma).

2.1.9.) Sequencing

Sequenase version 2.0 kits^{TM} for plasmid sequencing (United States Biochemicals, Cambridge Bioscience, Cambridge, U.K.). Sequenase version 2.0 kits^{TM} for direct sequencing of PCR products (Amersham).

Dynabeads^{TM} (Dynal Ltd., Merseyside, U.K).

2.1.10.) RNA extraction

RNAzol^{TM} B (Biogenesis Ltd., Bournemouth, U.K.).

2.1.11.) Tissue culture

All tissue culture components were obtained from GIBCO-BRL, unless otherwise stated.

Cell lines: COS-7 and CV-1 (European Collection of animal cell cultures Wiltshire, U.K.), EDR3 (G. Firestone, University of California, Berkeley, U.S.A.), 2s-Fasa and HEK293 (University of Edinburgh).

Calf thymus DNA (Sigma).

DMSO (Sigma).

DOTAP (Boehringer Mannheim, U.K.).

2.1.12.) DNA cloning

Restriction enzymes were obtained from Promega, except: *Ava*II, *Kpn*I and *Sfi*I (GIBCO-BRL).

Klenow fragment (5-10 U/μ l) and 10x Klenow reaction buffer (Promega).

Random hexamer primers (3 μg/μl in 3 mM Tris-HCl [pH 7.0], 0.2 mM EDTA) (GIBCO-BRL).

Calf intestinal phosphatase (CIP) supplied at a concentration of 20-30 U/μl (GIBCO-BRL).

DNA ligase and 10x Ligase buffer (Promega).

2.1.13.) Bacterial growth, colony screening and solutions for plasmid DNA extraction

Bacto-tryptone, yeast extract and agar (GIBCO-BRL).

Antibiotics: ampicillin, tetracycline, streptamycin and kanamycin (Sigma).

X-gal and IPTG (Sigma).

Lysozyme (Sigma).

2.1.14.) Reverse transcription of RNA

Random hexamer primers (as describerd in section 2.1.12.).

Molony Murine Leukaemia Virus (MMLV) Reverse Transcriptase, 10x MMLV reacton buffer and 0.75 M DTT (Stratagene Ltd., Cambridge, U.K.).

RNAsin (20 U/μl) (Promega).

2.1.15.) Western blotting

Glucocorticoid receptor monoclonal antibody MAb 250 (also referred to as antibody No. 7) was a gift from Dr. A-C Wikström, Department of Medical Nutrition, Huddinge University Hospital, Huddinge, Sweden.

High molecular weight protein markers (GIBCO-BRL).

ECL Western blotting kit and PVDF membrane (Amersham).

2.1.16.) Radiochemicals

All radioisotopes (nucleotides and ligands) were obtained from Amersham:

[α^{32}P]-dCTP: Supplied at S.A., 3000 Ci/mmole

[γ^{32}P]-ATP: Supplied at S.A., 3000 Ci/mmole

[α^{35}S]-dATP: Supplied at S.A., 1250 Ci/mmole

[1,2,4,6,7-^{3}H]-Dexamethasone: Supplied at S.A., 86.0 Ci/mmole

2.2.) Methods

2.2.1.) Animal maintenance

Prior to sacrifice all animals were maintained in house under standard living conditions (12 h light, 12 h dark), with free access to food and water in a temperature controlled environment.

2.2.2.) Extraction of genomic DNA

The protocol for the extraction of genomic DNA was taken from methods described by Sternberg *et al.*, (1990). Essentially, 100-200 mg of tissue was homogenised on ice in 5 ml PBS (phosphate buffered saline: 1 Sigma PBS tablet dissolved in 200 ml ddH_2O) and centrifuged at 2000 rpm, 10 mins at 4^oC. Pellets were washed twice by resuspension in 2 ml PBS and finally resuspended in 1 ml lysis buffer (50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.15 M EDTA, 1% SDS and 1 mg/ml proteinase K). The resulting mixture was incubated at 60^oC for 1h to digest unwanted proteins. Vigorous pipetting was avoided to minimise shearing of the DNA.

DNA was recovered by increasing incubation volumes to 4.5 ml with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), adding 0.5 ml 3M sodium acetate (pH 5.2) and precipitating with 3 volumes 100% ethanol. Precipitated DNA was transferred to 1.5 ml microfuge tubes, washed with 0.5 ml 100% ethanol, briefly air dried and redissolved in 0.5-1 ml TE (pH 8.0) at 4^oC over a period of at least 2 days.

Where DNA was expected to be of a lower integrity (from degraded tissue samples), proteinase K digests were protein extracted by combining with an equal volume of TE-equilibrated phenol, mixing gently and spinning at 5000 rpm, 10 mins at 4^oC. Taking care to avoid the interface, the supernatant was transferred to a fresh tube. Recovered supernatants were combined with an equal volume of chloroform/isoamyl alcohol (24:1 mixture), again mixed

gently and spun at 5000 rpm, 10 mins at 4°C. Supernatants were recovered as previously described, transferred to a fresh tube, mixed with 1/10 volumes of 3M sodium acetate (pH 5.2) and DNA precipitated with 3 volumes 100% ethanol. Resulting DNA pellets were washed with 100% ethanol, briefly air-dried and redissolved in TE (pH 8.0) at 4°C.

2.2.3.) Polymerase chain reaction (PCR) amplification of nucleic acids

General PCR strategy

Amplifications were performed using an Omnigene Thermal Cycler (Hybaid Ltd., U.K.) in 25µl reaction volumes by combining 10-100 ng of genomic DNA or 1-10 ng of plasmid DNA with 20 pmoles of each primer and 2U *Taq* DNA polymerase in a standard reaction mixture containing; 1x *Taq* polymerase buffer, 125 µM dNTPs, 1.5 mM Mg^{2+} and 0.01 mg/ml acetylated BSA. Reaction mixes were overlaid with 1-drop of mineral oil (approximately 25 µl) and heated to 95-97°C for 5-7 mins prior to PCR. In general, cycle profiles of: 95°C, 1 min (step 1), followed by: X°C, 30s, 72°C, 30s and 95°C, 30s (step 2), continued for 30 cycles and finally: X°C, 30s, 72°C, 5 mins final extension (step 3) were used, where X°C = Primer annealing temperature (normally about 5°C below the average of the primer T_ms) and was typically in the range of 56-60°C.

These conditions were optimal for the majority of PCR primers listed in Appendix 1 and were effective in the amplification of up to 2.5-3.0 kb of target sequence using *Taq* DNA polymerase. PCR reactions found to be inefficient under these conditions were further optimised, as described in chapter 3.

^{32}P-PCR

PCR products were ^{32}P-labelled by incorporating a single ^{32}P end-labelled primer (see section 2.2.7.) in subsequent PCR reactions. PCRs were performed as described previously, except DNA templates were initially denatured at 95°C for 5 mins prior to the addition of both *Taq* DNA

polymerase and ^{32}P-labelled primer. In pilot experiments, the ^{32}P-labelled primer was added in a volume of 2.0 µl labelling reaction mixture (corresponding to 10-20 pmoles).

RT-PCR

Aliquots of reverse transcription reactions (2 µl: purified by phenol/chloroform extraction) were PCR-amplified in 25 µl volumes using previously described methods. RNA/cDNA templates were initially denatured for 2 mins at 94-95 ^{o}C prior to PCR.

2.2.4.) Preparation and electrophoresis through agarose and polyacrylamide gels

Various percentage agarose gels and their application:

Percent agarose	Typical application
0.8% Low gelling temperature (LMP) agarose	Isolation of DNA fragments for ^{32}P-labelling or column purification (section 2.2.10.) prior to cloning
0.8% Normal agarose	Analysis of plasmid clones and genomic DNA
1.5% Normal	Analysis of restriction fragments and PCR products larger than around 200 bp. Isolation of large DNA fragments for purification by dialysis (section 2.2.10.) prior to cloning
2-3% Normal	Analysis of restriction fragments and PCR products generally smaller than 200 bp, carrying polymorphisms down to about 10 bp
4% Metaphor agarose	Analysis of PCR products down to 100 bp with resolution of polymorphisms down to 1-3 bp

The compositions of all electrophoresis and gel loading buffers were exactly as described by Sambrook *et al.*, (1989).

Agarose gels

Standard agarose gels at the appropriate percentages (depending on application, see above) were prepared as described by Sambrook *et al.*, (1989). Metaphor agarose gels were prepared as recommended by FCM Bioproducts, Rockland, U.S.A..

DNA samples were mixed with 0.5-1 volumes of 1x sucrose gel loading buffer (Sambrook *et al.*, 1989) prior to gel loading and electrophoresed in 1x TAE buffer, or 0.5x TAE for Metaphor gels to improve resolution. Normally, gels were run at 150-200 v, 40 mA. LMP gels were run at lower voltages (50-100 v, 20 mA), to prevent excessive heating and possible melting of the gel. DNA bands were visualised using an ultraviolet (UV) transilluminator, following staining with ethidium bromide at 0.5-1.0 μ g/ml.

Polyacrylamide gels

6% Polyacrylamide gels for sequencing and microsatellite typing were prepared by mixing 100 μl APS (25% w/v APS in ddH_2O) and 100 μl TEMED with 100 ml gel solution: 15 ml 40% acrylamide solution (195 g acrylamide and 5 g bis-acrylamide in 500 ml ddH_2O), 15 ml 5x TBS and 50 g urea, made up to 100 ml with ddH_2O. Gels were cast by standard methods. DNA samples were mixed with 0.66 volumes of 1x formamide gel loading buffer (sequencing samples) or 0.165 volumes (PCR amplified microsatellites) prior to loading onto pre-heated (50-60^{o}C) gels. Gels were run at 2000 v, 58 mA, 110 W for 1.5-2 h, or until the xylene cyanol dye front had reached about half way down the gel. DNA bands were visualised by transferring the gel to Whatman 3 mm paper, drying using a BioRad model 583 gel dryer (BioRad), at 80^{o}C for 2 h and exposing to Hyperfilm-MP for 4-16 h, at -80^{o}C with an intensifying screen. ^{35}S-containing gels were exposed at room temperature for 24-48 h.

2.2.5.) Measurement of nucleic acid concentrations

The concentrations of nucleic acid solutions (double stranded DNA, oligonucleotides longer than the average PCR primer length [21 mer] and

RNA) were measured spectrophotometrically at 260 nm (concentration alone), or at a ratio of 260/280 nm (to estimate for purity). Nucleic acid concentrations were deduced as described by Sambrook *et al.*, (1989).
The concentration of DNA bands in gels was estimated by comparing their fluorescent intensity with that of a band of similar size in a known concentration of marker ladder.

2.2.6.) Restriction endonuclease digestion of DNA

For routine restriction analysis (20 µl standard volume), 0.2-0.5 µg of DNA solution (PCR product, or potential recombinant plasmid clone from miniprep) was digested with 2-3 enzyme units/µg of DNA in a final volume of 20 µl containing 1x restriction enzyme reaction buffer. Incubations were routinely carried out at 37°C for 1-2 h, unless otherwise stated. 10 µl of the resulting digests were checked for restriction patterns on 1.5% agarose gels.

For generation of cloning fragments and Southern probes, 5-10 µg of DNA solution (plasmid clone) was digested with 1-2 enzyme units/µg of DNA in a final volume of 40 µl (Southern probe), or 80 µl (cloning fragments). 1 µl of resulting digests were checked on 1.5% gels for completeness of digestion. Reaction conditions were as previously described.

2.2.7.) ^{32}P-labelling of double and single stranded DNA

^{32}P end-labelling of single stranded oligonucleotides

PCR primers and single stranded oligonucleotide probes used in Southern blotting were ^{32}P-labelled at the 5'-end with [γ ^{32}P]-ATP (3000 Ci/mmole) using T4 polynucleotide kinase (Promega). Diluted primer (10µl, 10-20 pmoles/µl) or oligonucleotide probe (10µl, 70-80 ng/µl) was combined with 5µl 10x polynucleotide kinase (PNK) buffer (700mM Tris-HCl [pH 7.6], 100mM $MgCl_2$ and 50 mM DTT) and 29 µl water, boiled for 2 mins and rapidly cooled on ice. 5µl [γ ^{32}P]-ATP (diluted to ~500 cpm in water for the labelling of PCR

primers) and 1μl polynucleotide kinase were then added and the mixture incubated for 45 mins at 37°C.

Random primer labelling of Southern probes

cDNA fragments from plasmid digests were band excised from LMP agarose gel and melted at 70 °C. A 20 μl sample of molten DNA-agarose solution (approximately 100-200 ng DNA) was boiled for 5-10 mins, rapidly cooled on ice and combined with dATP, dGTP and dTTP (each at 0.0625 mM), 10-20 ng random hexamer primers, 5 μl [α^{32}P]-dCTP (3000 Ci/mmole) and 1 μl Klenow fragment and incubated in 1x Klenow buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT and 0.2 mM HEPES [pH 7.5]), at 37°C for 1 h. The reaction was stopped by the addition of 200 μl of Sephadex G50 column elution buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA [pH 8.0] and 0.5% SDS).

Probe purification and measurement of specific activity

The ^{32}P-labelled probe DNA was separated from unincorporated radioactive nucleotides through a Sephadex G50 column (see Sambrook *et al.*, 1989). Labelled probe DNA was eluted from the column using elution buffer (as described above) and fractions of peak activity collected and pooled.

Probe specific activity was determined by mixing an aliquot of purified probe with 3 ml of a toluene-based scintillant, Cocktail T (666 ml toluene, 332 ml Triton X-100, 5 g 2,5-Diphenyloxazole [PPO] and 0.15 g 1,4-Di-2-(5 phenyoxazoyl) benzene [POP]) and counting cpm using a B4430 Tricarb scintillation counter (Packard, U.K.). Specific activities of 10^7-10^8 cpm/μg were routinely obtained.

2.2.8.) Southern blot analysis

Alkali Southern blotting of PCR products from agarose gels

PCR products were separated and visualised in ethidium bromide stained agarose gels against 1 kb DNA markers. DNA samples were depurinated,

denatured and transferred to Hybond-N^+ nylon membrane using the methods described by Sambrook *et al.*, (1989).

Southern blotting of PCR products from polyacrylamide gels

Unlabelled PCR products resolved on standard 6% polyacrylamide gels were transferred to nylon membrane using the basic methods of Southern. The region of the gel predicted to contain the PCR products was overlaid with Hybond-N^+ nylon membrane (normally around 10x20 cm, exceeding the PCR 'product window' by at least 2-3 cm on upper and lower margins) and wetted with 2x SSC. Any bubbles were carefully removed and the membrane overlaid with two pieces of Whatman 3 mm blotting paper and a stack of absorbent paper towels. With the application of sufficient pressure, the PCR product DNA was allowed to transfer onto the nylon membrane for 6-16 h.

2.2.9.) Hybridisation of radiolabelled DNA probes to Southern filters

Using probes generated from random primer labelling

Prior to hybridisation with labelled probe, membranes were 'blocked' for 1 h in 25 ml prehybridisation buffer (5x SSC [Sambrook *et al.*, 1989], 5x Denhardt's solution [Sambrook *et al.*, 1989] and 0.5% SDS) containing 20 μg/ml of sonicated salmon sperm DNA. The salmon sperm DNA was denatured at 100°C for 5 mins before adding to the hot (65°C) prehybridisation buffer. After blocking of the membrane, 20 ml of prehybridisation buffer was removed and 100 μl (equivalent to roughly 20-50 ng) ^{32}P-labelled DNA probe solution added, following denaturation at 100°C for 5-10 mins. Hybridisations were carried out in 8 cm diameter hybridisation cylinders mounted in a rotating oven (Hybaid Ltd, U.K.) at 65°C for 16-18 h.

Hybridised filters were washed twice in 2x SSC, 0.1% (w/v) SDS for 10 mins at room temperature, once in 1x SSC, 0.1% (w/v) SDS for 15-30 mins at 65°C and if significant background counts still remained, once in 0.1x SSC, 0.1% (w/v) SDS for 10 mins at 65°C. The washed filter was wrapped in clingfilm to retain

moisture and exposed to autoradiographic film (Hybond-MP) for 2-3 h at -80°C with an intensifying screen.

Using single stranded oligonucleotide probes

Prior to hybridisation with labelled probe, membranes were 'blocked' for 1 h in 50 ml 3% casein prehybridisation buffer (1.5 g milk powder dissolved in 50 ml 2x SSC). Hybridisations were carried out (either immediately or after several days storage of filters at 4°C) at 60°C for 8-10 h in 10 ml 2x SSC (containing 2% w/v milk powder) supplemented with 25 µl ^{32}P-labelled oligonucleotide probe (equivalent to 30-40 ng DNA solution of specific activity: 10^5-10^7 cpm/µg). Hybridised filters were washed twice in 2x SSC, 0.1% SDS for 30 mins at 60°C, wrapped in clingfilm and exposed to autoradiographic film as described above.

2.2.10.) DNA cloning techniques

Purification of DNA fragments

For cloning purposes, DNA fragments from restriction enzyme digestion of plasmid DNA were recovered from agarose gels by electroelution into dialysis tubing as described by Sambrook *et al.*, (1989).

PCR products for sequencing (up to ~2 kb) and cloning (up to ~500 bp) were efficiently purified using QIAGEN PCR purification columns (QIAGEN, Dorking, Surrey, U.K.) according to the manufacturers recommendations.

Phenol/chloroform extraction of nucleic acids

All nucleic acid solutions were purified after proteinaceous digestions, as described by Sambrook *et al.*, (1989).

Ligation of DNA fragments

For the directional cloning of DNA fragments carrying different cohesive termini, the appropriate ratio of molecular ends for an efficient ligation

had to be optimised. For single fragment ligations, vector : insert ratios of 1:1 and 1:3 (and occasionally 3:1) were routinely tested. The mass ratio of vector : insert(s) was dependent on the size (in bp) of fragments to be ligated. For multiple fragment ligations, similar ratios of vector to insert were used, with mass ratios decreasing with decreasing size of insert fragment. In general, 20-30 ng of linearised vector DNA was ligated with X ng of insert DNA fragment(s) [depending on their size, in bp] in one of the ratios described above, by combining 1 μl 10x ligase buffer (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT and 5 mM ATP), 0.5 μl (1.5-2 weiss units) T4 DNA ligase, X μl DNA solution (for each DNA fragment, dependent on number of fragments and their size and concentration) and Y μl ddH_2O (dependent on X) in a final volume of 10 μl. The DNA ligase was added last and the reaction components gently mixed by stirring with a pipette tip. Ligation reactions were incubated at 16°C for 16 h and then stored at 4°C until required for transformation.

Dephosphorylation of linearised plasmid DNA

Purified linear plasmid DNA for cloning was dephosphorylated using calf intestinal phosphatase (CIP) at a concentration of 0.01 U/pmole of 5' protruding DNA ends (where 1 pmole of DNA ends represents the number of ends generated from the linerisation of 1 μg of a 4.3 kb plasmid). Reactions were incubated at 37°C for 30 mins and terminated by heating to 70°C for 30 mins. Inactivated CIP was removed by phenol/chloroform extraction.

CIP was diluted to a concentration of 0.2 U/μl using CIP dilution buffer (30 mM triethanolamine [pH 7.6], 3 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, supplied with the enzyme) prior to use.

Klenow fill-in of protruding 5' ends

The filling in of 5' protruding termini with unlabelled dNTPs was achieved as follows: 1-4 μg of DNA (in a volume of 20 μl) was digested to completion with a suitable restriction enzyme generating 5' overhangs. The digested DNA was then phenol/chloroform extracted, ethanol precipitated and resuspended in 20 μl ddH_2O. The DNA solution was combined with 40 mM

dNTPs, 20 μg/ml acetylated BSA and 1 U of Klenow/μg of DNA. Reactions were incubated in 1 x Klenow buffer (50 mM Tris-HCl [pH 7.2], 10 mM $MgSO_4$ and 0.1 mM DTT) at room temperature for 1 h and terminated by heating for 10 mins at 70°C. Denatured Klenow was removed by phenol/chloroform extraction.

T-vector (pT7Blue) cloning of PCR products

For a standard ligation reaction, 50 ng (0.03 pmole) of pT7Blue T-vector (AMS Biotechnology, Oxford, U.K.) was ligated with 10-50 ng of amplified PCR product in a volume of 10 μl as recommended by the supplier. Ligation reactions were incubated at 16°C for 2-16 h. Normally, PCR product DNA was recovered by precipitation using 2 M ammonium acetate and 2 volumes of 100% ethanol on ice to minimise the precipitation of lower molecular weight material such as unincorporated PCR primers.

2.2.11.) Bacterial transformation and colony screening

All bacterial growth media and related solutions were sterilized by autoclaving unless otherwise stated.

Preparation of competent bacterial cells

Bacterial cells of *E. coli* strain DS941 (obtained from Glasgow University Department of Genetics) were made competent with rubidium chloride (RbCl) using a method obtained from Glasgow University Department of Genetics, originally described by Hanahan, (1983).

Transformation of bacterial strains with plasmid DNA

NovaBlue *E. coli* competent cells from the T-vector DNA cloning kit (AMS Biotechnology, Oxford, U.K.) were transformed with T-vector as recommended by the supplier.

For *E.coli* strain DS941, the transformation procedure was similar to that described for NovaBlue cells, with the following modifications: 50 μl of

competent cells were mixed with 2-5 µl ligation mixture. Cells were heat shocked for exactly 2-mins at 42°C and transferred to ice for a further 2 mins prior to the addition of 250 µl 2x YT medium. Cultures were shaken at 180-200 rpm in an orbital shaker for 1 h at 37°C prior to plating.

Rapid screening of bacterial transformants

Following transformation of competent bacterial cells, resulting colonies were screened for recombinants by one of the following methods:

Blue/white colony screening: following T-vector transformation, bacterial cells carrying a disrupted *lacZ* gene were selected as white colonies following staining with X-gal.

PCR (boil) method: colonies to be analysed were retained/duplicated on a second independent LB-agar plate (reference plate) containing 50 µg/ml ampicillin, by touching the chosen colony with the end of a sterile yellow pipette tip and then crossing the surface of the agar on the reference plate. Bacterial colony duplicates were grown for 16-18 h at 37°C and stored at 4°C. The remainder of the colony was lifted into 1 ml LB medium supplemented with 50 µg/ml ampicillin and grown for 6-16 h, with shaking (200 rpm) at 37°C. 10 µl of bacterial culture was transferred to a 1.5 ml microfuge tube, diluted with 90 µl ddH_2O and boiled for 10 mins. The bacterial lysate was spun at 14,000 rpm, for 5 mins at room temperature to pellet cell debris. The resulting supernatant was used directly in PCRs. PCR products were verified against a positive control PCR amplified from the original template DNA run in parallel

SCOP analysis (S. Rusconi personal communication) was routinely used for the rapid initial sizing of potential recombinants which were not pre-selected by any other means (such as blue/white colony screening). Typically, 100-200 colonies could easily be analysed in one day by this method.

Bacterial colonies were retained for further reference as described in the PCR-boil method. The remainder of each colony was mixed with 20 µl LETR-buffer (2 mg lysozyme 100mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 8.0) and 0.1 mg/ml RNaseA) in a 1.5 ml microfuge tube and incubated on ice for 15-20 mins. 2-drops of TE (pH 8.0) equilibrated phenol were then added to each incubation. Samples were vortexed briefly and spun at 14,000 rpm, for 5 mins at room temperature. 10 µl of the resulting supernatant was mixed with agarose gel loading buffer and run on a 1.5% agarose gel against a plasmid clone of known size, acting as a size marker. Colonies carrying recombinant plasmids of the correct size were identified from the reference plate and analysed further (e.g. miniprep, followed by restriction analysis).

Storage of bacterial transformants

All bacterial clones and subclones were stored as recommended by Sambrook *et al.*, (1989).

2.2.12.) Preparation of plasmid DNA

All bacterial plasmids were prepared by the alkaline lysis method described by Sambrook *et al.*, 1989.

Minipreparations

These were adjusted to 5 ml to generate enough plasmid DNA (3-5 µg/ml of original culture volume for a high copy number plasmid) for rapid restriction analysis or sequencing.

Midipreparation (100 ml)

For reasons of speed and expected product purity, plasmid midipreparations were also carried out using the QIAGEN midi-prep kit and accompanying protocol (QIAGEN). Using the corresponding QIAGEN tip lure recommended for this culture volume, plasmid DNA yields of 300-500 µ g were achieved.

Maxipreparation (500 ml)

Large scale preparations of plasmid DNA were used in conjunction with a subsequent purificaton step (equilibrium centrifugation in cesium chloride [CsCl]-ethidium bromide gradients) designed to produce high quality supercoiled DNA suitable for transfection of mammalian cells.

The basic protocol for maxipreparation of plasmid DNA was the same as that described by Sambrook *et al.*, (1989).

Nucleic acids were recovered at 10-12,000 rpm for 30 mins in a Beckman JC M2 centrifuge (JA20 rotor). Supernatants were decanted off and resulting pellets washed with 0.5 ml 100% ethanol. Tubes were inverted to drain and briefly dry nucleic acid pellets (5-10 mins, room temperature) before dissolving in 3 ml TE buffer (pH 8.0).

Cesium chloride density gradient centrifugation

Cesium gradients were prepared by mixing crude plasmid DNA solutions with 2.6-2.8 g of solid CsCl and 100 µl of a 10 µg/ml solution of ethidium bromide. Filled ultracentrifuge tubes were adjusted to a weight of 5.9-6.0 g (to ensure buoyant plasmid bands were retained in the middle of tubes following centrifugation) using a solution of CsCl in TE (pH 8.0) [2g/ml]. Plasmid DNA was banded at 70,000 rpm for 16 h at 4°C, or at 100,000 rpm for 4 h at 4°C in an Optima^{TM} TLX ultracentrifuge (Beckman, U.K.). Banded supercoiled DNA was recovered from gradients and ethidium bromide removed as described by Sambrook *et al.*, (1989).

The purified DNA was pooled and concentrated by ethanol precipitation and centrifugation at 10-12,000 rpm for 30 mins at 4°C in a Beckman JC M2 centrifuge. Where yields were sufficiently high (usually for high molecular weight, high copy number plasmids), the precipitated DNA could be physically transferred to a microfuge tube and pelleted at 14,000 rpm for 10 mins at room temperature. DNA pellets were washed in 0.5 ml 100% ethanol, air dried for about 10 mins and resuspended in 1-2 ml TE (pH 8.0).

Plasmid DNA prepared in this way was successfully stored at 4°C with no compromise to its integrity.

2.2.13.) Dideoxy chain termination sequencing of DNA

The sequencing of double or single stranded DNA was based on the dideoxy chain termination method in conjunction with the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals, Cambridge Bioscience, Cambridge, U.K.), or an appropriately modified version.

Sequencing double stranded plasmid DNA

Typically, 18 µl of plasmid DNA solution (2-10 µg, depending on the source of DNA) were denatured in a 1.5 ml microfuge tube using 2 µl of denaturation buffer (2 M NaOH, 1 mM EDTA) for 5 mins at room temperature. Denatured, single stranded DNA was recovered by precipitation with one tenth the volume of 3M sodium acetate (pH 5.2) and 3-volumes of 100% ethanol at -80°C for 1 h. DNA was then pelleted in a microfuge at 14,000 rpm for 10 mins at room temperature. The pellet was washed with 200 µl of 100% ethanol, briefly air dried and sequenced as described in the Sequenase version 2.0™ protocol. Sequenced products were separated on 6% polyacrylamide 8M urea sequencing gels. Prior to gel loading, product samples from each terminating nucleotide were denatured for 2 mins at 80-85°C and rapidly cooled on ice.

Sequencing of PCR products

Using Dynabeads: PCR products were immobilised onto magnetic Dynabeads® M 280 streptavidin, denatured and sequenced as recommended by the supplier. All manipulation and wash steps required a magnetic particle concentrator (MPC, Promega) to pellet the magnetic beads.

By direct method: double stranded PCR products were sequenced directly, without the need to remove complementary DNA strands, using a Sequenase version 2.0™ kit specifically designed for this purpose (Amersham). Prior

to sequencing, PCR products (non-purified) were treated with the hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phoshpatase to remove unincorporated dNTPs, primers and spurious single stranded products. Purified DNA templates were sequenced and electrophoresed as recommended by the supplier.

2.2.14.) RNA extraction

Total RNA was extracted using RNAzol B under conditions recommended by the supplier. Resulting RNA pellets were washed in 200 µl of 100% ethanol, dried briefly at room temperature and resuspended in a suitable volume of ddH_2O (DEPC-treated as described by Sambrook *et al.*, 1989), supplemented with DEPC-treated EDTA (pH 7.6) to a final concentration of 1 mM. RNA samples were adjusted to a final concentration of 1-2 µg/ml and stored at -80°C in one tenth the volume of DEPC-treated 3M sodium acetate (pH 5.2) and 3x the final volume of 100% ethanol.

2.2.15.) Reverse transcription of RNA

Total RNA was reverse transcribed into RNA/cDNA hybrids for RT-PCR by combining the following in a 1.5 ml microfuge tube: 0.01 M DTT, 0.2 U, RNAsin, 4 mM dNTPs, 5-10 µg Total RNA, 5-10 pmoles gene specific primer or random hexamer primers, 20 U MMLV-RTase and 32.75 µl, ddH_2O in a total volume of 50 µl. Reaction components were incubated in 1x RT Buffer at 37-42°C for 50-70 mins, depending on the length of the required transcript. Completed reactions were terminated by phenol/chloroform extraction and stored frozen at -20°C prior to RT-PCR.

2.2.16.) Tissue culture

Growth and maintenance of tissue culture cells

Both COS-7 and CV-1 cells were grown in DMEM (with 0.11 g/l Na-pyruvate), supplemented with 1% penicillin-streptomycin solution, 1% L-glutamine solution, FCS (10% for COS-7 and 2.5% for CV-1 and HEK293) and NBCS, 2.5% (for CV-1 and HEK293). 2s-Fasa cells were grown in Mccoy's 5-A medium and EDR3 in a 50 : 50 mixture of Glasgow medium and DMEM. Both were supplemented with 1 % penicillin-streptomycin solution, 1% L-glutamine solution and 10% FCS. All cells were grown routinely on 10 cm tissue culture dishes at 37^{o}C, in a water-saturated 5% CO_2 atmosphere.

When passaging cells, medium was aspirated off and the cells rinsed in 5 ml of room temperature TBS (0.14 M NaCl, 0.0027 M KCl and 0.025 M Tris-HCl [pH 7.4]). The TBS was aspirated off and the cells treated with 2 ml of trypsin-EDTA solution. Following aspiration of the trypsin solution cell plates were incubated at 37^{o}C for 2-5 mins until cells were dislodged from the plate (determined by gentle tapping). 4-8 ml of growth medium was then added and adherent cells and cell clumps disaggregated by several passages through a pipette (10 ml), whilst forcing the tip against the base of the tissue culture dish. Cells were normally replated at a density of 0.25-0.5x10^{6}. In routine maintenance, surplus cells were discarded. For bulk cell growth for transfection experiments, lifted cells were distributed between further tissue culture dishes and the culture volume adjusted to the appropriate level (10 ml for a 10 cm dish, 5 ml for a 6 cm dish).

Freezing and resuscitation of tissue culture cells

Cells were frozen to -80^{o}C at an approximate density of 0.1-0.2x10^{6}/ml. Cells lifted from tissue culture plates were pelleted for 5 mins at 1000 rpm, 4^{o}C. Pelleted cells were resuspended in freezing medium (FCS supplemented with 10% DMSO), allowed to freeze slowly, overnight (16-18 h) at -80^{o}C and then transferred to liquid nitrogen for indefinite long term storage.

Cells were resussitated by thawing as quickly as possible at 37°C and plating in 10 ml of growth medium in a 10 cm culture dish. Cells were normally revived completely within 2-3 days.

2.2.17.) Transfection of tissue culture cells with plasmid DNA

Densitometry of plasmid clones

The DNA concentrations of the GR clones used in all quantitative assays (pSTC series) were determined by densitometry. Plasmid DNAs (100 ng of each clone) were run on a 1% agarose gel. DNA was visualised using a DNA imager (Oncor, Appligene, Durham, U.K.) and the final image saved on a Macintosh compatible disc as a TIFF file. The file was then converted to a PICT file using the program: Graphic Converter (designed by: Thorsten Lemke, Peine, Germany) and scanned densitometrically using the program: Scan Analysis 68000 (Biosoft, Cambridge, U.K.). Peak heights (graphically) and peak areas (numerically), measured for each DNA band were compared for each clone analysed.

Transient transfection into COS-7 cells was carried out using the transfection reagent DOTAP as recommended by the supplier. Cells were grown for a further 48 h prior to harvest.

For the $CaPO_4$ coprecipitation method, plasmid DNA of appropriate quantity (depending on dish size: 20 µg for a 10 cm dish, 10 µg for a 6 cm dish) was prepared as follows: for a 10 cm culture dish, a total of 20 µg of plasmid DNA was combined with 70 µl ddH_2O, 150 µl T.E. (pH 7.6), 250 µl Ca^{2+} (4x) solution (0.5 M $CaCl_2$, 0.05 M HEPES [pH 7.0]) and 500 µl Pi (2x) solution (0.75 mM Na_2HPO_4, 0.75 mM NaH_2PO_4, 0.05 M HEPES [pH 7.0] and 0.28 M NaCl in ddH_2O). For a 6 cm culture dish, a total of 10 µg of plasmid DNA was combined with 35 µl ddH_2O, 75 µl T.E. (pH 7.6), 125 µl Ca_2^+ (4x) solution and 250 µl Pi (2x) solution.

All components, except 2x Pi solution were mixed and incubated for 5-60 mins until equilibrated to room temperature. Pi solution was then added and reactions incubated for a further 5-15 mins (depending on the age of the Pi solution). $CaPO_4$-DNA precipitates were added to cells plated at 80-90% confluence in DMEM containing 3% FCS. Optimum transfection was achieved with $CaPO_4$-DNA crystals of a similar size to the nucleoli of the cell nucleus. Precipitates were incubated with cells for 12-16 h under standard conditions (37^oC, 5% CO_2,) and removed with x2 washes of TBS. Cells were harvested after a further 44-48 h growth in DMEM containing 3% FCS.

2.2.18.) Lac Z staining of tissue culture cells

Cells transfected with clones encoding *lac Z* (and therefore expressing β-galactosidase) were assayed 48 h after the removal of the transfection mixture for the presence of functional enzyme as follows: growth medium was aspirated off and cells rinsed twice with PBS (1 Sigma PBS tablet per 200 ml ddH_2O [pH 7.4]: 3 ml for a 6 cm dish, 5 ml for a 10 cm dish). Fixation solution (2% formaldehyde, 0.2% glutaraldehyde: 10.8 ml 37% formaldehyde and 0.8 ml 50% glutaraldehyde in 200 ml PBS) was then added (2 ml for a 6 cm dish, 4 ml for a 10 cm dish) and cells incubated for 2-5 mins (max.) at 4^oC. Fixative was removed and cells again rinsed twice with PBS (3 ml for a 6 cm dish, 5 ml for a 10 cm dish). Cells were stained in staining solution (37^oC, 1x staining solution [5 mM $K_3[Fe_2(CN)_6]$ and 5 mM $K_4[Fe_3(CN)_6]$ in 1x PBS] supplemented with 2 mM $MgCl_2$ and X-gal at 1 mg/ml [dissolved in N,N-dimethylformamide]), (2 ml for a 6 cm dish, 4 ml for a 10 cm dish) by incubating 2-6 h at 37^oC. The staining solution was shaken well before use. Once blue colouring was apparent in positive cells, the staining solution was replaced with PBS (3 ml for a 6 cm dish, 5 ml for a 10 cm dish) and plates stored at 4^oC to enhance pigmentation. The fraction of cells expressing β-galactosidase were estimated by averaging numbers of blue stained cells per 10 fields of view (magnification (4/0.1) for three transfection plates per clone. Cells were photographed using a Nikon microscope mounted with a Nikon FX 35A camera (Nikon, Kingston Upon Thames, Surrey, U.K.).

2.2.19.) β-galactosidase assay

From 6 cm tissue culture plates of CV-1 cells transfected with different GR clones, 50 μg of cell extract (prepared by NP40 lysis as described in section 2.2.22.) were mixed with 181 μl of 0.1 M sodium phosphate buffer (0.082 M Na_2HPO_4, 0.018 M NaH_2PO_4 [pH 7.5]), 66 μl of 1x ONPG solution (4 mg/ml ONPG in 0.1 M sodium phosphate buffer [pH 7.5]) and 3 μl of 100x Mg^{2+} solution (0.1 M $MgCl_2$, 4.5 M 2-mercaptoethanol). Samples were vortexed briefly and incubated at 37°C for 30-60 mins until a yellow colour developed. Reactions were stopped by adding 500 μl of a 1M $Na_2(CO_3)$ solution. Optical densities of terminated reactions were measured at 420 nm. Each assay was performed in triplicate and an average O.D. reading determined for each GR clone.

2.2.20.) Determination of protein concentrations in cell extracts

Protein concentrations in all tissue culture cell extracts and tissue homogenates were determined using the Bio-Rad (Bradford) protein assay kit as recommended by the supplier.

2.2.21.) Steroid binding assays

Using liver cytosol extracts

Rat liver cytosol extracts were prepared from freshly sacrificed animals. Livers were perfused via the hepatic portal vein with 20 ml of saline (room temperature) to remove blood. 1-2 g of tissue (on ice) was finely minced using a pair of scissors and homogenised in 3-volumes of ice cold molybdate extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 2 mM DTT, 50 mM NaCl, 0.1 M Na-molybdate and 10 % glycerol [pH 7.2]). Homogenates were spun at 10-12,000 rpm for 20 mins at 4°C (Beckman JC M2 centrifuge) to remove cellular and tissue debris, followed by ultracentrifugation at 50,000 rpm, for 1 h at 4°C (Beckman optima™ TLX ultracentrifuge) to pellet microsomes and DNA. Protein concentrations were determined by Bradford assay.

Using sonicated cell extracts

Both cytosolic and nuclear proteins were extracted from cell lines expressing GR (either naturally or from transfected cDNAs). Cells (normally 30-40x10^6) were harvested using trypsin-EDTA, pelleted and resuspended in ice cold molybdate buffer, supplemented with 10 mM DTT. Cells were sonicated on ice at an amplitude of 6 microns. Cell debris and DNA were pelleted by ultracentrifugation at 50,000 rpm, for 1 h at 4°C (Beckman optima™ TLX ultracentrifuge) and protein concentration measured as described for liver cytosol.

Receptor steroid binding assays were performed by equilibrating 50 µg aliquots of cell extracts with ^{3}H-dexamethasone (1.5 nM final concentration) and increasing concentrations of unlabelled dexamethasone or corticosterone in ice cold molybdate buffer [final concentrations: 1580 nM, 500 nM, 158 nM, 50.0 nM, 15.8 nM, 5.0 nM, 1.58 nM, 0.5 nM, 0.158 nM and 0.05 nM]) and incubating for 24-48 h at 4°C. ^{3}H-dexamethasone binding to receptor (GR) was determined by removing unbound steroid ligand using 100 µl of an ice cold solution of activated charcoal (5% w/v methanol washed activated charcoal [Sigma] and 0.1% w/v Dextran T70 [Pharmacia] in molybdate buffer) and spinning at 2,800 rpm for 15 mins at 4°C. 125 µl of recovered supernatant was mixed with 250 µl of scintillant (Optiphase Supermix: Wallac, Milton Keynes, U.K.) and cpm measured over a 5 minute period using a 1450 Microbeta plus liquid scintillation counter (Wallac).

Scatchard analysis

Calculations of dissociation constants (Kd) and receptor binding capacities (Bmax, also referred to as R1) were made from homologous and heterologous competition curves (Scatchard analysis) using the curve fitting program, LIGAND (Munson and Rodbard, 1980).

Statistics

Raw data obtained from studies of linkage of GR and other genotypes with a number of physiological variables in different strains of rat were analysed by analysis of variance using Numann-Keul's correction test for multiple

comparisons. Kd (dex), Kd (B) and R1 values generated from analysis of steroid binding by GR were compared by parametric and non-parametric statistics where appropriate.

2.2.22.) Immunodetection of membrane-bound proteins

Dot blot analysis

Protein samples, serially diluted from 100 μg down to 0.01 μg (i.e., 100, 10, 1, 0.1 and 0.01 μg total protein) in a volume of 50 μl were spotted onto a strip of PVDF membrane and dried at room temperature for 30-60 mins. Filters were then blocked using a 3% casein solution (3%w/v milk powder, 0.1% Tween-20 in PBS]) for 1 h at room temperature. Filters were probed using specific monoclonal antibody (section 2.1.15.) at various concentrations. Specific primary antibody binding was detected using the ECL immunodetection kit.

Western blotting

For large numbers of samples (10-15), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting was carried out using a 15x20 cm midigel system (separation using a vertical midi electrophoresis tank [GIBCO-BRL], and blotting using a PROTEAN II xi electroblotting system [BIO-RAD]). For the rapid turnover of smaller numbers of samples (rapid analysis), a minigel system, comprised of a vertical minigel tank (Sigma) and the Mini PROTEAN II electroblotting system (BIO-RAD) was used. Samples were generally run on 8% gels alongside high molecular weight protein markers.

Sample preparation by NP40 lysis

Soluble proteins (from cytosol and nucleus) were recovered from tissue culture cells by NP40 lysis. Harvested cells were pelleted in 1.5 ml microfuge tubes (15 s, 14,000 rpm) washed in 1 ml ice cold PBS (pH 7.4), repelleted (15 s at 14,000 rpm) and lysed in 40-50 μl NP40 lysis buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT and 0.1-0.5% NP40) for 15 mins at 4°C. Cell debris and cell nuclei were pelleted at 14, 000 rpm for 15-20 mins at 4°C.

Nuclear proteins were extracted using TGEN-50. Nuclei from lysed cells were washed twice in ice cold PBS (pH 7.4) and resuspended in an estimated 4-times the volume of TGEN-50 (10 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 50 mM NaCl and 0.5 % NP-40). Nuclei were lysed at 4°C for 15 mins. Nuclear debris was removed at 14,000 rpm for 5 mins at 4°C. Protein concentrations were determined by Bradford assay.

Whole cell preparations (~$1x10^4$-$1x10^5$/loading) were analysed for GR expression by pelleting at 14,000 rpm for 15 s at room temperature, washing in 1 ml of ice cold PBS (pH 7.4), repelleting (14,000 rpm for 15 s) and lysing in 20 µl of gel loading buffer. Samples were denatured at 95-100°C for 5-mins, cooled to room temperature and DNA and cell debris spun out for 3-5 min at 13,000 rpm and the supernatant loaded onto the gel.

SDS gel preparation, sample loading and electrophoresis

SDS-polyacrylamide gels were prepared as follows: for minigels, 14.8 ml of 8% resolving gel solution (8% acrylamide solution, 0.375 M Tris-HCl [pH 8.8]) was polymerised using 50 µl 10% APS and 25 µl TEMED. The gel solution was overlaid with 100% ethanol (about 1.5 cm depth) to encourage a flat polymerised gel surface.

The resolving gel was overlaid with 4.9 ml of 6.5% stacking gel solution (6.5% acrylamide solution, 0.15 M Tris-HCl [pH 6.8]), polymerised using 30 µl 10% APS and 15 µl TEMED. Midigels (15x20 cm) were prepared in much the same way, except the resolving gel (50 ml) was polymerised using 100 µl 10% APS and 50 µl TEMED, and the stacking gel (30 ml) was polymerised using 50 µl 10% APS and 30 µl TEMED.

Prior to gel loading, protein samples (10-20 µg of liver cytosol extract or 20-30 µg of tissue culture cell extract: each at 5-10 µl final volume) were mixed with twice their volume (10-20 µl) of 1x SDS-gel loading buffer (0.0625 M Tris HCl [pH 6.8], 0.5% w/v SDS, 0.7 M 2-Mercaptoethanol, 10% glycerol and 0.001% w/v bromophenol blue), heated at 95-100°C for 5-mins to denature, cooled to room temperature and loaded onto a gel pre-run at 100 v, 20 mA

(for about 30 mins). Both midi and mini gels were run at 100 v, 20 mA in 1x Tris-glycine eletcrophoresis buffer (0.1 % w/v SDS, 0.025 M Tris-HCl and 0.19 M glycine [pH 8.3]) until the bromophenol dye front was about half way down the gel. The voltage was then adjusted to 150 v and finally, to 200 v until the dye front had run off the bottom of the gel.

Electroblotting of proteins onto solid supports

Proteins separated on SDS-polyacrylamide gels were transferred onto PVDF membrane prior to probing with antibody. PVDF membrane cut to the exact dimensions of the gel was 'activated' by soaking in 100% methanol for one minute exactly and washing twice in ddH_2O for 2 mins each. 'Activated' membrane was further soaked in 1x Tris-glycine electrophoretic transfer buffer (2.5 mM Tris-HCl, 19.2 mM glycine, 2% methanol [pH 8.3]) for a minimum of 10 mins along with x4 pieces of Whatman 3 mm blotting paper cut to the same dimensions as the gel sandwiching pads (part of the electroblotting apparatus, used to promote a tight contact between gel and membrane). Soaked PVDF membrane was placed on top of the gel and good contact between gel and membrane and exclusion of any bubbles achieved by gently rolling accross the surface of the overlaid membrane using a glass pipette. Gel plus membrane was sandwiched on either side with two pieces of the pre-soaked Whatman 3 mm blotting paper and one gel sandwiching pad, whilst submerged in transfer buffer. The entire gel sandwich was assembled in the blotting tank inside a transfer casette and submerged in 1x transfer buffer. Proteins were transferred from negative to positive at a constant current of 250 mA for 1 h-40 mins (mini gel), or 3-4 h (midi gel) for efficient GR transfer, in ice cold conditions to prevent excessive heating.

ECL detection of proteins bound to solid supports

Membrane bound proteins were detected using the ECL immunodetection kit. Blotted membranes were blocked for 1 h in 3% casein blocking solution and probed with specific primary monoclonal antibody (10 µl for a midi-sized membrane [1:500 dilution] and 5 µl for a mini-sized membrane [1:750 dilution]) in a 5 ml volume of PBS-Tween. Incubation with secondary

antibody and detection of specific membrane-bound proteins was carried out exactly as described in the ECL immunodetection protocol.

Chapter 3
Results 1

Codon variation in the glucocorticoid receptor in inbred strains of rat

3.1.) Introduction

A number of studies in man and rat have indicated that abnormal adrenocortical hormone activity may contribute to the development of hypertension and obesity. In this chapter, the possible involvement of altered GR function has been considered in several inbred rat strains. The phenotypic evidence that glucocorticoid hormone action is affected in rats with a genetic predisposition to developing hypertension and obesity is reviewed.

Under normal physiological conditions, the circulating levels and eventual activity of glucocorticoids fall under the regulatory control of GR. In conditions of natural glucocorticoid excess, the release of corticosteroids from the adrenal and transcription of the GR gene itself are feedback inhibited by GR (Fraser, 1992). With abnormal GR function (typically the result of a mutated receptor protein) the feedback inhibition of corticosteroid release and GR down-regulation (which normally occurs in conditions of glucocorticoid excess) is compromised. This inability to feedback inhibit pituitary ACTH secretion results in an increase in the synthesis of glucocorticoid and mineralocorticoid hormones, which may affect corticosteroid related phenotypes such as blood pressure (Mantero *et al.*, 1983; Bianchi *et al.*, 1984; Fraser *et al.*, 1994). Abnormal glucocorticoid activity has also been proposed to play a role in the expression of obesity in Zucker rats. A detailed overview of the phenotypes of rat strains SHR and Zucker obese is given chapter 1 (MHS in chapter 5) and will only be considered further in this chapter in the context of steroid binding affinity.

It has previously been shown (Kenyon *et al.*, 1994; Panarelli *et al.*, 1995) that the glucocorticoid receptor (GR) from MHS and SHR strains of rat show significant differences in their affinities for steroids (cortisol, aldosterone

and dexamethasone and the glucocorticoid antagonist, RU486) compared with their normotensive controls (MNS and WKY, respectively). For the MHS/MNS model, the hypertensive strain MHS revealed consistently lower affinities for these steroids, producing higher Kd values. Measurements of receptor-steroid binding in liver cytosol extracts from non-adrenalectomised animals produced Kd values 1.39, 2.19, 2.06 and 4.12-fold greater for dexamethasone, corticosterone, RU486 and aldosterone respectively, in MHS than in MNS. For SHR and WKY strains, GR steroid binding characteristics were essentially reversed, the hypertensive strain (SHR) having generally higher affinities for natural steroids: 1.16 and 1.37-fold lower Kd values for corticosterone and aldosterone respectively than in WKY. Affinities for dexamethasone were not significantly different between strains. Similar studies in Zucker rats has yielded receptor affinities for corticosterone 100-fold lower in obese compared with lean rats, reduced to a difference of 2-3-fold following adrenalectomy (White and Martin, 1990; C. Kenyon, unpublished observations).

The total numbers of hepatic receptors between MHS and MNS and SHR and WKY rats were comparable under the chosen assay conditions and for the Milan strains, this was consistent with the observations made by Stewart *et al.*, (1993), in which levels of GR mRNA from liver and kidney were not found to be significantly different between these strains. For Zucker rats, the obese strain showed a 40-50 % reduction in the number of hepatic receptors which was normalised following adrenalectomy (White and Martin, 1990; C. Kenyon, unpublished results). Although changes in glucocorticoid activity can result from regulatory gene mutations which may alter the turnover of GR transcripts or from post translational modifications affecting the level of functional protein, taken together, the findings described above for the rat models of hypertension were more suggestive of abnormalities resulting from changes in the primary GR sequence. It was therefore logical to consider a search for mutations in the structural genes for GR, since these are frequently associated with changes in affinity for ligand (Brown *et al.*, 1990; Hurley *et al.*, 1991; McPhaul *et al.*, 1991). The potential relationship between any gene and disease phenotype must also take into account the possibility of mutations elsewhere, other

than in the candidate gene itself, but in close linkage on the same chromosome arm. There is always the possibility that unknown mutations within the same locus of DNA are responsible for the observed disease phenotype.

The question of functional differences of GR can be addressed using different but complementary methods. Primary sequence information would allow for identification of sequence changes which might explain diffences in receptor function. Primary sequence data would also be useful in providing genetic markers for use in linkage studies.

The aims of this section of work were therefore focused in three main areas.

1.) To define a specific genetic marker for the rat glucocorticoid receptor locus (*Grl*). Such markers form the basis of rat breeding experiments in which genetic linkage with one or more physiological variables of the disease phenotype is sought (discussed further in chapter 5). The requirement for a genetic marker for the rat *Grl* was aided by the prior knowledge of a polyglutamine encoding triplet $(CAG)_n$ repeat sequence (microsatellite) in the 5'-end of the GR coding sequence (identified from the cDNA sequence: Miesfeld *et al.*, 1986). Trinucleotide repeat polymorphisms could themselves be considered as potential sources of differential GR function, either at the steroid binding or gene regulatory levels and were thus exploited early on in linkage studies.

2.) To determine whether there was variation between pairs of rat strains (MHS and MNS, SHR and WKY, Zucker lean and obese) in the codons for GR which may have provided an explanation for the observed differences in steroid binding affinity.

3.) To carry out a detailed analysis of the GR triplet $(CAG)_n$ repeat sequence from a large number of inbred rat strains. This was done to provide information on the extent of heterogeneity in the GR polyglutamine tract in inbred rats, which would be potentially useful when choosing

appropriate rat strains for breeding, or GR alleles for experiments focusing on GR function.

Part 1

3.2.) Methods for resolving rat *Grl* triplet $(CAG)_n$ repeat polymorphisms

From nucleotide position n223 in exon 2, the rat GR contains a series of glutamine residues encoded by a CAG trinucleotide repeat. Because of the inherent polymorphic nature of simple sequence repeats, this region was chosen as the starting point in the search for a genetic marker of the rat glucocorticoid receptor locus for use in linkage studies (see chapter 5). A number of potential methods were available for resolving triplet $(CAG)_n$ repeats, only two of which (high percentage agarose gel electrophoresis and sequencing) were well established. A preliminary assessment of each method was made to find the one most suitable for routine typing of the rat GR. All methods were based on PCR, so an initial verification that amplified sequences were of rat GR origin was required.

3.2.1.) PCR amplification of rat *Grl* triplet repeats, Southern blotting and probing with a rat GR cDNA fragment

The rat GR polyglutamine tract was amplified from SHR (Spontaneously Hypertensive Rat) strain liver DNA. Reactions were performed in a 50 µl volume using rGR gene specific PCR primers, pA and pG (Appendix 1) and 1.5 mM Mg^{2+}. Primer annealing temperature was optimised at 60°C. For general PCR strategy see section 2.2.3. PCR products (395 bp) were resolved on a 1.5% agarose gel. Different DNA concentrations (50-500 ng) and cycle numbers (30-35 cycles) were used to help maximise the probability of PCR amplification (Fig. 3.1a.).

For verification of the specificity of primer annealing, amplified products were transferred onto nylon membrane (Hybond-N^+) using Southern

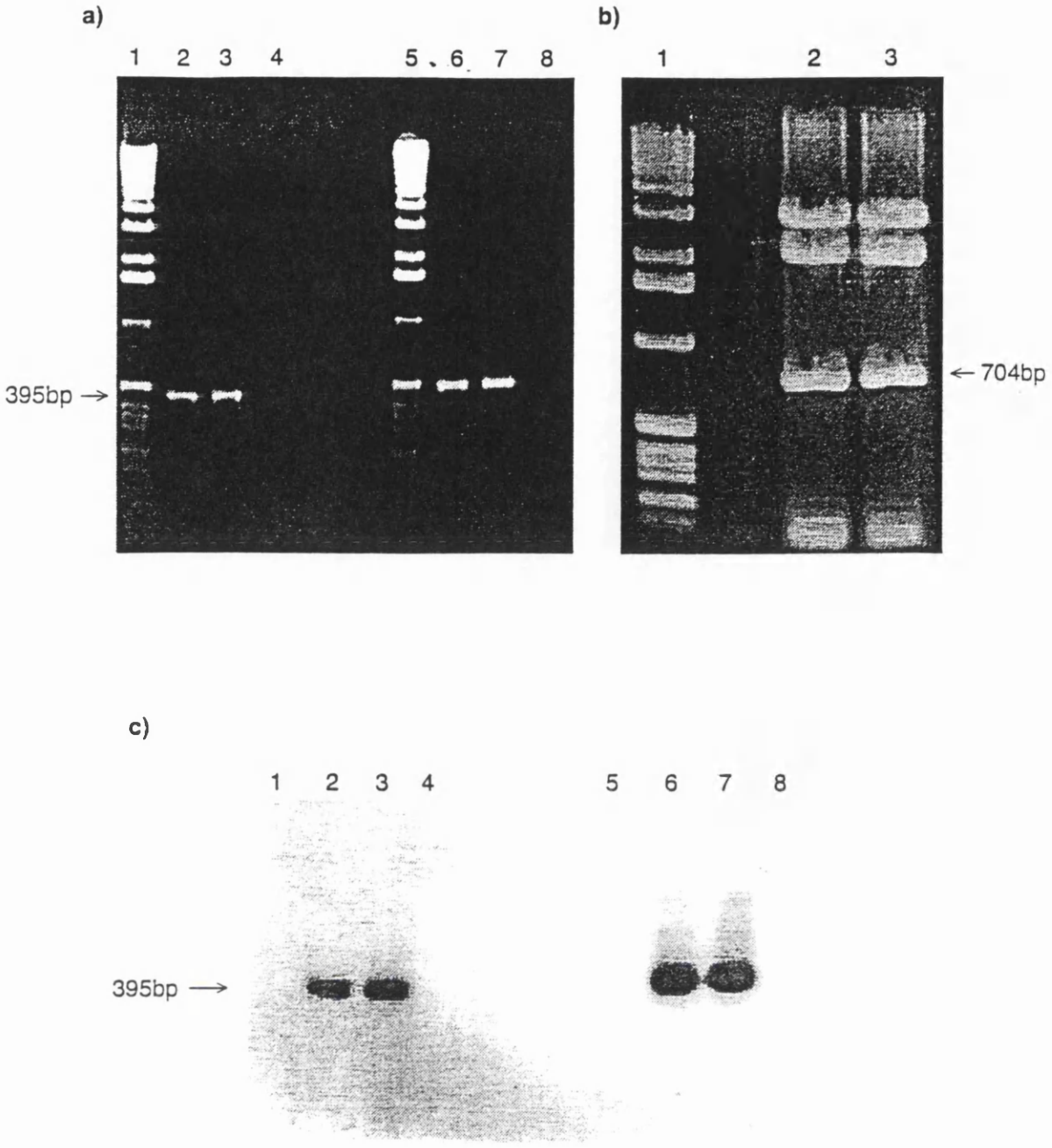

Fig. 3.1. Rat *Grl* polyglutamine tract amplified from strain SHR, using PCR primers pA and pG.

a.) PCR was carried out for 30-cycles (lanes 2 and 3), or 35-cycles (lanes 6 and 7): from 50 ng (lanes 2 and 6) and 500 ng (lanes 3 and 7) template DNA. Lanes 4 and 8: negative (no DNA) controls. Lanes 1 and 5: 1 Kb DNA ladder.

b.) Preparation of 704 bp cDNA probe from rat rGR clone pRBal117. A large scale *XbaI*/*BglI* digest (5μg) of clone pRBal117 was band separated on a 1% agarose gel (lanes 2 and 3). The 704 bp band was excised and ^{32}P-labelled using random oligonucleotide primers. Lane 1: 1 kb ladder.

c.) Southern blot of rat (SHR) amplified *Grl* polyglutamine tract. The hybridised filter was probed using the ^{32}P-labelled 704 bp *XbaI*/*BglI* fragment of clone pRBal117. Specific bands of the expected size (395 bp) were identified in lanes 2, 3, 6 and 7.

blotting methods as described in section 2.2.8. Blotted DNA fragments were probed using a 704 bp ^{32}P-labelled (section 2.2.7.) cDNA fragment (Fig. 3.1b.) spanning the extreme 5'-end of the rat GR coding sequence, including the triplet $(CAG)_n$ repeat. The probe was generated by *XbaI/BglII* digestion (section 2.2.6.) of rat GR cDNA clone pRBal117 (Fig. 3.2.). The hybridised filter revealed single PCR product bands of the expected size (395 bp) and intensity, with respect to the number of PCR cycles (Fig. 3.1c.).

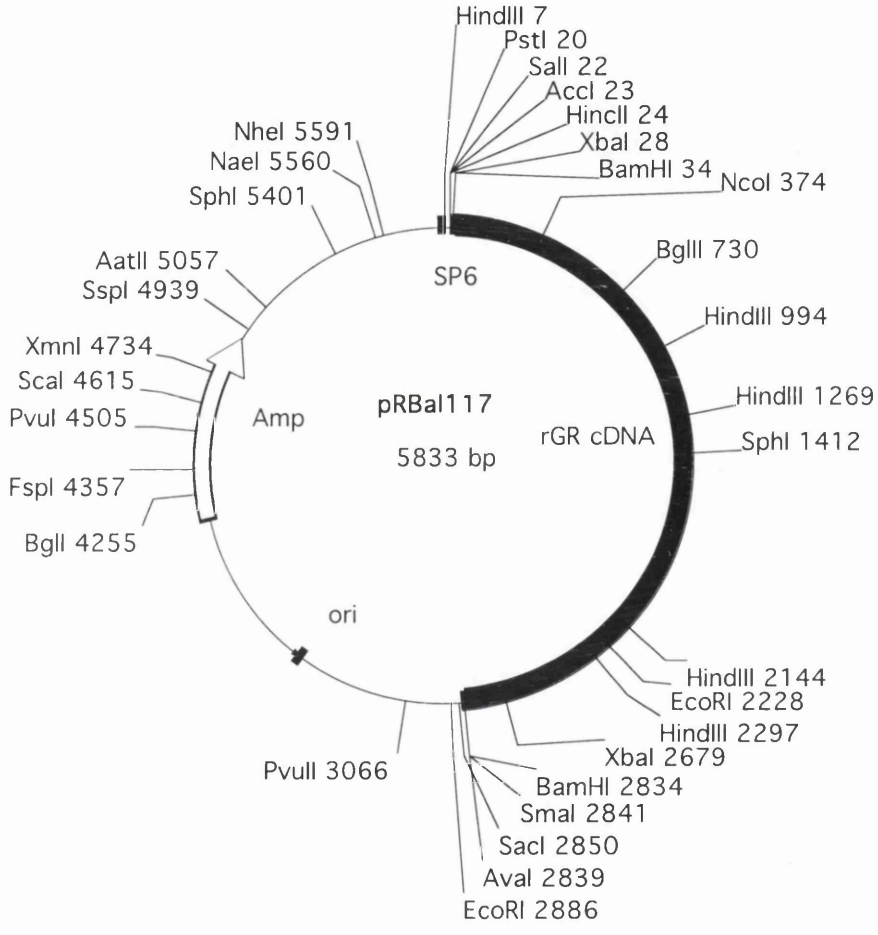

Fig. 3.2. Restriction map of rat GR cDNA clone, pRBal117 (Miesfeld *et al.*, 1986). The clone pRBal117 is a 5.8 kb plasmid based on the bacterial cloning vector: pSP64 (Promega, U.K.). The entire rGR coding sequence, 360 nucleotides of the 3' UT and 24 nucleotides of the 5' UT are cloned into the *Bam*H1 site of the vector polylinker in sense orientation.

3.2.2.) Analysis of *Grl* triplet $(CAG)_n$ repeats in rat strains: SHR, WKY, MHS, MNS and SD, using high percentage agarose gels

Having demonstrated the specificity (stringency) of annealing of PCR primers pA and pG in SHR, further analysis of GR polyglutamine tracts was

carried out. Genomic DNA, prepared from the livers of parental rats of strains: SHR, WKY, MHS, MNS and Sprague Dawley (SD) was PCR amplified using primers pA and pG under exactly the same conditions as described in section 3.1.1. PCR products (5 μl sample volumes) were resolved on a 3% agarose gel, run at 150 v/100 mA, in 0.5 x TAE buffer (1 x in gel) to improve resolution.

3.2.3.) T-vector cloning and sequencing of amplified *Grl* triplet $(CAG)_n$ repeats from: SHR, WKY, MHS, MNS and SHR-SP

The T-vector, pT7Blue (AMS Biotechnology, U.K., Ltd.) is a 2887 bp cloning vector, specifically manipulated for cloning PCR products. The vector is linearised through an *Eco*RV site within the polylinker, part of the *lac Z* gene which encodes the α-subunit of β-galactosidase. The 5' T-overhang which is generated by this cleavage is ideally suited for the ligation of PCR products, which are normally terminated with 3' A-nucleotides by most of the commonly used thermostable DNA polymerases (e.g. *Taq* polymerase).

The T-vector cloning system relies on *Lac-Z* inactivation. Insertion of a cloned DNA fragment within the *Eco*RV-cut *Lac-Z* region disrupts the open reading frame (ORF). Because the vector no longer encodes a functional β-galactosidase α-peptide (which normally complements the *LacZ* ω-fragment, expressed by the bacterial host-strain) the enzymic conversion of the chromogenic substrate X-gal to 5-bromo-4-chloro-3-indolyl and galactose, is prevented.

GR microsatellites from SHR, WKY, MHS, MNS and SHR-SP, amplified using PCR primers pA and pG, were cloned from single PCR reactions into the T-vector as described in (section 2.2.10.). Following 16-20 h ligation ($16^{o}C$) and competent Novablue-*E.coli* transformation, (section 2.2.11.), cultured bacterial plates supplemented with ampicillin (50 μg/ml), tetracycline (20 μg/ml) X-gal (1.75 μg) and IPTG (0.08 mM), were subjected to blue/white colony selection. A number of white colonies were selected, representatives

of each rat strain and preserved as 'potentially positive' recombinants by streaking onto LB-ampicillin agar plates. Colony streaks were grown for 16-20 h (37^{o}C) and stored at 4^{o}C prior to further analysis.

Selected white colonies (Table 3.1.) carrying either full length pA/pG generated PCR product inserts or some other product of the PCR reaction, were further grown in 1 ml LB-ampicillin liquid cultures (see section 2.2.11.). Each was analysed for the presence of the correctly sized insert (395 bp) by a second round of PCR amplification (in a consistent 50 µl reaction volume), using the same primers as those used in the initial PCR (pA and pG). Diluted samples (1/10) were boiled for 5 min and 1 µl aliquots used directly in PCR (see section 2.2.11.). Amplified products were run on 1.5% agarose gels against the 1 kb ladder. PCR reactions for each transformation (per rat strain) included a no-culture negative control to monitor for cross contamination. These were usually found to be negative, but when contamination was identified, all PCR solutions were discarded and replaced. Clones producing a signal of the correct size (395 bp) were retained by streaking onto LB-ampicillin plates and storing at 4^{o}C for further analysis by sequencing. Clones carrying full length inserts were innoculated into 100 ml LB-ampicillin medium (midiculture) and grown 16-20 h (37^{o}C), with shaking. Bacterial plasmids were recovered according to the Qiagen midiprep protocol (see section 2.2.12.). Clone DNA was analysed on 1% agarose gels for quality (absence of nicking) and efficiency of removal of RNA. $O.D._{260}$ was measured at a 1/100 dilution and plasmid concentrations adjusted to 1 µg/µl in T.E. (pH 8.0). Normally 4-6 µg of plasmid template DNA was re-precipitated and denatured prior to sequencing by the methods of Sanger from the reverse PCR primer, pG which doubled as a convenient sequencing primer. Three to four positive clones were sequenced per rat strain (see method for plasmid sequencing, section 2.2.13.).

3.2.4.) Typing *Grl* triplet repeat lengths in a wider variety of rat strains using a ^{32}P-based PCR approach

Due to the problems encountered in generating reliable sequence data from T-vector cloned GR polyglutamine tract (discussed in section 3.3.1b. of this chapter), a different typing strategy was adopted. This new approach combined directly the speed and efficiency of PCR with the high resolving powers of polyacrylamide gel electrophoresis, coupled to the added sensitivity of autoradiographic detection. Higher resolution was achieved by reducing the average size of PCR products, from 395 bp down to a range of about 100-120 bp. This required the design of a new set of universal PCR primers, flanking the poyglutamine tract as closely as possible. The new primers were designated: p9 (forward) and p19 (reverse) (their positioning in the GR coding sequence is listed in Appendix 1). The incorporation of radioactivity into PCR products was achieved by end-labelling p9 using [γ^{32}P]-ATP as described in section 2.2.7.

Pilot typing experiments were carried out on parental rats from a small number of selected strains: SHR, WKY, MHS, MNS, WKY_{Gla}, $SHR\text{-}SP_{Gla}$ (Glasgow strains) and Lyon strains: LL (low), LN (normal) and LH (high) blood pressure). Glucocorticoid receptor poyglutamine tracts were amplified in 25 µl reaction volumes by combining 5-10 pmoles each of PCR primers: p9 (^{32}P-labelled) and p19, with 50-100 ng genomic DNA and 2 mM Mg^{2+} and PCR cycling for 30-cycles under the general conditions described in materials and methods (section 2.2.3.). Primer annealing temperature was 60^{o}C.

A further 61 inbred strains together with 29 of their substrains (Table 3.3.) (obtained from Prof. B. van Zutphen, University of Utrecht, The Netherlands. [see section 2.1.3.]) were typed using an optimised ^{32}P-based PCR protocol. PCRs were optimised by reducing the concentration of DNA and Mg^{2+} used in each reaction down to about 20 ng/µl and 1.5 mM respectively. The labelled PCR primer (p9) was also prepared at a 3x higher specific activity, so that the quantity of primer mixture added to each

reaction was reduced making interference of PCR by labelling reaction components less likely. ^{32}P-labelled PCR products were resolved on 6% polyacrylamide, 8 M urea gels and respective bands identified autoradiographically (section 2.2.4.).

3.2.5.) Development of a suitable marker for ^{32}P-based microsatellite typing

A series of T-vector clones (Table 3.2.) containing rat GR polyglutamine tracts of known length and sequence (see section 3.2.1b. and Fig. 3.6.), were used as DNA markers to calibrate the sizes of PCR products of unknown length.

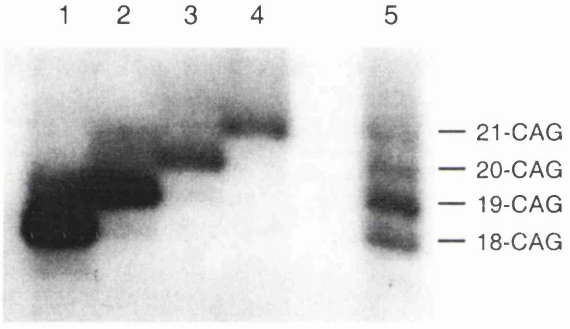

Fig. 3.3. Microsatellite markers.
^{32}P-PCR amplifications were performed from T-vector cloned rat GR polyglutamine tracts. Clones: T3/3 C3, T1/2 C2, T3/2 C5 and T4/1 C14 (Table.3.3.) were amplified using PCR primers p9 (labelled with γ-^{32}P) and p19 (Appendix 1). PCR products of: 18, 19, 20 and 21 CAG-repeats were resolved either separately (lanes 1-4), or from a pooled mixture of these PCR products, producing a banding ladder (lane 5) on a standard 6% polyacrylamide 8M urea gel. Numbers on the right represent the length in $(CAG)_n$ repeats of the individual marker bands.

PCR products generated from these T-vector clones: T3/3 C3 (18-CAG), T1/2 C2 (19-CAG), T3/2 C5 (20-CAG) and T4/1 C14 (21-CAG repeats), also amplified using p9 [^{32}P] and p19, were pooled to produce a composite DNA ladder with a resolution down to at least one codon spacing between bands (see Fig. 3.3.). The method used for determination of triplet $(CAG)_n$ repeat length is described in section 3.3.1b.

3.2.6.) Resolving rat GR polyglutamine tract polymorphisms using 4% metaphor gels

In addition to the high sensitivity and resolving powers of ^{32}P gel typing systems described in section 3.2.4, consideration was also given to overall speed and efficiency and the possibility of minimising, or even avoiding the use of ^{32}P in the routine typing of rat GR polyglutamine tracts. Methodology for resolving microsatellite polymorphisms in simple or multiplex PCR systems using 4% metaphor agarose gels has been developed (Shiels *et al.*, 1995). Amplified polyglutamine tracts from strains: MHS (21-CAG repeats), MNS (20-CAG repeats), SHR (19-CAG repeats), a rat GR clone (18-CAG repeats; see chapter 6 for details) and BC (7-CAG repeats), were resolved on gels run in 0.5 x TAE (1 x in gel) and kept as cold as possible.

3.3.) Results

3.3.1.) Typing rat GR triplet $(CAG)_n$ repeat lengths by different methods (3.3.1a.-3.3.1d.)

3.3.1a.) Using high percentage agarose

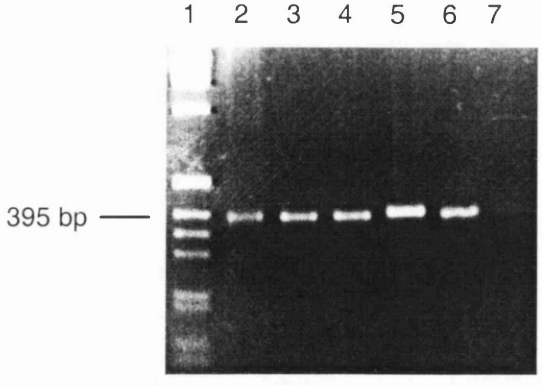

Fig. 3.4. A search for genetic variation in rat GR polyglutamine tracts. Polyglutamine tracts were amplified from the GR of parental rats of strains: SHR (lane 2) WKY (lane 3) MHS (lane 4) MNS (lane 5) and SD (lane 6) using PCR primers, pA and pG. PCR products (395 bp) were resolved on a 3% agarose gel. Lane 1: 1 kb DNA ladder. Lane 7: No DNA negative control.

Only minor differences in the migrational properties of pA and pG amplified GR polyglutamine tracts from rat strains: SHR, WKY, MHS, MNS and SD were identified using 3% agarose (Fig. 3.4.), indicating the need for a more sophisticated approach for typing rat GR alleles.

3.3.1b.) T-vector cloning and sequencing

Table 3.1. lists the number of blue and white colonies obtained following the transformation of NovaBlue *E.coli* with T-vectors ligated with pA/pG-generated PCR products from each of the rat strains. All clones, sequenced as described in section 3.2.3, contained PCR product inserts of rat GR origin (determined by the positive identification of flanking sequences). To conform to a previous report (Gearing *et al.*, 1993), repeat lengths were calculated as the total number of CAG codons in respective polyglutamine tracts, interrupted by one alanine codon at the 5'-end. For the remainder of this thesis, CAG-repeat alleles will be referred to, where appropriate, in the following way: Grl^{CAGX}, where X refers to the length of the $(CAG)_n$ repeat. As an example, Figure 3.5. below details the rGR 7-repeat allele, Grl^{CAG7}.

Ser Thr Ser Asn Val Gln Gln Arg Gln Gln Gln Gln Gln Pro Gly Leu Ser Lys
5' >TCC ACA AGC AAT GTG CAG CAG CGA CAG CAG CAG CAG CAG CCA GGC TTA TCC AAA< 3'

Fig. 3.5. The coding sequence of the 7-repeat rGR polyglutamine microsatellite (Grl^{CAG7}).
The sense DNA strand nucleotide sequence of the CAG repeat (underlined), plus five flanking codons either side is shown. The respective amino acid sequence is given above in the three letter code.

Figure 3.6. and Table 3.2. show examples of the rat GR polyglutamine tract sequences identified, together with evidence of minor length variabilities. Sequences presented are of the antisense DNA strand. Glutamine polymers are therefore expressed as GTC-repeats [5'->3', see Fig. 3.6.] and not the conventional CAG-repeats, obtained from the sense DNA strand. Unexpectedly, variation was identified, not only between different rat strains, but also between clones isolated from PCRs from the same strain. The assignment of a single polyglutamine tract length to a given rat strain glucocorticoid receptor was therefore unreliable.

Rat strain microsatellite cloned	Transformation Set	No. of colonies selected (pool of 3 x10cm plates)	
		BLUE	WHITE
SHR	T1/2	2	6
WKY	T3/2	10	8
	T4/3	5	12
MHS	T4/2	15	24
MNS	T4/1	8	24
SHR-SP	T3/3	4	5
	T4/4	14	12
T-vector positive control insert		10	63

Table. 3.1. Blue/white colony selection following ligation of PCR amplified rat GR polyglutamine tracts into the T-vector.
Ligated vectors were used to transform competent Novablue *E.coli.* (AMS Biotechnology, U.K., Ltd.). Each transformation set was derived from a single PCR reaction from respective rat strain genomic DNAs. The numbers of blue and white colonies for each set were the proceeds from three 10 cm bacterial plates. Ligation efficiency was monitored using the T-vector kit (AMS Biotechnology, U.K., Ltd.) positive control insert.

These inconsistencies were considered to be caused by mutations arising during the PCR reaction, or during the growth of clones in *E. coli* prior to sequencing. The majority of mutations could be explained by the phenomenon of 'slippage' (Strand *et al.*, 1993; Tautz and Schlotterer, 1994) a process in which a replicated DNA strand in the region of a polymeric tract of repeat sequence is aligned out of register with the template strand during DNA synthesis. This is an unavoidable process which can occur *in vitro* or *in vivo.* In each set of transformations, contamination was considered an unlikely explanation because of the implementation of a reliable negative control.

A second observation from the sequencing of T-vector clones was the low frequency of polyglutamine tracts interrupted by a single point mutation (T -> C transitions, converting Gln_{90}->Arg_{90}), (Table 3.2., clones: T4/2 C2, T4/4 C3 and Fig. 3.6., sequence E).

Clone	CAG repeat number	Rat strain (PCR template)
A T1/2 C1	20	SHR
C2	19	SHR
B T3/2 C5	20	WKY
C7	19	WKY
C15	19	WKY
C T3/3 C3	18	SHR-SP
D T4/1 C14	21	MNS
C18	21	MNS
C19	21	MNS
E T4/2 C1	16	MHS
C2	5-CCG-14	MHS
C11	Nonsense	MHS
C14	19	MHS
F T4/3 C2	20	WKY
C6	20	WKY
G T4/4 C3	5-CCG-14	SHR-SP
C5	18	SHR-SP
C7	19	SHR-SP

Table. 3.2. Variation in the number and composition of CAG repeats in the rat glucocorticoid receptor polyglutamine tract cloned into the T-vector (Cambridge Bioscience, U.K.).
Each of the letters A-G, represents a set of T-vector clones derived from one PCR amplification of a single rat genomic template.

The low frequency and sporadic occurrence of these mutations implied that they were artefacts incorporated during PCR and were analysed no further.

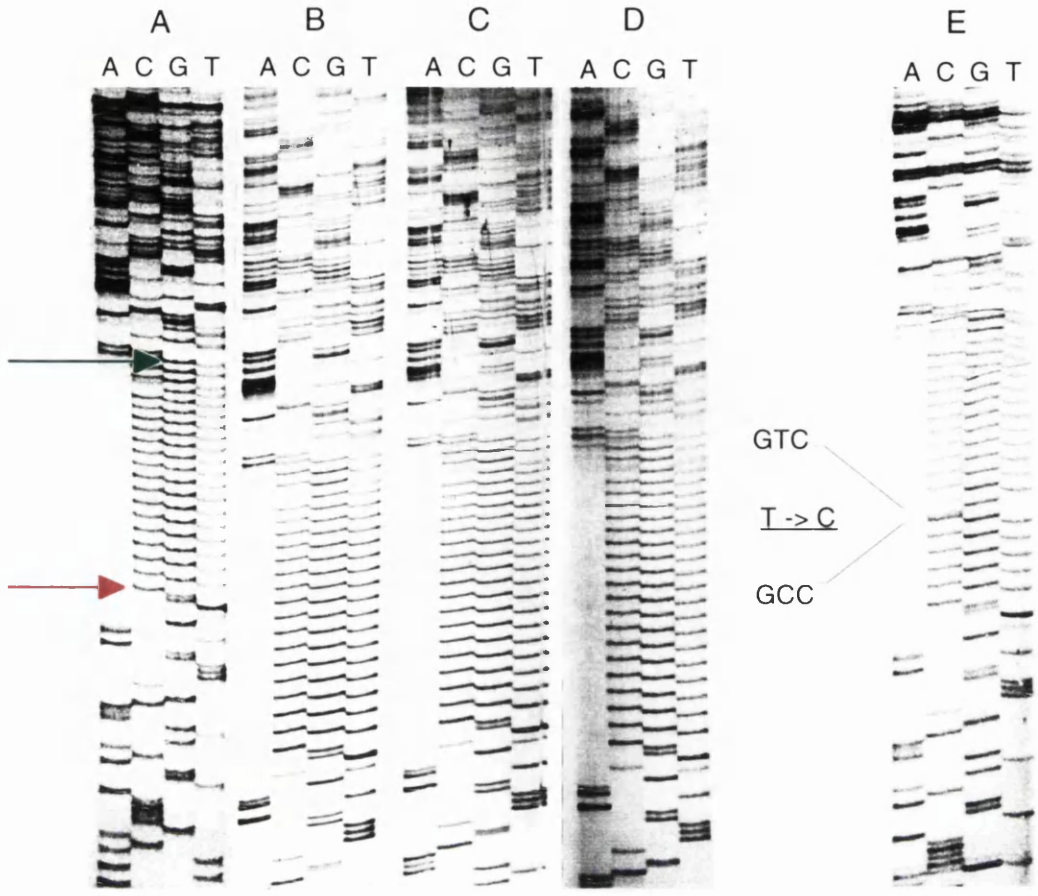

Fig. 3.6. Sequencing of rat GR polyglutamine tracts from T-vector clones. Sequencing was carried out using the antisense primer: pG (Appendix 1). Sequences: A (C, T3/3 C3), B (A, T1/2 C2), C (B, T3/2 C5) and D (D T4/1 C14) (Table.3.3.) demonstrate variability in polyglutamine tract length. Polymer lengths were determined by counting the numbers of GTC codons (including the single GCT) in the antisense DNA strand from top (green arrow) to bottom (red arrow). Sequence E shows a probable PCR artifact in which a T->C transitional point mutation (nucleotides relative to antisense DNA strand: letters underlined) converts Gln_{90} (GTC- antisense) of the polyglutamine tract to Arg_{90} (GCC-antisense). Letters above sequence lanes represent terminating nucleotides. In each case the antisense DNA strand is shown (5' -> 3', top to bottom). GR sequences flanking polyglutamine tracts confirms the stringency of PCR-primer annealing.

3.3.1c.) Typing of Grl triplet $(CAG)_n$ repeat polymorphisms in the rat models of essential hypertension: a.) MHS/MNS and SHR/WKY and b.) SHR-SP_{Gla}/WKY_{Gla} and Lyon strains by ^{32}P-PCR

a.) In MHS/MNS and SHR/WKY

The migration of PCR products, independently amplified from the DNA of several parental rats of strains MHS (n=5), MNS (n=6), SHR (n=3) and WKY (n=3), were compared on 6% polyacrylamide gels. A size difference of 3 bp was identified between MHS and MNS and SHR and WKY. These results

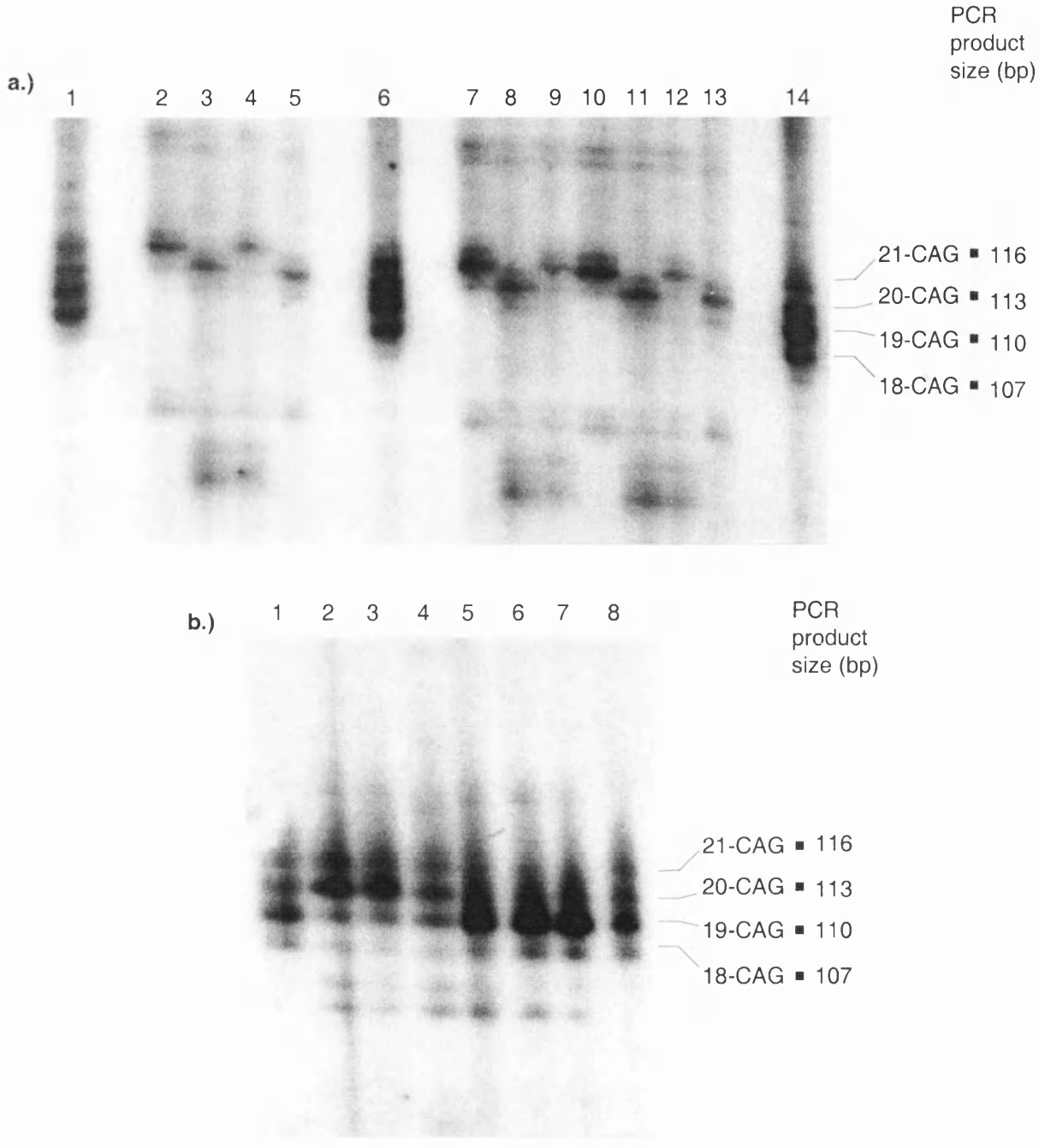

Fig. 3.7. Typing gel showing polymorphism of the GR polyglutamine tract between rat strains: MHS, MNS and SHR, WKY.

a.) MHS and MNS. Lanes 2, 4, 7, 9, 10 and 12: MNS (21-CAG repeats). Lanes 3, 5, 8 and 11: MHS (20 CAG-repeats). Lane 13: Sprague Dawly (20-CAG repeats). Lanes 1, 6 and 14: Pooled microsatellite markers.

b.) Between strains SHR and WKY. Lanes 2-4: WKY (20-CAG repeats). Lanes 5-7: SHR (19-CAG repeats). Lanes 1 and 8: microsatellite markers.

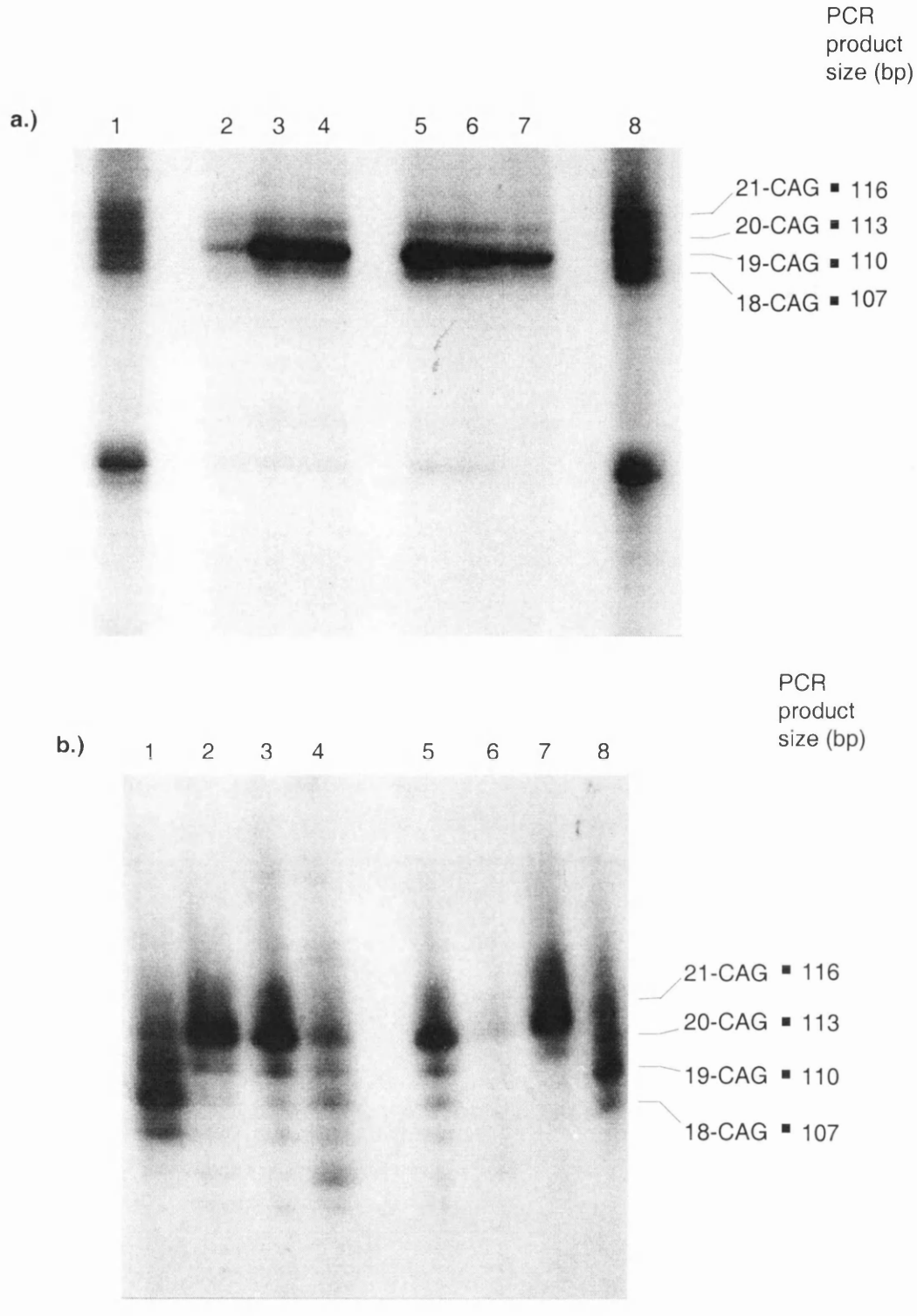

Fig. 3.8. Typing gels showing an absence of polymorphism of the GR polyglutamine tract between strains: SHR-SP$_{Gla}$ and WKY$_{Gla}$ and LL, LN , and LH

a.) SHR-SP$_{Gla}$ and WKY$_{Gla}$ (Glasgow strains). Lanes 2-4: WKY; A1641, A1661 and A 1662 (19-CAG repeats). Lanes 5-7: SHR-SP; C2128, C2129 and C 2134 (19-CAG repeats). Lanes 1 and 8: microsatellite markers.

b.) Lyon strains: LL (low) LN (normal) and LH (high) blood pressure. Lanes 2 and 3: LL. Lanes 4 and 5, LN. Lanes 6 and 7, LH (all 21-CAG repeats). Lanes 1 and 8: microsatellite markers.

suggested a polyglutamine tract which was shorter by one codon in MHS and SHR compared with their normotensive controls: MNS 21, MHS 20 and WKY 20, SHR 19-CAG repeats (Fig. 3.7a and b, respectively). PCR products from individuals of the strains MHS and MNS were loaded onto the gel in an alternate pattern to demonstrate the reproducibility of the polymorphism between PCR product bands (and hence, polyglutamine tracts. Fig. 3.7a.). Differences in the length of polyglutamine tracts (in codons) was determined by comparing the migrational distance of sample PCR products with that of reference bands of the size marker (Fig. 3.7a: lanes 1, 6 and 14 and 3.7b: lanes 1 and 8) (refer also to section 3.2.5.). Because no examples of T-vector clones were found with changes in the arrangement of nucleotides in flanking sequences, any differences in the size of PCR products must have been attributable solely to mutations in the polyglutamine tract. The expected GR polyglutamine tract lengths predicted for each strain (MHS, MNS, SHR and WKY) by the typing of whole ^{32}P-labelled PCR products was confirmed by the direct sequencing of RT-PCR and genomic PCR products (see part 2 of this chapter).

b.) In SHR-SP$_{Gla}$/WKY$_{Gla}$ and Lyon strains

Individuals of strains: SHR-SP$_{Gla}$, WKY$_{Gla}$ and Lyon strains, LL (low), LN (normal) and LH (high) blood pressure, were also typed. No GR polyglutamine tract polymorphisms were identified between strains (Fig. 3.8.). For the SHR-SP$_{Gla}$ and WKY$_{Gla}$ strains (Fig. 3.8a.) the CAG repeat number was 19. For the Lyon strains (Fig. 3.8b.) the CAG repeat number was 21.

3.3.1d.) Typing of selected strains using 4% Metaphor gels

Figure 3.9., reported in Shiels *et al.*, (1995), shows the successful fractionation of variant rat GR triplet $(CAG)_n$ repeat PCR products on high percentage metaphor agarose gel. Allelic variants of 21, 20, 19 and 18-CAG repeats (rGR T-vector clone, T 3/3 C3; section 3.2.5. and Table 3.2) and 7-CAG repeats (rat strain BC) were resolved successfully on 4% metaphor down to a resolution of 1-codon spacing from single or mixed sample gel loadings. Resolving capabilities were similar to that of 6-8% polyacrylamide.

Despite the possible application of metaphor gels in microsatellite typing, these gels were not used routinely because of the extreme fragility of high percentage gels, becoming an increasing problem with the build up of heat in 'fast' gel runs. Gels were broken too easily resulting in the need for repetition.

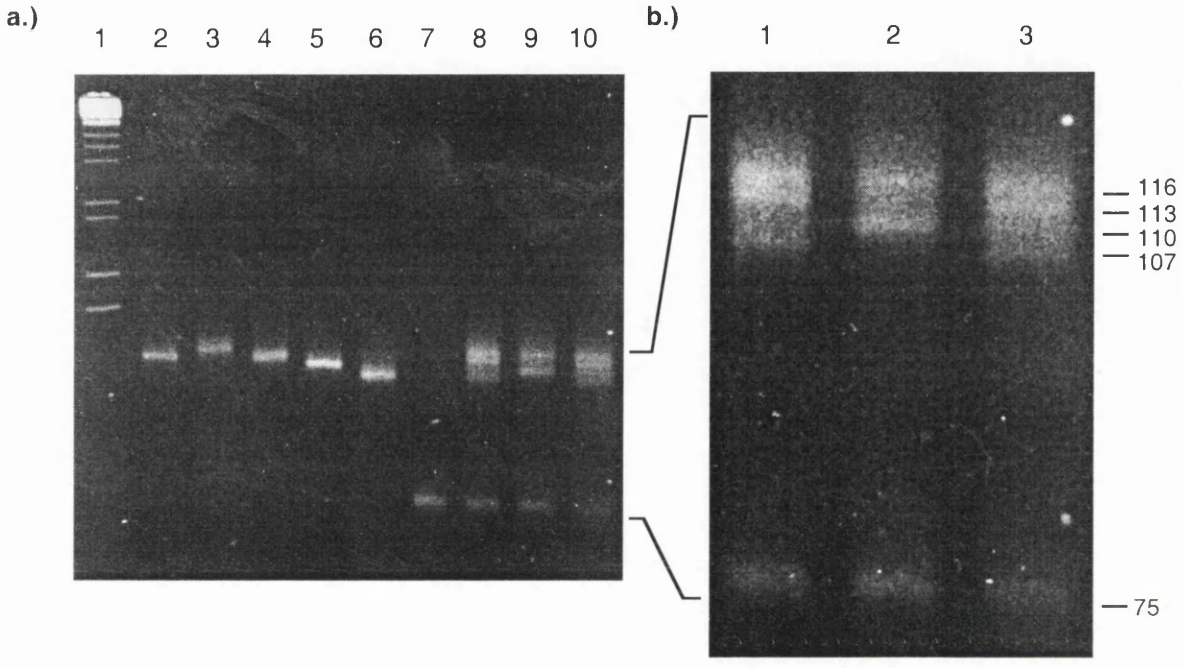

Fig. 3.9. Rat GR triplet $(CAG)_n$ repeat variants resolved on a 4% metaphor gel.
a.) GR alleles, cloned and sequenced from different rat strains and found to have the following numbers of CAG repeats were resolved on a 4% metaphor gel: 20 (MHS, lane 2), 21 (MNS, lane 3), 20 (MHS, lane 4), 19 (SHR, lane 5), 18 (GR clone [see text for details], lane 6) and 7 (BC, lane 8). Lanes 8 and 10 show mixtures of 21, 20 and 7 repeat variants. Lane 9 shows a mixture of 21, 19 and 7 repeat variants. Lane 1: 1 kb ladder.
b.) Lanes 8-10 from a.), amplified to show the resolution of bands with two or one codon spacing. The numbers and arrows at the right hand side shows the length in bp of the five allelic PCR products.

The integrity of gels could be maintained by running much more slowly (2-4 h: 10 cm gel), which kept them cooler. However, even under these conditions, the resolution of bands in mixed sample loadings was not always clear. The use of ^{32}P in a polyacrylamide gel system was therefore preferred because of high sensitivity and resolution. In addition, 3-4 times as many samples could be assayed at any one time compared with the metaphor gel.

3.3.2.) Polymorphisms of the rat glucococrticoid receptor polyglutamine tract in 61 inbred strains and substrains

A total of 61 inbred strains and 29 of their substrains (Table 3.3.) were analysed using the method described in section 3.1.4.

Rat Strains and Sub-strains*	*Grl* allele
A2/Colle; ALC/colle; BBWB/Mol; BDII/Han; GC/Kun; MNS/Gib; MW/Hsd; SPRD/Mol; SPRD-Cu3/Han; SR/*2; SS/JrIpvc; WF/*4; WOK.1A/K	*Grl CAG21*
ACI/*2; AGUS/OlsHsd; AO/OlaHsd; AS/Ztm; AUG/OlaHsd; AVN/Orl; BN/*10; BN.lx/Cub; BP/lxCub; BS/Ztm; BUF/Han; CAP/Kuv; COP/OlaHsd; DA/Han; DZB/G; E3/Han; F344/Han; LE/Han; LEP/Cub; LEW/*12; MHS/Gib; OM/Han; PAR/Wsl; PD/Cub; PVG/OlaHsdCpb; R/*3; SD/Rij; SHD/Ztm; SDL/Ipvc; WAG/Rij; WIST/N; WKY/*2; WOK.1W/K	*Grl CAG20*
LH/Ztu; OKA/Wsl; SHR/OlaHsd; SHR-SP/RmRiv	*Grl CAG19*
Amorat/Wsl; Aristorat/Wsl; BH/Ztm; LOU/CHan	*Grl CAG17*
BC/Cpbu; BDE/Han; BDIV/Ifz; BDVII/Cub; BDIX/Han; BDX/Cub; NAR/SaU; U/A	*Grl CAG7*

Table 3.3. CAG-repeat alleles of the glucocorticoid receptor in inbred strains of *Rattus norvegicus*.
Asterisks indentify those strains or substrains which were obtained and tested from several different laboratories, this is noted by showing the 'holder' as '/*', followed by the number of different suppliers. Thus R (CAG^{20}) was obtained and tested from three separate laboratories (R/*3). A catalogue of all holders is given by Otsen, (1995).

Figure 3.10. shows a typical ^{32}P-typing gel obtained for a sample of inbred strains and substrains. In general, the clarity of PCR product bands was

better than previously seen for the typing of strains: MHS, MNS, SHR, WKY, SHR-SP_{Gla}, WKY_{Gla}, LL, LN and LH (Figs. 3.7. and 3.8.).

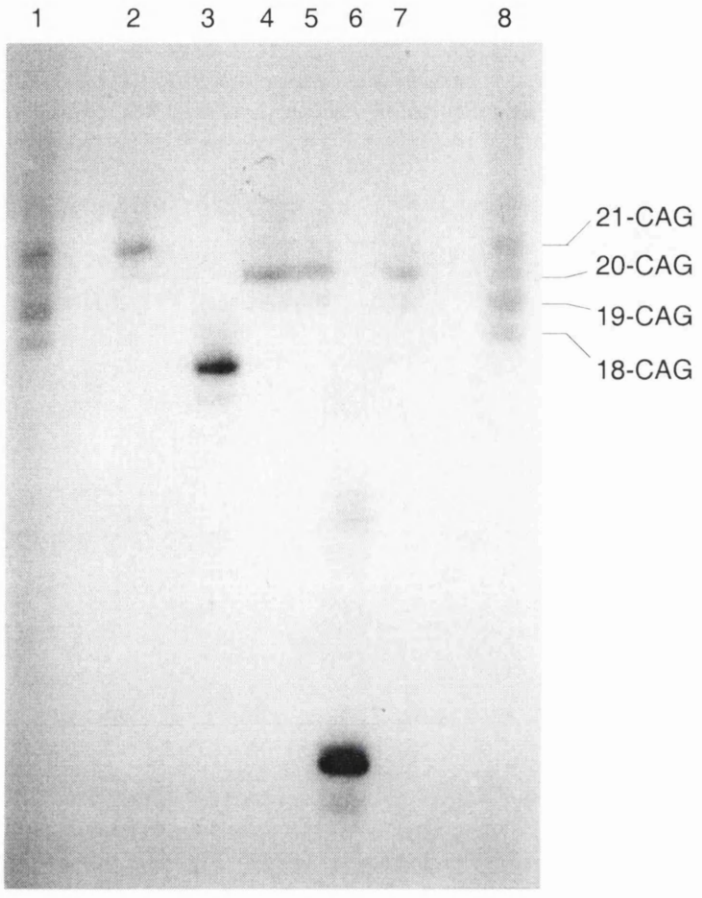

Fig. 3.10. Typical typing gel for GR polyglutamine tract lengths in inbred strains and substrains.
Rat strains: MW (lane 2), ARISTORAT (lane 3), SDH (lane 4), OM (lane 5), BDIX (lane 6) and LEW (lane 7). Lanes 1 and 8: Cloned microsatellite markers.

The majority of rat strains were reliably 'typed' by comparing PCR product migration with that from the cloned markers (lanes 1 and 8). The smallest PCR product, from rat strain BDIX (lane 3) revealed a novel PCR product size (74 bp) which could not be predicted by comparison with the markers. The length of this polyglutamine tract was determined by direct sequencing of the PCR product using pG (see Fig. 4.8.). Other 7-CAG repeat lengths (see Table 3.3.) were determined by comparison with similarly sized PCR products of previously determined sequence (results not shown). 98% of strains tested were found to be homozygous at the GR locus as expected, with polyglutamine tracts of: 7, 17, 19, 20 and 21 CAG repeats (Table 3.3. and Fig.

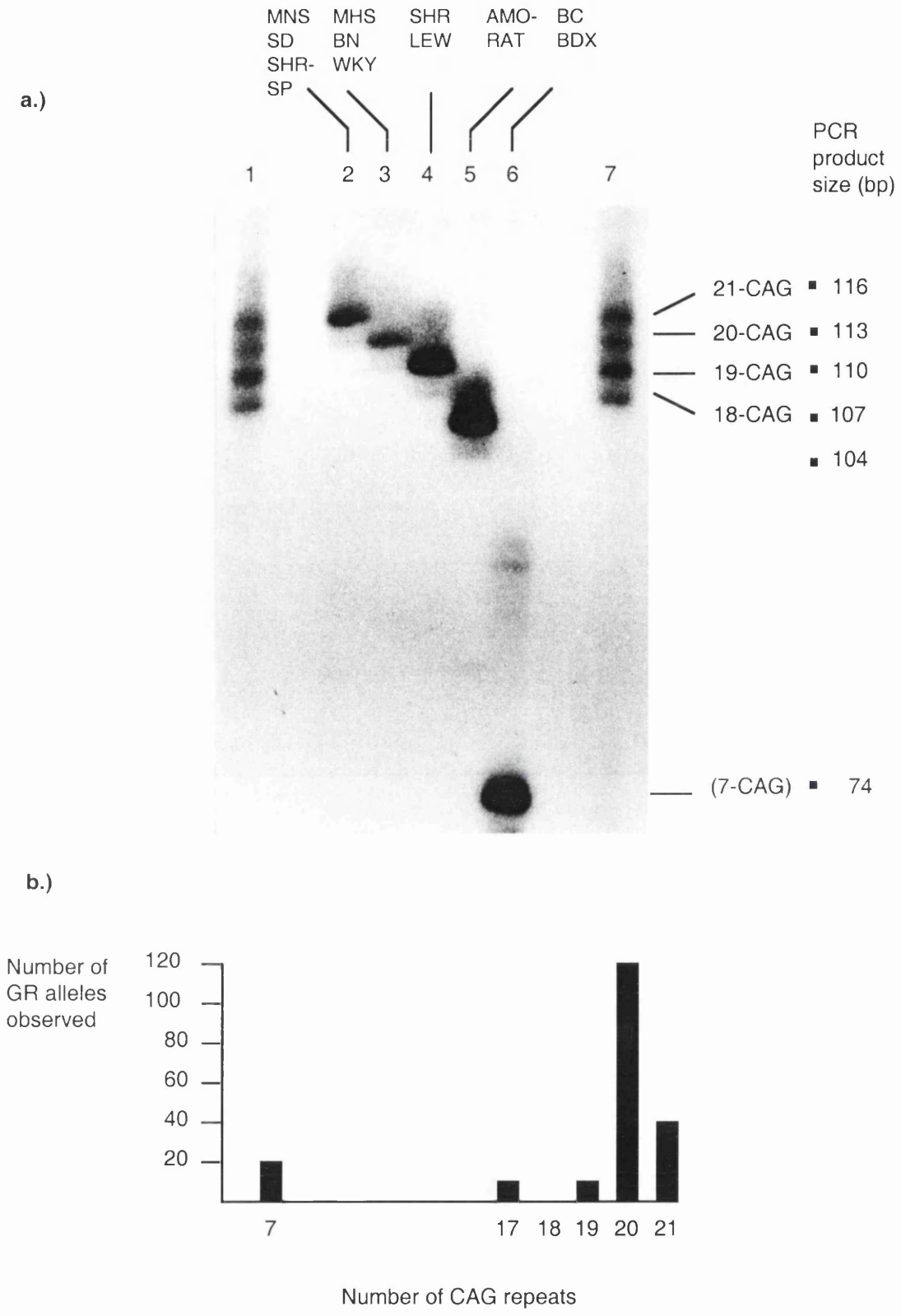

Fig. 3.11. The range of triplet $(CAG)_n$ repeat variants in inbred rat strains.
a.) Polyacrylamide typing gel showing examples of GR alleles from different rat strains. Abbreviations above each lane cite strain and substrain examples with GR triplet $(CAG)_n$ (polyglutamine) tract lengths of: 21-CAG (lane 2) 20-CAG (lane 3) 19-CAG (lane 4) 17-CAG (lane 5) and 7-CAG (lane 6). Lanes 1 and 7: previously cloned and sequenced rat GR microsatellite markers. The length of each PCR product (bp) is given to the right of the figure.
b.) Histogram showing a discontinuous distribution of GR allele triplet CAG repeat lengths.

3.11a.). Two substrains of ACI and LEW were apparently heterozygous for 20 and 21 CAG repeat alleles. However, no breeding studies have been carried out to confirm heterozygosity.

An important observation made in the 'typing' of all rat strain GR triplet $(CAG)_n$ repeats by ^{32}P-PCR was the appearence of shadow bands above and below the main PCR product band. This is a common feature of microsatellite typing by this technique (Jacob *et al.*, 1991). These artefactual bands are understood to arise from stuttering during the PCR reaction (Tautz and Schlotterer, 1994) when newly synthesised DNA strands sit down out of register with the template strand in the region of the polymeric tract (see section 3.3.1b.).

3.4.) Distribution pattern of the different GR alleles found in inbred strains

The entire range of rat GR polyglutamine tract lengths identified in the 61 inbred strains and substrains typed (section 3.3.2.) is shown in Figure 3.11a. The strain notations above each of the lanes 2-6, represent examples of rat strains and substrains with GR polyglutamine tracts of each of the respective lengths: MNS, SD and SHR-SP (21-CAG), MHS, BN and WKY (20-CAG), SHR and LEW (19-CAG), AMORAT (17-CAG) and BC and BDX (7-CAG repeats) (refer also to Table 3.3.). Seven of the strains were represented by more than one substrain, and these had consistent *Grl* CAG-genotypes.

Figure 3.11b., shows the incidence and distribution pattern of GR alleles with differing polyglutamine tract lengths. The rarest allele was the 17-CAG repeat allele, present in only 2% of individuals tested, whilst the most common was the 20-repeat allele present at 61%. No GR alleles with a polyglutamine tract length of between 8 and 16, or less than 7 or greater than 21 CAG-repeats were identified. This lead to a clear discontinuous allelic distribution.

3.5.) Discussion of *Grl* triplet $(CAG)_n$ typing results

The primary aim of this section of work was to establish genetic markers for *Grl* for use in rat breeding experments and to optimise methods for genotyping. This was achieved by identifying polymorphism in the triplet $(CAG)_n$ repeat of the rGR coding sequence. The established markers were used by the groups of Bianchi (Milan rat model, Milan) and Samani (SHR/WKY model, Leicester) in studies of linkage between *Grl* and glucocorticoid related phenotypes in rat models of hypertension, discussed in chapter 5.

An analysis of the rat GR from other strains was also undertaken to determine the extent of heterogeneity in the polyglutamine repeat region. To date, CAG repeat lengths in the rat GR of 7, 17 and 21 have been reported in the literature, the result of a minimal analysis of Sprague Dawley and Wistar rat genomic DNAs obtained from a variety of sources (Gearing *et al.*, 1993). In the body of work reported here, 61 inbred strains and substrains (a total of 90 rats) were analysed for *Grl* triplet $(CAG)_n$ repeats identifying a clear discontinuous distribution between alleles.

Knowledge of the range of natural GR triplet repeat variants would be useful in selecting the structurally more interesting alleles for studies of GR function in tissue culture and *in vivo* and hence, in choosing the appropriate rat strains for breeding experiments. Differences in GR genotypes may be used as a basis for searching for positive associations between *Grl* and glucocorticoid related phenotypes. Based on the screening of rat strains for *Grl* triplet $(CAG)_n$ repeat lengths (section 3.3.), a marked difference between two of the strains, BC (7-CAG repeat allele) and Lewis (20-CAG repeat allele) was identified. These strains are of interest because of differences in cholesterol metabolism and immune responsiveness (the immune system in the Lewis rat is compromised because of a hyposensitive HPA-axis). The possibility that these phenotypes are associated with differences in GR genotype are currently under investigation (C. Kenyon, personal communication). Finally, the distribution and incidence of the

different GR alleles may be useful as a basis for understanding the evolution of triplet $(CAG)_n$ repeat sequence in rat *Grl* (see discussion to chapter 4).

Part 2

3.6.) Methods for determining variation in glucocorticoid receptor coding sequences

The coding sequence of the glucocorticoid receptor from inbred rat strains: MHS, MNS, SHR, WKY and Zucker, lean and obese were determined from mRNA using methods of RT-PCR coupled with direct sequencing of PCR products. The objective was to compare GR sequences between the strain pairs of each model, in order to identify mutations which might explain the differences in steroid binding affinity. Importantly, the Milan and Zucker lean and obese rats used for both steroid binding analysis (Kenyon *et al.*, 1994; Panarelli *et al.*, 1995 and Kenyon, unpublished results, respectively) and primary sequence determination reported here were from the same colonies. Milan rats were obtained from stocks held in Sheffield, Zucker rats from Harlan Olac. SHR and WKY rats were from the same colony as those used by Samani in linkage studies. (see materials and methods and chapter 5 for further comment).

Total RNA was isolated from 100 mg samples of rat liver using RNAzol B, following the method outlined in section 2.2.14. The integrity of RNA routinely obtained is shown in Figure 3.13a. Approximately 5 μg samples of total RNA were reverse transcribed into complementary DNA/RNA hybrid molecules using MMLV reverse transcriptase, as described in section 2.2.3. Figure 3.13b. shows the reproducibility, between reverse transcription reactions and between rat strains. Full length reverse transcripts of the rGR coding sequence (over 2.3 kb) were initiated from the rGR gene specific antisense primer rGR1 (Appendix 1). The hybridisation site of rGR1 is shown in Figure 3.12. Pools of smaller reverse transcripts of variable

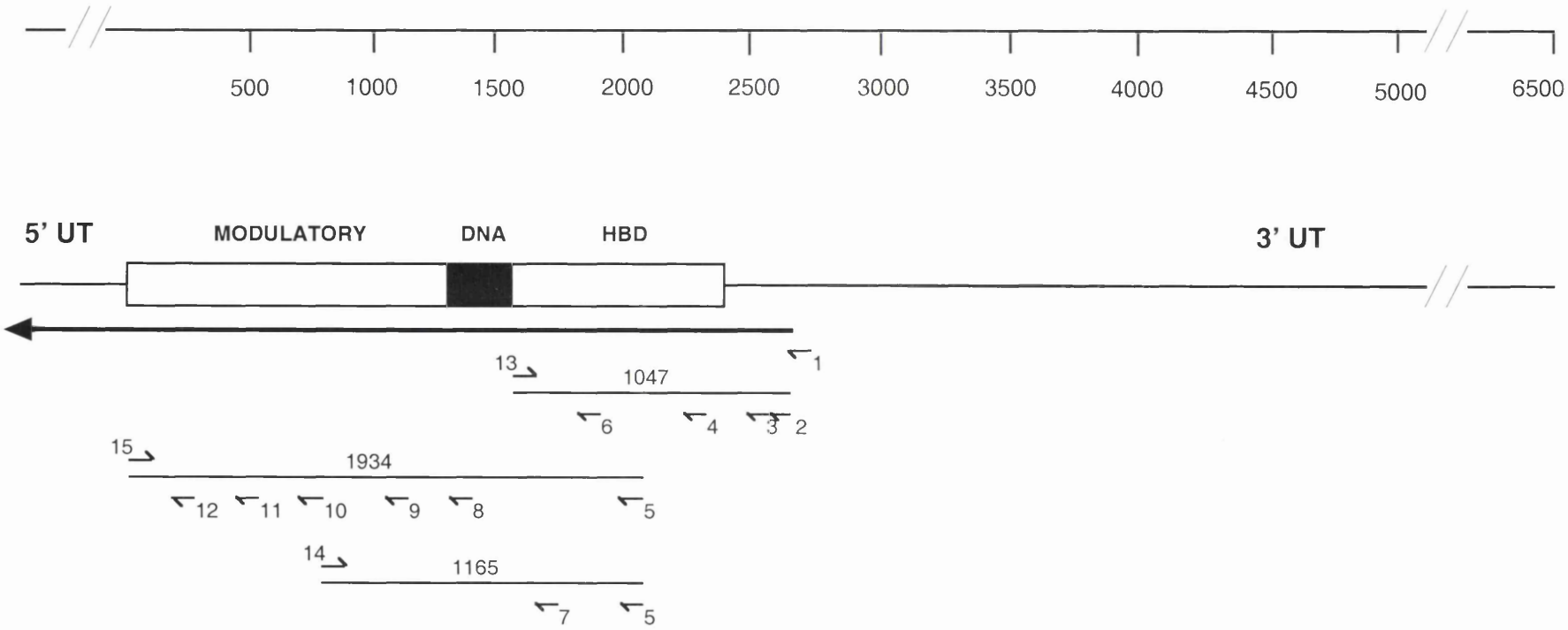

Fig. 3.12. Strategy for determination of rat glucocorticoid receptor (GR) coding sequences. The entire GR coding sequence (2385 bp) was reverse transcribed (heavy line) from total RNA using GR gene specific primer, rGR1 (1) located in the 3' UT. PCR products (thin overlapping lines), generated from RNA/cDNA hybrids were amplified using primer pairs: 13 and 2, 15 and 5, and 14 and 5 (some of which were also used for sequencing). Overlapping PCR product DNAs were sequenced using a variety of GR cDNA specific primers, designated: 1; rGR1, 2; rGR2, 3; rGR25, 4; rGR28, 5; rGR10, 6; rGR6, 7; rGR20, 8; rGR21, 9; rGR22, 10; rGR23, 11; pG, 12; rGR29, 13; rGR5-BIO, 14; rGR31-BIO, and 15; rGR19-BIO (see Appendix 1). Regions of the GR transcript were: 5' UT and 3' UT; corresponding to 5' and 3' untranslated sequences, respectively, Modulatory; modulatory domain, DNA; DNA binding domain and HBD; hormone binding domain. Numbers above each PCR product represent size in bp.

length (500 bp, up to around 1 kb), routinely used to verify small stretches of sequence, were initiated from random hexamer primers (GIBCO-BRL, U.K.) at multiple positions along the GR transcript (results not shown).

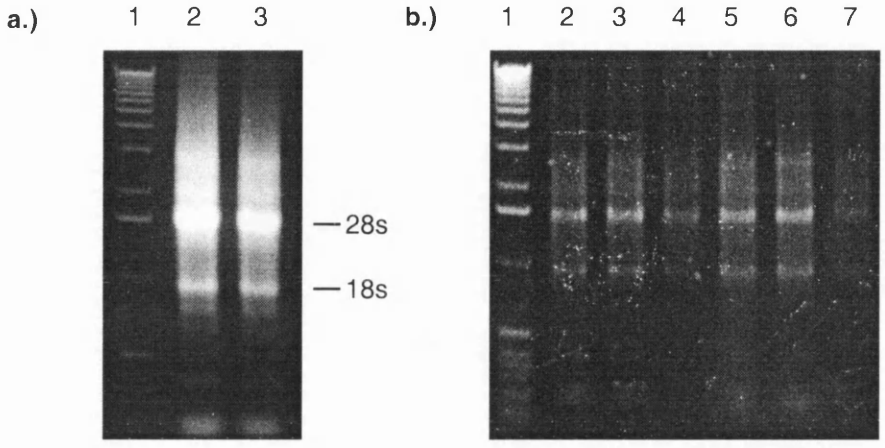

Fig. 3.13. Input and output of reverse transcription reactions.
a.) Examples of the integrity of RNA isolated from rat liver: lane 2, strain MHS; lane 3, strain MNS. In each case, 1-1.5 μg total RNA was loaded per lane. Lane 1, 1 kb DNA ladder.
b.) Duplicate reverse transcription reactions using total RNA from strains MHS (lanes 2 and 3) and MNS (lanes 5 and 6). Lanes 4 and 7 are negative, no MMLV controls. In each case, 2 μl of RT-reactions (1/25th) were loaded per lane. Lane 1, 1 kb DNA ladder.

PCR products for sequencing, derived from the reverse transcribed rGR message, were generated from a series of independent overlapping PCR primer pairs (Fig. 3.12.), which in combination covered the entire GR coding sequence. Examples of the product yield and specificity of typical RT-PCR reactions are shown in Figure 3.14.

Under conditions described in section 2.2.3., PCR products of 250-500 ng (see section 2.2.5.), were biotin labelled through the forward PCR primer. They were immobilised onto dynabeads through the 5' biotin label and denatured and sequenced from multiple positions using specific primers complementary to rGR cDNA, as described in section 2.2.13.

Sequencing of PCR amplifications from each primer set was carried out at least 2-3 times, each PCR template being derived from independent reverse transcription products. The reference notation (see Appendix 1) for each of the numbered PCR and sequencing primers described above are illustrated

in Figure. 3.12. For comparative purposes, the rat GR cDNA clone, pRBal117 (A gift from R. Miesfeld, University of Arizona, Arizona, U.S.A. [See Fig. 3.2.]), containing a previously cloned and sequenced rat GR cDNA from rat

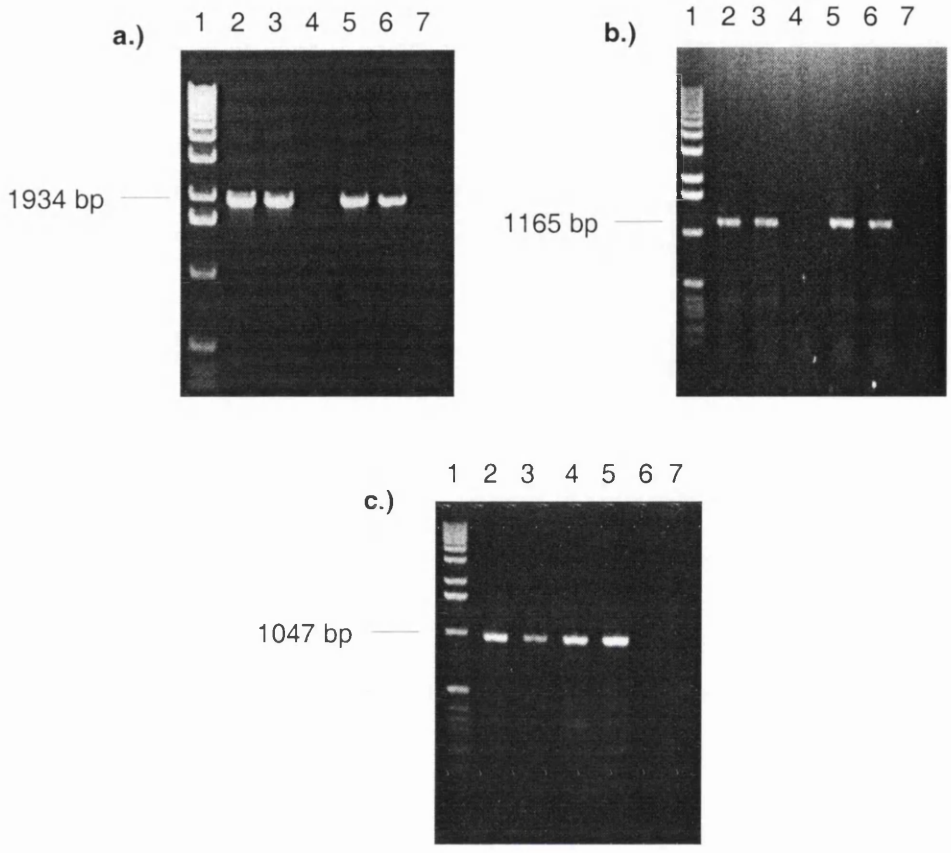

Fig. 3.14. Examples of RT-PCR products generated from rat GR RNA/cDNA hybrid templates from strains MHS and MNS.
Gels show examples of consistent, but moderate yields of high specificity RT-PCR products generated using primer pairs:
a.) rGR19-BIO and rGR10 (1934 bp product).
b.) rGR31-BIO and rGR10 (1165 bp product).
c.) rGR5-BIO and rGR2 (1047 bp product).
Product bands, each the proceeds of independent reverse transcription and PCR reactions, represent loadings of 2 µl per 25 µl RT-PCR. Samples on each gel are loaded in pairs for each rat strain: MHS, lanes 2 and 3 (a., b. and c.); MNS, lanes 5 and 6 (a. and b.); lanes 4 and 5 (c.). Lanes: 4, (a. and b.) and 6 (c.) MHS, no MMLV negative controls. Lanes: 7 (a., b. and c.) MNS, no MMLV negative controls (refer to text for details). Lanes: 1 (a., b. and c.), 1 kb DNA ladder.

hepatoma cell line 6.10.2 (Miesfeld *et al.*, 1986), was re-sequenced using the same methods, PCR and sequencing primers as those used to sequence the GR from other rat strains.

Negative controls, the purpose of which were to monitor for the possibility of contamination of RNA with DNA sequences were used in every set of reverse transcription reactions (for examples, see Fig. 3.14.). Contamination by DNA was considered to be from two possible sources; either genomic DNA from liver cells from the RNA preparation process (not usually a problem when sequencing across several spliced intron/exon boundaries from large multi-exon genes such as GR; roughly 80 kb in human (Encio and Detera-Wadleigh, 1991) to over 100 kb in the rat (Jacobson, 1991), or from contaminating plasmid DNA containing cloned GR sequences. Controls routinely gave negative results in PCR. Where contamination arose, all components, including the MMLV reverse transcriptase were replaced.

3.7.) Results

3.7.1.) Nucleotide sequence differences in the rat glucocorticoid receptor

Sequencing of the glucocorticoid receptor coding sequence from rat strains: MHS, MNS, SHR and WKY, identified two types of mutation.

a.) *The polyglutamine repeat*

The CAG repeat, n223-288 in the modulatory domain of rGR (nucleotide coordinates relative to met1: taken from the published cDNA sequence of Miesfeld *et al.*, 1986) showed length differences of one codon between MHS (20-CAG) and MNS (21-CAG) and SHR (19-CAG) and WKY (20-CAG) (Fig. 3.15.). No difference was found in the triplet $(CAG)_n$ repeat length between Zucker lean and obese strains.

b.) *Nucleotide substitutions*

In addition to differences in the length of the polyglutamine tract, comparison of nucleotide sequences between these rat strains and rat GR cDNA clone pRBal117 revealed three silent transitional mutations at nucleotide positions: n 198 (TTC -> $TTT)_{Phe66}$, n 531 (TTT -> $TTC)_{Phe177}$ and n

711 (GAT -> GAC)$_{Asp237}$. These three point mutations were found in the hypertensive strains, MHS and SHR (see Figs. 3.16., 3.17., 3.18. and 3.21.).

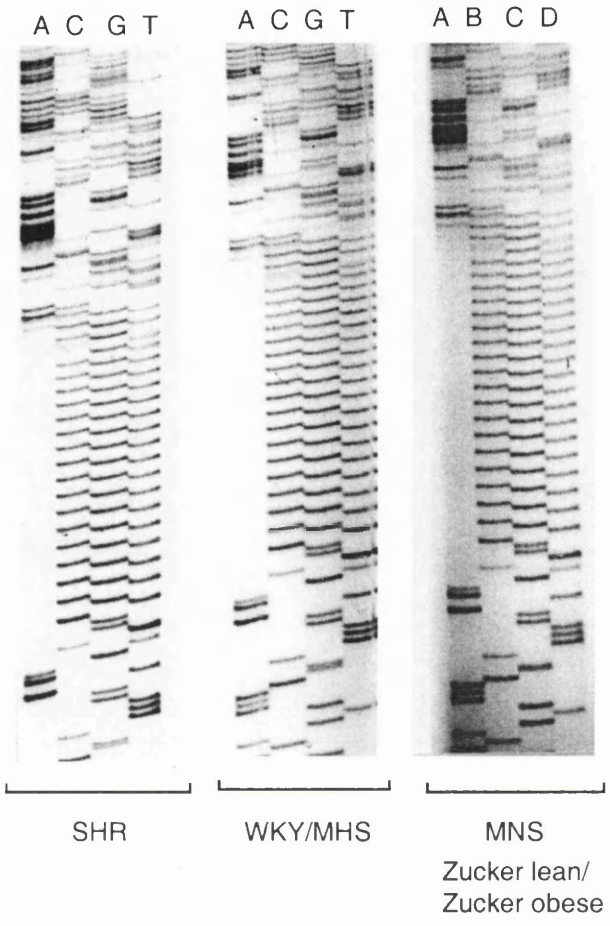

Fig 3.15. Polymorphisms of the GR polyglutamine tract between strains of the rat models of human essential hypertension: MHS/MNS and SHR/WKY.
Triplet $(CAG)_n$ repeat numbers identified were: 19 (SHR), 20 (WKY and MHS) and 21 (MNS). Letters above each sequence lane represent terminating nucleotides. Sequences shown are of the antisense DNA strand, reading 5' -> 3', top to bottom. Sequences were initiated from primer pG (Appendix 1).

None were present in MNS, which in turn was identical in sequence to the cloned rat GR cDNA of pRBal117. Arbitrarily, the MNS GR sequence was taken as the base, or wild type sequence, against which all others were compared (Fig. 3.16). The strain WKY, was mutated only at the third nucleotide position: n 711 (Figs. 3.16. and 3.18.).

No coding sequences differences were identified between the Zucker lean and obese strains, which in turn were identical to that of the MNS strain (see Fig. 3.16.).

```
MNS                                                                                                  gggc tcacattaat atttgccaAT GGACTCCAAA  12
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GAATCCTTAG CTCCCCCTGG TAGAGACGAA GTCCCTGGCA GTTTGCTTGG CCAGGGGAGG GGGAGCGTAA TGGACTTTTA  92
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TAAAAGCCTG AGGGGAGGAG CTACAGTCAA GGTTTCTGCA TCTTCGCCCT CAGTGGCTGC TGCTTCTCAG GCAGATTCCA  172
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AGCAGCAGAG GATTCTCCTT GATTTCTCGA AAGGCTCCAC AAGCAATGTG CAGCAGCGAC AGCAGCAGCA GCAGCAGCAG  252
MHS   ---------- ---------- -----T---- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- -----T---- ---------- ---------- ---------- ---------- ----------

MNS   CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG CAGCAGCCAG ACTTATCCAA AGCCGTTTCA CTGTCCATGG GGCTGTATAT  332
MHS   ---------- ---------- ---------- ---    ---- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---    ---- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ----------        ---- ---------- ---------- ---------- ----------

MNS   GGGAGAGACA GAAACAAAAG TGATGGGGAA TGACTTGGGC TACCCACAGC AGGGCCAACT TGGCCTTTCC TCTGGGGAAA  412
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   CAGACTTTCG GCTTCTGGAA GAAAGCATTG CAAACCTCAA TAGGTCGACC AGCGTTCCAG AGAACCCCAA GAGTTCAACG  492
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TCTGCAACTG GGTGTGCTAC CCCGACAGAG AAGGAGTTTC CCAAAACTCA CTCGGATGCA TCTTCAGAAC AGCAAAATCG  572
MHS   ---------- ---------- ---------- --------C- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- --------C- ---------- ---------- ---------- ----------

MNS   AAAAAGCCAG ACCGGCACCA ACGGAGGCAG TGTGAAATTG TATCCCACAG ACCAAAGCAC CTTTGACCTC TTGAAGGATT  652
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TGGAGTTTTC CGCTGGGTCC CCAGGTAAAG ACACAAACGA GAGTCCCTGG AGATCAGATC TGTTGATAGA TGAAAACTTG  732
MHS   ---------- ---------- ---------- ---------- ---------- --------C- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- --------C- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- --------C- ---------- ----------

MNS   CTTTCTCCTT TGGCGGGAGA AGATGATCCA TTCCTTCTCG AAGGGGACAC GAATGAGGAT TGTAAGCCTC TTATTTTACC  812
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GGACACTAAA CCTAAAATTA AGGATACTGG AGATACAATC TTATCAAGTC CCAGCAGTGT GGCACTGCCC CAAGTGAAAA  892
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   CAGAAAAAGA TGATTTCATT GAACTTTGCA CCCCCGGGGT AATTAAGCAA GAGAAACTGG GCCCAGTTTA TTGTCAGGCA  972
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AGCTTTTCTG GGACAAATAT AATTGGTAAT AAAATGTCTG CCATTTCTGT TCATGGTGTG AGTACCTCTG GAGGACAGAT  1052
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GTACCACTAT GACATGAATA CAGCATCCCT TTCTCAGCAG CAGGATCAGA AGCCTGTTTT TAATGTCATT CCACCAATTC  1132
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   CTGTTGGTTC TGAAAACTGG AATAGGTGCC AAGGCTCCGG AGAGGACAGC CTGACTTCCT TGGGGGCTCT GAACTTCCCA  1212
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GGCCGGTCAG TGTTTTCTAA TGGGTACTCA AGCCCTGGAA TGAGACCAGA TGTAAGCTCT CCTCCATCCA GCTCGTCAGC  1292
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AGCCACGGGA CCACCTCCCA AGCTCTGCCT GGTGTGCTCC GATGAAGCTT CAGGATGTCA TTACGGGGTG CTGACATGTG  1372
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GAAGCTGCAA AGTATTCTTT AAAAGAGCAG TGGAAGGACA GCACAATTAC CTTTGTGCTG GAAGAAACGA TTGCATCATT  1452
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GATAAAATTC GAAGGAAAAA CTGCCCAGCA TGCCGCTATC GGAAATGTCT TCAGGCTGGA ATGAACCTTG AAGCTCGAAA  1532
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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MNS   AACAAAGAAA AAAATCAAAG GGATTCAGCA AGCCACTGCA GGAGTCTCAC AAGACACTTC GGAAAATCCT AACAAAACAA  1612
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TAGTTCCTGC AGCATTACCA CAGCTCACCC CTACCTTGGT GTCACTGCTG GAGGTGATTG AACCCGAGGT GTTGTATGCA  1692
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GGATATGATA GCTCTGTTCC AGATTCAGCA TGGAGAATTA TGACCACACT CAACATGTTA GGTGGGCGTC AAGTGATTGC  1772
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AGCAGTGAAA TGGGCAAAGG CGATACCAGG CCTGAGAAAC TTACACCTGG ATGACCAAAT GACCCTGCTA CAGTACTCAT  1852
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   GGATGTTTCT CATGGCATTT GCCCTGGGTT GGAGATCATA CAGACAATCA AGTGGAAACC TGCTCTGCTT TGCTCCTGAT  1932
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   CTGATTATTA ATGAGCAGAG AATGTCTCTA CCCTGCATGT ATGACCAATG TAAACACATG CTGTTTGTCT CCTCTGAATT  2012
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   ACAAAGATTG CAGGTATCCT ATGAAGAGTA TCTCTGTATG AAAACCTTAC TGCTTCTCTC CTCAGTTCCT AAGGAAGGTC  2092
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TGAAGAGCCA AGAGTTATTT GATGAGATTC GAATGACTTA TATCAAAGAG CTAGGAAAAG CCATCGTCAA AAGGGAAGGG  2172
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AACTCCAGTC AGAACTGGCA ACGGTTTTAC CAACTGACAA AGCTTCTGGA CTCCATGCAT GAGGTGGTTG AGAATCTCCT  2252
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   TACCTACTGC TTCCAGACAT TTTTGGATAA GACCATGAGT ATTGAATTCC CAGAGATGTT AGCTGAAATC ATCACTAATC  2332
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   AGATACCAAA ATATTCAAAT GGAAATATCA AAAAGCTTCT GTTTCATCAA AAATGActgc cttactaaga aaggttgcct  2412
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

MNS   taaagaaagt                                                                          2422
MHS   ---------- ---------- ---------- ---------- ---------- ---------- ----------
SHR   ---------- ---------- ---------- ---------- ---------- ---------- ----------
WKY   ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

Fig. 3.16. Alignment of rat glucocorticoid receptor (GR) nucleotide sequences.
Coding sequences of GR derived from RNA/cDNA hybrids from strains: MHS, MNS, SHR and WKY are shown. The base GR sequence (2422 bp) is that of strain MNS, which was found to be identical to rat GR cDNA clone pRBal117 (Miesfeld *et al.*, 1986), in which a number of mistakes were identified (letters shown in bold type). Gaps (line 332) represent missing glutamine codons, resulting in a shortening of respective polyglutamine tracts and presumably translated GR protein. Upper case lettering in bold type identify the sites of silent point mutations. Lower case lettering in bold type: 5' and 3' untranslated sequences. Codons underlined show the major start and stop codons of the full length receptor protein. Numbers to the right hand side represent nucleotide positions in relation to the ATG start site (line 12).

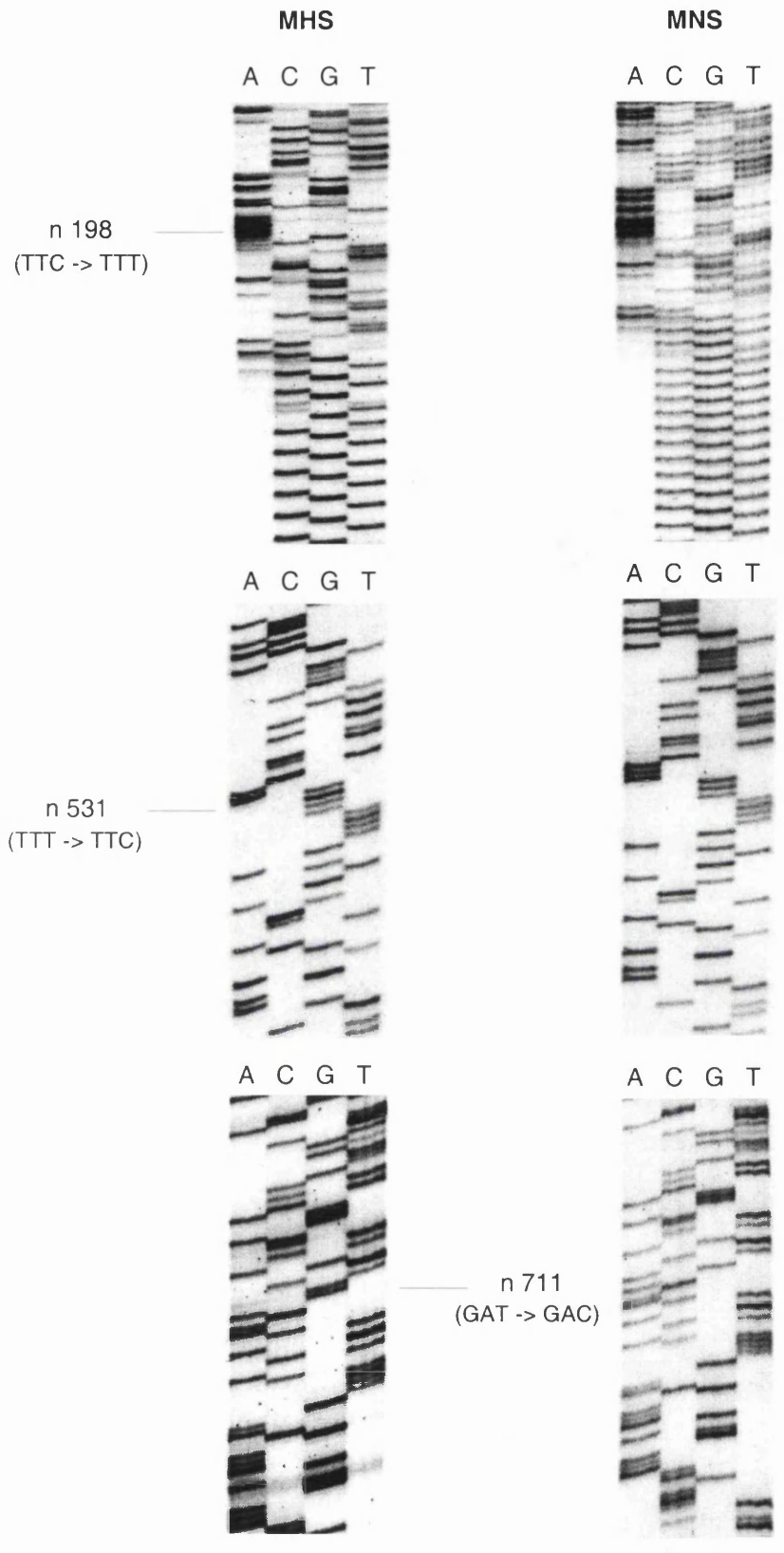


Fig. 3.17. Nucleotide sequence differences in the glucocorticoid receptor between rat strains: MHS and MNS.
Three silent point mutations were identified in the GR coding sequence of hypertensive rat strain MHS (sequences on the left) at nucleotide positions:
a.) n 198, **b.)** n 531 and **c.)** n 711. None of these mutations were present in the normotensive control strain MNS (sequences on the right).

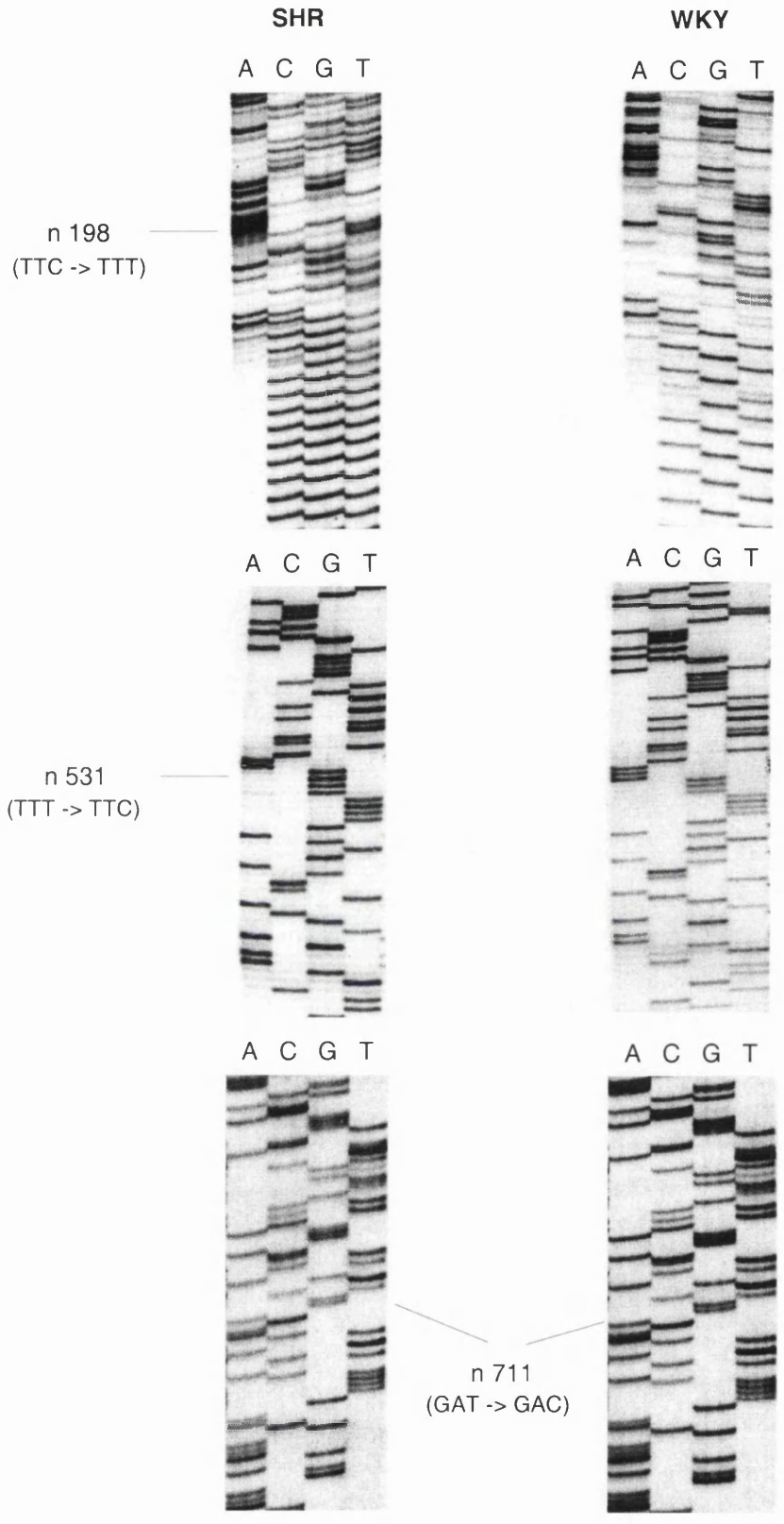


Fig. 3.18. Nucleotide sequence differences in the glucocorticoid receptor between rat strains: SHR and WKY.
Three silent point mutations were identified in the GR coding sequence of hypertensive rat strain SHR (sequences on the left) at nucleotide positions:
a.) n 198, **b.)** n 531 and **c.)** n 711. One of these mutations (n 711) was found to be present in the normotensive control strain WKY (sequences on the right).

Sequencing across longer CAG repeats (e.g., from pG: Appendix 1) produced sequence which was often stuttered in appearance; compare upper blocks of sequence in Figs. 3.17. and 3.18. with Fig. 3.19a., derived from a GR 7-repeat allele (see section 3.2.5. and chapter 4 for further details of rats with *Grl*^{CAG7} alleles). Point mutations at nucleotide position n198 (in MHS and SHR) were therefore verified by sequencing across the opposite (sense) DNA strand from p9, detailed in Appendix 1 (Fig. 3.19b.).

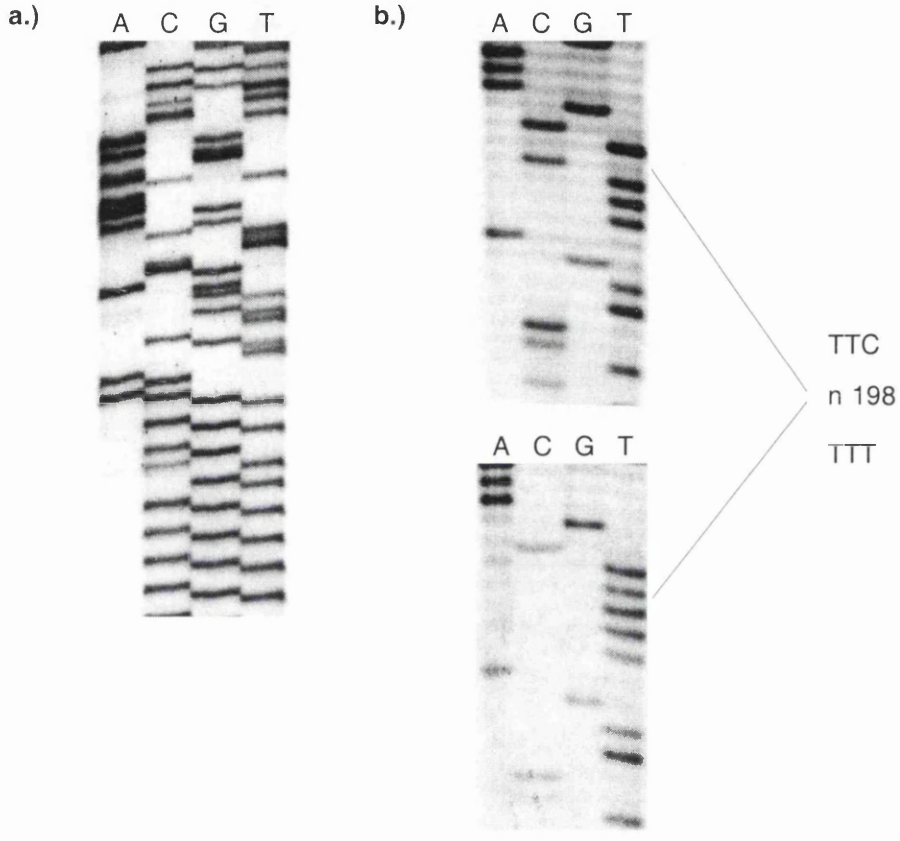


Fig. 3.19. Clarification of GR sequences.
a.) The clarification of sequence above the rGR trinucleotide repeat derived from a *Grl*^{CAG7} rat strain (see text for details).
b.) Varification of silent nucleotide substitution n198, by sequencing of opposite (sense) DNA strand (orientation is 5' -> 3', top to bottom). Top sequence set, strain MNS; bottom sequence set, strain MHS. Letters above each sequence lane represent terminating nucleotides.

c.) *Errors in the Miesfeld sequence*

Re-sequencing of pRBal117 in duplicate revealed 10 nucleotide differences when compared to the published sequence (Miesfeld *et al.*, 1986), which was also derived, in part, from pRBal117. All of these differences were present

in each rat strain and were therefore regarded as genuine sequencing errors.

These errors may have arisen in the original sequence because of technological difficulties at the time sequencing was carried out (1985-1986). Sequencing was from M13 clones (S. Rusconi, personal communication) which require more complicated sequencing protocols than those currently available. The differences identified are listed in Table 3.4.

Five of the nucleotide differences identified in pRBal117: n 293 (G -> A), n 676 (A -> G), n 778 (A -> G), n 1799 (T -> C) and n 1804 (G -> C), predicted a change in amino acid at the corresponding position in the rGR protein. These were, respectively: Gly_{98} -> Asp_{98}, Ser_{226} -> Gly_{226}, Asn_{260} -> Asp_{260}, Leu_{600} -> Pro_{600} and Leu_{602} -> Phe_{602} (Figs. 3.20. and 3.21.).

Position (in nucleotides) relative to met1	New codon sequence	Change in amino acid
66	CAA -> CAG	
293	GGC -> GAC	Gly -> Asp
676	AGT -> GGT	Ser -> Gly
778	AAC -> GAC	Asn -> Asp
879	CTA -> CTG	
1799	CTA -> CCA	Leu -> Pro
1804	TTG -> TTC	Leu -> Phe
1821	CTC -> CTG	
1876	TTG -> CTG	
1905	AGC -> AGT	

Table 3.4. Errors identified in rat GR cDNA clone pRBal117. The rat GR cDNA of clone pRBal117 (Miesfeld *et al.*, 1986) was re-sequenced in duplicate for the purpose of comparison with novel GR sequence obtained from rat strains: MHS, MNS, SHR and WKY. Inconsistencies with the published sequence were identified at 10 nucleotide positioins which are recorded relative to met 1 in the GR coding sequence.

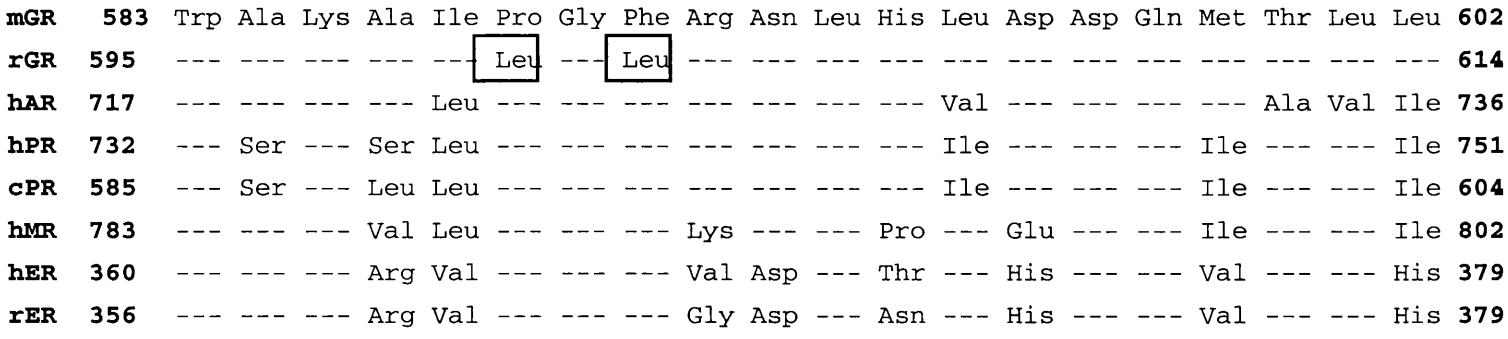


Fig. 3.20. Region of homology in the hormone binding domain of selected members of the steroid hormone receptor superfamily (taken from Danielsen *et al.*, 1986). Dashes represent identity with the mouse GR (mGR) sequence. The same receptor type from different species is shown, only if the sequences differ in this region. Prefixes: m (mouse), r (rat), h (human) and c (chicken) denote species specificity. The two boxed leucines indicate those residues with mistaken identity in the published rat GR sequence (Miesfeld *et al.*, 1986). When corrected, (Pro$_{600}$ substituted for Leu$_{600}$ Phe$_{602}$ for Leu$_{602}$) the rat sequence in this domain shows complete identity with both mouse and human GRs.

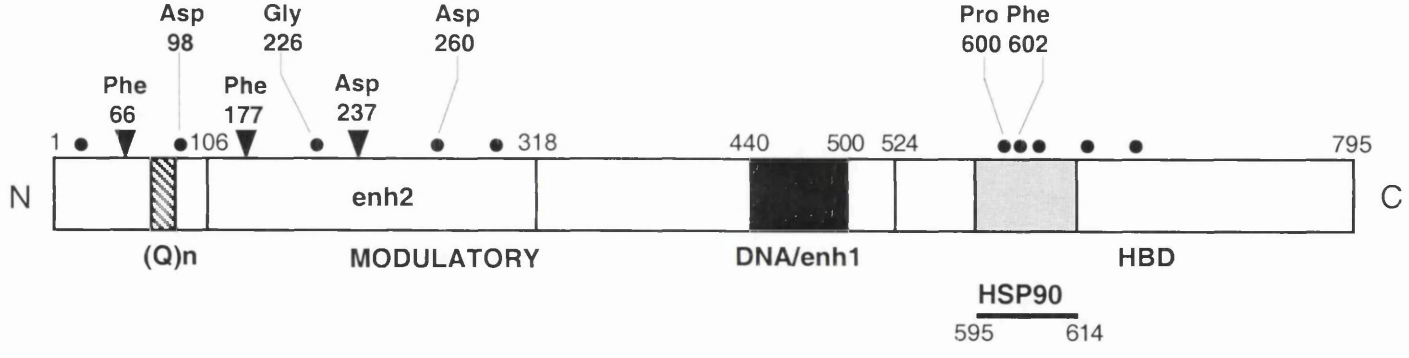


Fig. 3.21. Localisation of silent mutations and sequencing errors in the rat glucocorticoid receptor protein.
Silent point mutations in the GR coding sequence from rat strains: MHS, SHR and WKY are located by arrowheads. These were identified at positions: n 198 (Phe), n 531 (Phe) and n 711 (Asp) flanking the polyglutamine tract. All three mutations were present in the hypertensive strains MHS and SHR. In normotensive control strains, mutations were absent apart from n 711, present in WKY. Polyglutamine tracts (hatched box: residues 75-96, in the MNS strain) were polymorphic between MHS (20-CAG) and MNS (21-CAG) and SHR (19-CAG) and WKY (20-CAG). Heavy dots mark the positions of sequencing errors identified in the published sequence of the rat GR cDNA (Miesfeld et al., 1986), five of which: n 293 (G -> A)^{Asp98}, n 676 (A -> G)^{Gly226}, n 778 (A -> G)^{Asp260}, n 1799 (T -> C)^{Pro600} and n 1804 (G -> C)^{Phe602} predict a change in the encoded amino acid in the GR protein (presented with the correction above the amino acid position). Functional domains are: Modulatory; modulatory domain, DNA/enh1; DNA binding and transcriptional enhancer (1) domain, HBD; hormone binding domain, enh2; transcriptional enhancer domain (2). Stippled region marks the position (residues 595-614) of the HBD bound by HSP90 and conserved in all members of the steroid receptor superfamily (refer to Fig. 3.20).

3.8.) Discussion of sequencing results

The reason for this section of work was to try to find an explanation for the apparent differences in steroid binding affinities of GR between the strains of the rat models of hypertension: MHS/MNS and SHR/WKY, as reported by Kenyon *et al.*, (1994) and Panarelli *et al.*, (1995). Structural mutations in GR would support its possible involvement (and hence, of the glucocorticoid receptor locus [*Grl*]) as a candidate gene in the development of the hypertensive phenotype. Glucocorticoid receptor coding sequences from inbred rat strains: MHS, MNS, SHR and WKY, which to my knowledge have not been reported elsewhere, identified three point mutations in the hypertensive strains MHS and SHR, which were absent in the normotensive controls (apart from mutation n 711 in strain WKY). However, all point mutations were in the wobble codon position and were silent (Fig. 3.16.). Thus, from single nucleotide substitutions, no change in GR function would be predicted. These mutations were used as genetic markers for rat GR haplotype analysis, described in chapter 4.

Polymorphism was also found in the triplet $(CAG)_n$ repeat of the GR coding sequence, which could be of potential importance for GR function. Polymeric tracts consisting of repeat codons for glutamine, or glutamine rich sequences are a common feature of several transcription factors (Ross *et al.*, 1993; Gerber *et al.*, 1994). Many belong to the steroid receptor superfamily, of which the rat glucocorticoid receptor (rGR) and human androgen receptor (hAR) have been most intensely studied. Both have a variable length polyglutamine tract in the amino terminal domain and repeat expansions in the androgen receptor are well established (LaSpada *et al.*, 1992). The idea that relatively long polyglutamine tracts (20 residues or more) may be implicated in the functioning of transcriptional regulators is supported by earlier studies involving the transcription factors Sp1 and the human TATA binding protein TFIID (Courey and Tjian, 1988; Kao *et al.*, 1990) for which a possible role in gene regulation has been assigned.

Because of the potential functional significance of polyglutamine tracts, particularly at the level of gene transcription and also, in the case of GR, in the binding of hormone, further investigations of the possible effects of polyglutamine tract length on rGR function (steroid binding properties, detailed in chapter 6 and transcriptional regulatory properties) were considered. The coding sequence of GR from Zucker lean and obese rats was identical at all positions and the same as that of strain MNS. This suggests that differences in receptor activity in the Zucker strains *in vivo* must depend on influences other than on primary receptor sequence.

Re-sequencing of the rat GR cDNA clone pRBal117 identified ten sequencing errors when compared to the original report of Miesfeld *et al.*, (1986). These differences were confirmed in reverse transcribed PCR amplified sequence from RNA of rat strains MHS, MNS, SHR, WKY and Zucker lean and obese. All novel rat GR sequences identified in this section of work have been submitted to the EMBL sequence databases under the accession number Y12264. These results lead to five coding changes in the published rGR sequence (Miesfeld *et al.*, 1986), Gly_{98} being amended to Asp_{98}, Ser_{226} to Gly_{226}, Asn_{260} to Asp_{260}, Leu_{600} to Pro_{600} and Leu_{602} to Phe_{602} (Fig. 3.16. and 3.21.). Two of these differences, Leu_{600} to Pro_{600} and Leu_{602} to Phe_{602} map within a part of the hormone binding domain that is conserved among all other members of the nuclear hormone receptor family, and is implicated in the binding of HSP90 (Danielsen, 1986: see Figs. 3.20. and 3.21.). Amino acid insertions within this subdomain (corresponding to residues 595-614 in rat GR) disrupt hormone binding (Giguere *et al.*, 1986). Deletion of this region results in a protease-sensitive receptor which associates only weakly with HSP90 and has some constitutive transcriptional activity (Housely *et al.*, 1990). Figure 3.20. depicts the amino acid sequence of this domain as originally reported by Danielsen, (1986). These amendments lead to residues 595-614 of rat GR showing a complete conservation of amino acid sequence with GR of mouse and human, and a lower but still extensive homology with other members of the nuclear hormone receptor family, and confirms its functional significance.

Chapter 4
Results 2

Typing of *Grl* triplet $(CAG)_n$ repeat lengths in wild rats and the determination of *Grl* haplotypes in selected wild rats and inbred strains

4.1.) Introduction

Through PCR analysis of the number of CAG codons, myself and others have found strain variation in the length of the polyglutamine tract of rat GR (Gearing *et al.*, 1993; Heeley *et al.*, 1996a). The data for inbred strains is presented in chapter 3 of this thesis. It was therefore of interest to determine whether the discontinuous distribution of GR CAG-repeat alleles seen in inbred rat strains was an accurate representation also found in unselected rats, or an artefact resulting from the chance fixation of GR alleles through random choice of strains for breeding. I therefore typed 155 wild *R. norvegicus* from Scotland and England.

As there was no evidence of a structural mutation in the coding sequence of GR from MHS or other rat strains which might explain the suggested differences in glucocorticoid receptor activity or phenotype between strain pairs, the likelyhood of genetic variation elsewhere in *Grl* was considered. Part of this section of work was therefore based on a haplotype analysis centred on the genetic variation in and around the CAG repeat of rGR, both in the Milan strains (for which linakge analysis was available. See Chapter 5) and in a range of other inbred and wild rats.

4.2.) Methods for determination of *Grl* genotypes

4.2.1.) Typing of *Grl* triplet $(CAG)_n$ repeats

Tissue samples and preparation of genomic DNA

English wild *R. norvegicus* liver samples were obtained from the Central Science Laboratory, Ministry of Agriculture Fisheries and Food (MAFF), Slough, Berkshire. Scottish wild *R. norvegicus* were collected from local

regional pest control departments of Scottish district councils by periodic donation over a number of weeks.

The origin of each set of rat samples is shown in Figure 4.1. The following shorthand was adopted for reference to each location: St (Stirling); Fk (Falkirk); Fi (Fife); Gla (Glasgow); Be (Berkshire); Ha (Hampshire); Sh (Shropshire); Su (Sussex); Wi (Wiltshire); Ox (Oxfordshire); Es (Essex); Yo (Yorkshire); Do (Dorset). Numbers accompanying each notation in the remainder of this chapter refer to individual rats from the corresponding location. Genomic DNA was prepared from liver tissue using methods referenced in (section 2.2.2.). As much care as possible was taken to avoid unnecessary shearing and to generally maintain the intactness of DNA from all locations. DNA samples in 0.5x TE (pH 7.6) were left to resuspend over 2-3 days at 4^{o}C. Examples of the integrity of samples is shown in Figure 4.2. There was variability in the level of intactness of DNA from different locations in England. For example, DNA from Hampshire (Fig. 4.2a., lanes 2-11) was generally more degraded than that from Dorset (Fig. 4.2b., lanes 2-11). Some samples showed extreme degradation (e.g., Fig. 4.2a, lanes 3, 5 and 11) which were considered unsuitable because of likely effect on the efficiency of PCR amplification. Alternatively, some of these rat tissues may have contained contaminants which were ineffectively removed by phenol/chloroform extraction (see below). As a result, not all rats could be typed for *Grl* CAG repeats. Nine to ten rats were originally obtained for each site in England, a total of 94 animals, only 61 of which were typable. In contrast, all Scottish rats were typed successfully (see Table 4.1.). The degradation of genomic DNA was probably related to the time after death before which rat carcasses were collected. (E. Gill MAFF; personal communication). Many of the tissue samples obtained from MAFF came from rats which had been killed by Warfarin poisoning.

DNA loadings per lane (Fig. 4.2a. and b.) were of the order, 0.5-1.0 μg, measured using a spectrophotometer. Apparent differences in DNA loadings between lanes was most likely due to either: inconsistencies in spectrophotometer readings, resulting from differences in the degree of homogeneity of DNA samples, or more likely, because of differences in the

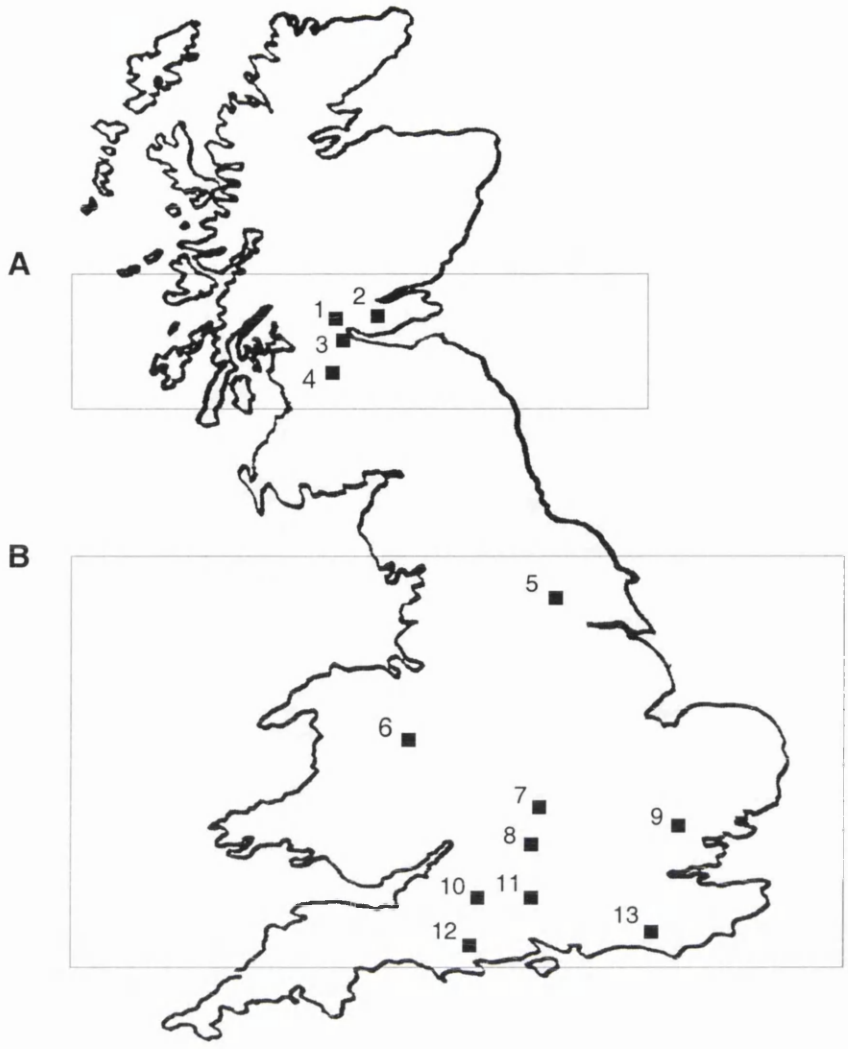


Fig. 4.1. Origins of wild rats used for typing of *Grl* triplet $(CAG)_n$ repeat variants: A, from Scotland; B, from England.
The county, or town (where known) of individual sample sites are shown: 1, Stirling; 2, Fife; 3, Falkirk; 4, Glasgow; 5, Yorkshire; 6, Shropshire; 7, Oxfordshire; 8, Berkshire; 9, Essex; 10, Wiltshire; 11, Hampshire; 12, Dorset; 13, Sussex.

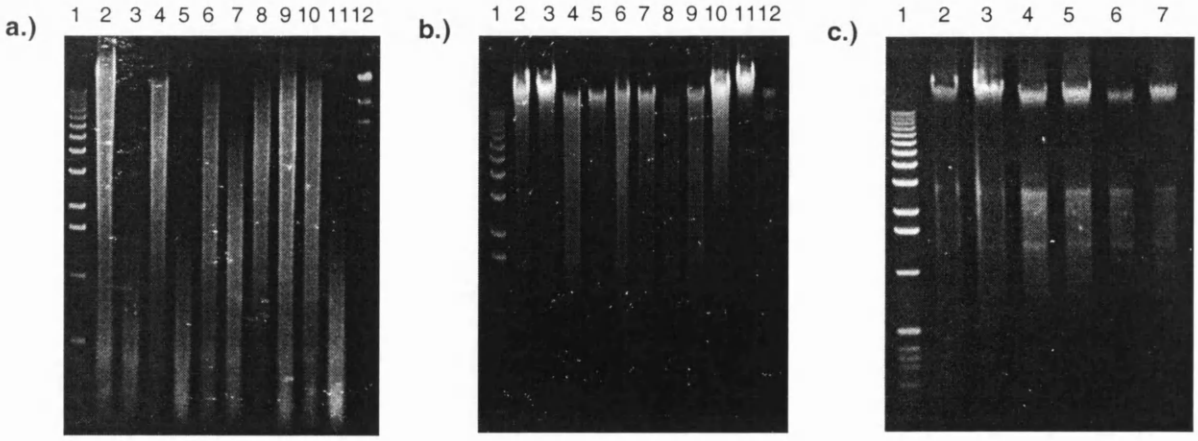


Fig. 4.2. Samples of wild rat genomic DNA from different geographical locations resolved on 0.8% agarose gels.
a.) Hampshire rat DNAs (lanes 2-11), 0.5-1 μg/lane.
b.) Dorset rat DNAs (lanes 2-11), 0.5-1 μg/lane.
c.) Random sample of Glasgow rat DNAs (lanes 2-7), 200-400 ng/lane.
See text for details of sample loadings per lane. Lanes 1 (a, b, c), 1 Kb DNA ladder (GIBCO-BRL). Lanes 12 (a and b), *Hind*III DNA size markers.

intactness of DNA samples. For the extraction of Scottish rat DNAs, where carcasses were much fresher (in the majority of cases, no older than 1-2 days prior to DNA extraction) the integrity of DNA samples was much higher. Fig. 4.2c. shows a random selection of DNAs prepared from Glasgow rats. DNA loaded per lane was of the order, 200-400 ng.

Typing of Grl triplet $(CAG)_n$ repeats

The conditions and PCR primers used for typing wild rat *Grl* triplet $(CAG)_n$ repeats were initially as described in chapter 3 for inbred strains.

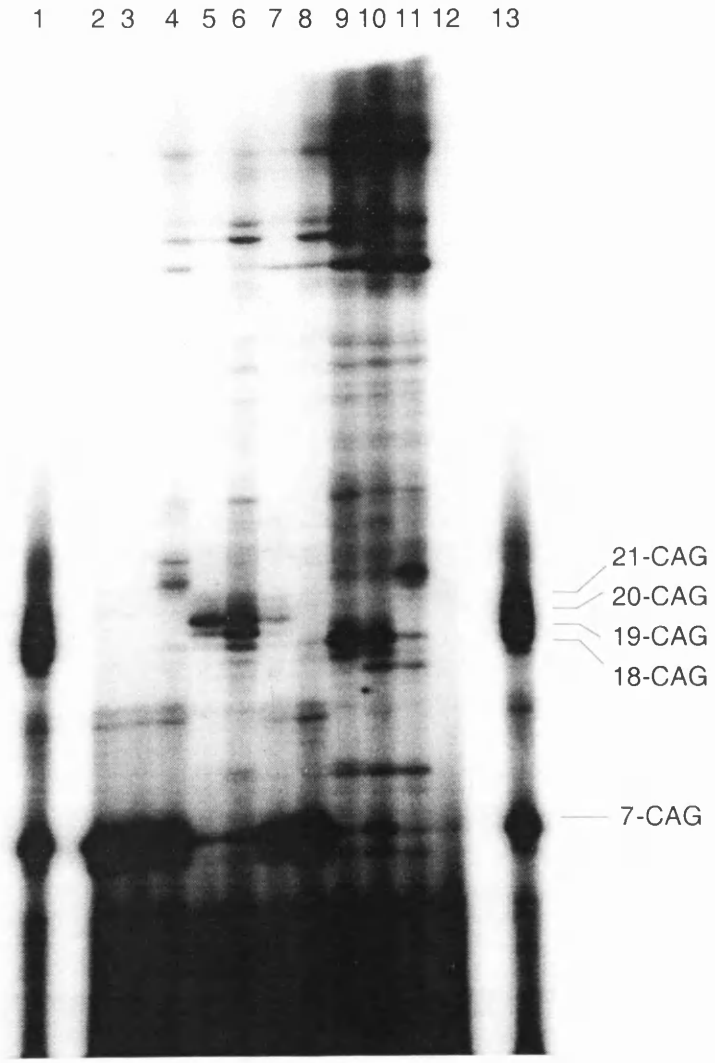


Fig 4.3. Pilot experiment showing the typing of *Grl* triplet $(CAG)_n$ repeats in a sample of wild rats.

DNA samples were initially amplified using exactly the same conditions as those described in chapter 3 for inbred strains. Amplifications were as follows: lane 2, Do91; lane 3, Do92; lane 4, Do94; lane 5, Wi48; lane 6, Yo80; lane 7, Su32; lane 8, Gla2, lane 9, Gla21; lane 10, Gla42; lane 11, Gla7 (see table 4.1.). Lane 12, no DNA negative control. Lanes 1 and 13, microsatellite markers.

Figure 4.3. shows an example of a typing experiment for *Grl* $(CAG)_n$ repeats for aelection of rats from England and Scotland. In each case, there are ambigu sities in the form of additional PCR bands. Notably, many of the amplifications revealed bands much higher in the gel than that predicted from the size markers. These additional DNA bands could represent somatic mutation of *Grl* triplet $(CAG)_n$ repeats. However, no such banding was observed in the typing of inbred strains, which suggested that these additional bands were artifacts, resulting from excess genomic DNA in PCR. Attempts were therefore made to optimise PCR conditions to take account of this problem. As a starting point, the samples used in the pilot experiment (Fig. 4.3) were diluted 10-fold. Prior to dilution, DNAs were left to resuspend for a further week at $4^{o}C$ to ensure homogeneity and OD_{260} values were re-determined.

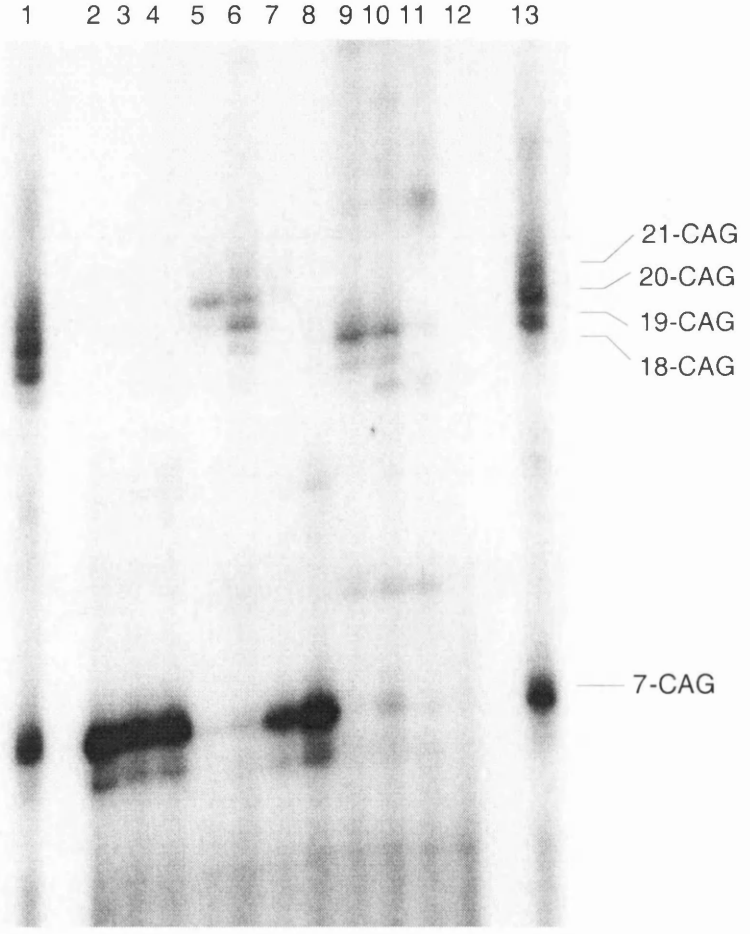


Fig. 4.4. Wild rat PCR amplifications performed under optimised conditions.
DNA per PCR reaction was in the range 2-5 ng. Gel loading was exactly as decribed in Fig. 4.3. (see table 4.1. for corresponding genotypes).

PCRs were performed using the diluted DNA samples exactly as in pilot experiments.

Figure 4.4. shows the resulting increase in product specificity. PCRs were further optimised by reducing cycle number from 30 to 28. Figure 4.5. below shows examples of the enhanced specificity achieved.

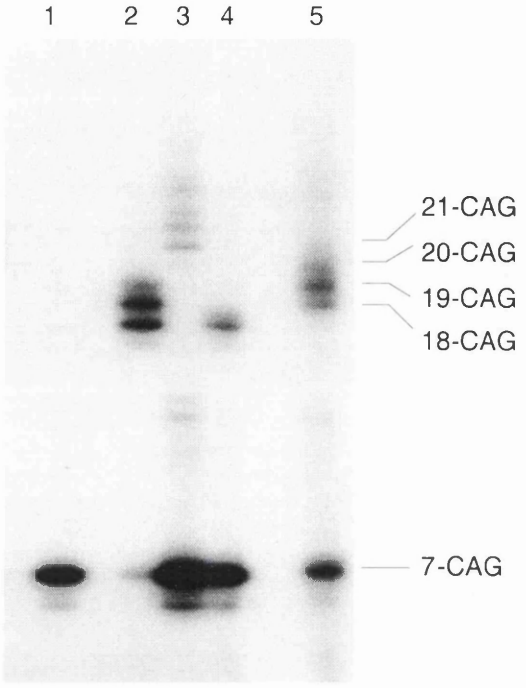


Fig. 4.5. Typing of wild rat $(CAG)_n$ repeats under further optimised PCR conditions.
Lane 1, Do91; lane 2, Su39; lane 3, Do94; lane 4, Su41. Lane 5, microsatellite markers.

The spurious banding in Figure 4.5., lane 3 (Do94) is likely to be an artifact resulting from an excess of DNA used in PCR, since the magnitude of the specific 7-CAG repeat product band is elevated compared with those in other lanes. Similar spurious bands were occasionally seen for clone DNA amplifications (marker bands), for which somatic mutation is not a consideration (results not shown). From this point, PCR conditions were considered optimal for the typing of the remaining wild rats.

Routine analysis of larger numbers of GR CAG repeat lengths was based on a Southern blotting approach, in which non-radioactive PCR products (amplified under optimised PCR conditions) were transferred to nylon membrane (Hybond-N^+, Amersham, U.K.) for 6-16h and then probed using

a ^{32}P-end labelled polyglutamine oligonucleotide probe (5' AGCAGCAGCAGCAGCAGGC 3': see Appendix 1 for coordinates in rGR cDNA) complementary to the 3' end of the rGR polyglutamine microsatellite (see section 2.2.7. for labelling conditions). Hybridisations were carried out at 60^{o}C for 8-10h in 10ml 2x SSC containing 2% w/v milk powder. Hybridised filters were washed in 2x SSC, 0.1% SDS for 30 mins (or until the background was sufficiently reduced) at 60^{o}C and exposed to autoradiographic film for 2-3h at -70^{o}C. Microsatellite markers were the same as described in chapter 3.

4.2.2.) *Grl* Haplotype analysis

Determination of *Grl* haplotypes was based on the nucleotide sequence in and around the *Grl* triplet $(CAG)_n$ repeat.

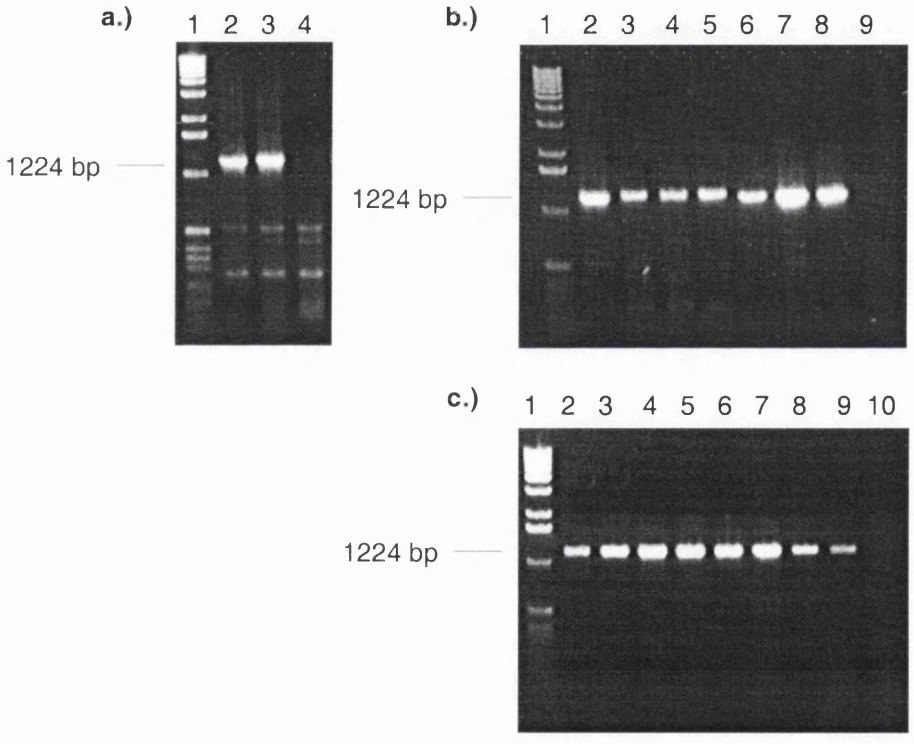


Fig. 4.6. Amplification of rGR sequences for haplotype analysis.
PCR products of 1224 bp were amplified for 47 rat DNAs. Amplifications were performed in duplicate following optimisation to provide enough DNA for sequencing (See text for details).

a.) Pilot experiment: non-optimised PCR with additional banding below the expected PCR band was performed using 1.5 mM Mg^{2+}, 35 PCR-cycles and 58^{o}C primer annealing temperature. Lanes 1 and 2, amplified BDE rat strain genomic DNA (see Table 4.5.); lane 4, no DNA negative control.

b.) Selection of PCRs performed under optimised conditions of 1.2 mM Mg^{2+}, 37 PCR-cycles and 60^{o}C primer annealing temperature. Lane 2, Gla32; lane3, Gla44; lane 4, Gla45; lane 5, Gla6; lane6, Gla10; lane 7, Fi3; lane 8, Fi6. Lane 9, no DNA negative control.

c.) Amplification of selected rat DNAs under optimised PCR conditions. Lanes 1 and 2, BDE strain; lanes 4 and 5, St1; lanes 6 and 7, St2; lanes 8 and 9, St3 (see table 4.5.). Lane 10, no DNA negative control.
Lanes 1 (a, b, c), 1 kb DNA ladder (GIBCO-BRL, U.K.).

Data obtained up until this point for inbred and wild rats indicated a high degree of triplet repeat polymorphism, together with three silent nucleotide substitution sites identified in chapter 3. PCR products (1224 bp) containing all potential mutation sites were amplified using primers rGR19 and rGR21 (Appendix 1).

4.2.3.) Sequencing

Novel triplet $(CAG)_n$ repeat lengths were sequenced from PCR products using the dynabead method described in section 2.13.

Duplicate PCR amplifications were performed for a total of 47 homozygous rat DNA templates (14 inbred rats, 33 wild rats; see table 4.5.). Pilot experiments were carried out in 25µl of standard reaction mixture (see section 2.2.3.) by combining 50-100ng genomic DNA with 20 pmoles of each PCR primer, 1.5 mM Mg^{2+} and 0.01 mg/ml acetylated BSA. The PCR reaction profile described in section 2.2.3. was performed for 35-cycles, with an annealing temperature of 58°C. PCR products were less specific than required for uninterrupted sequencing (see Fig. 4.6a.). The stringency of PCR reactions was therefore optimised by reducing Mg^{2+} concentration from 1.5 mM to 1.2 mM. Annealing temperature was also increased from 58 to 60°C, requiring an additional x2 PCR cycles (37-cycles, total). Fig. 4.6b. and c. shows succesfully optimised PCRs.

Grl haplotypes were determined by direct sequencing of double stranded Exonuclease I and Shrimp Alkaline Phosphatase-treated PCR products using a modification of the sequenase version-2 DNA sequencing kit (Amersham International, U.K.), described in section 2.2.13. Sequencing was initiated from three separate sites in the amplified DNA using primers: pG rGR22, rGR23 (Appendix 1). Gels were run exactly as described in chapter 3.

4.3.) Results

4.3.1.) *Grl* triplet $(CAG)_n$ repeat variation in wild rats

The absence of CAG repeat lengths of 2-6, 8-16, and of more than 21 in *Grl* alleles of inbred strains may reflect that only a limited number of alleles were fixed when establishing laboratory strains from wild rat populations. CAG repeat lengths of wild caught rats from Scotland and England were therefore analysed.

Origin (where caught)	Total number of rats from each location	Number of rats with specific GR genotype
Stirling	12	**St1-12 (*7*)**
Falkirk	3	**Fk1, 3 (*7*)**; Fk2 (*7, 21*)
Fife	9	**Fi3, 6 (*7*)**; Fi5, 7, 9 (*7, 22*); Fi2 (*7, 23*); Fi1, 4, 8 (*18, 22*)
Glasgow	70	**Gla2, 10, 31, 43, 53 (*7*)**; **Gla9, 21, 44, 65, 67, 70 (*18*)**; **Gla32, 45, 50, 51 (*20*)**; **Gla13, 14 (*21*)**; **Gla8, (*22*)**; **Gla7, 16, 26, 47, 62-64, 66 (*23*)**; Gla 42, 46, 55, 58 (*7, 18*); Gla6, 48, 52 (*7, 20*); Gla41 (*7, 22*); Gla3, 19, 20, 23, 30, 33, (*7, 23*); Gla1, 12, 15, 17, 49 (*18, 20*); Gla69 (*18, 21*); Gla11, 18, 22, 25, 27, 34, 35, 37, 40, 54, 56, 61, 68 (*18, 23*); Gla4, 5 (*19, 20*); Gla57 (*20, 22*); Gla24, 28, 29, 36, 38, 39, 59, 60 (*20, 23*)
Berkshire	8	**Bk27 (*7*)**; Bk2, 6 (*7, 18*); Bk26 (*7, 19*); Bk24 (*17, 19*); Bk22 (*17, 20*); Bk20, 25 (*19, 20*)
Hampshire	9	**Ha18 (*7*); Ha11, 14 (*19*); Ha9 (*20*)**; Ha20, 12, 15-17 (*7, 20*)
Shropshire	6	**Sh29 (*7*); Sh30, 89 (*23*)**; Sh31, 88 (*7, 23*); Sh90 (*19, 23*)
Sussex	8	**Su32, 36, 37 (*7*)**; Su41 (*7, 17*); Su34, 40 (*7, 22*); Su39 (*17, 18*); Su35 (*20, 21*)
Wiltshire	7	**Wi44 (*18*); Wi48-50 (*20*)**; Wi45 (*18, 20*); Wi42, 47 (*19, 20*)
Oxfordshire	7	**Ox59 (*19*)**; Ox61, 62, 66 (*7, 20*); Ox63 (*7, 21*); Ox64 (*20, 23*); Ox65 (*21, 22*)
Essex	2	Es74 (*7, 17*); Es75 (*7, 20*)
Yorkshire	5	**Yo84 (*20*); Yo77 (*23*)**; Yo80 (*7, 19*); Yo82 (*20, 22*); Yo81 (*21, 23*)
Dorset	9	**Do91, 92, 94, 96, 98-100 (*7*)**; Do95 (*7, 22*); Do93 (*19, 21*)

Table 4.1. Incidence of *Grl* genotypes found in Scottish and English wild rats.
Each rat genotype is prefixed by its origin, given in shorthand notation. References highlighted in bold type show homozygous animals (n = 67). Number of heterozygotes was found to be 88. Bracketed numbers in italics represent GR alleles.

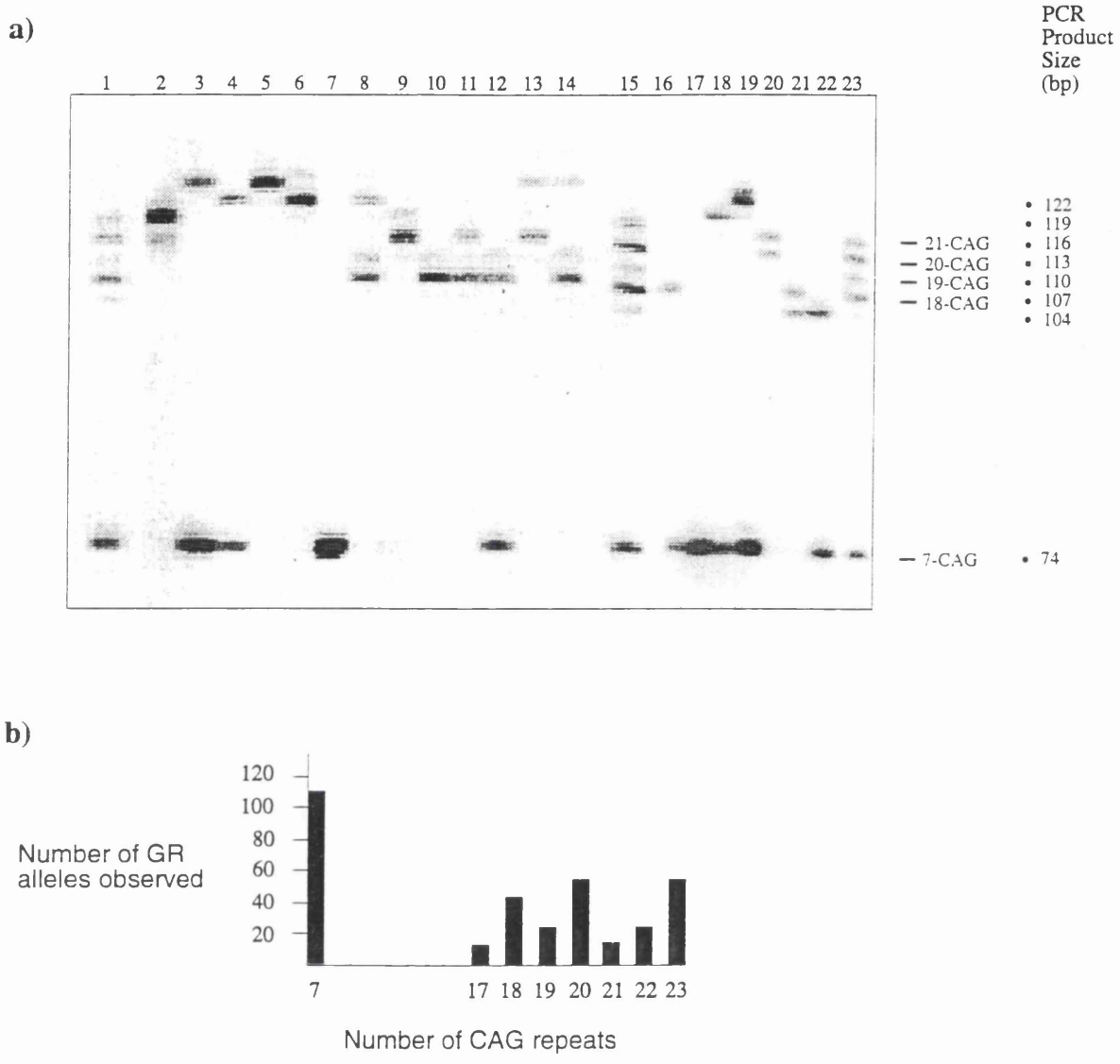


Fig. 4.7. The range of CAG repeat lengths in wild rats caught in Scotland and England. **a)**: ^{32}P-labelled PCR products resolved on a denaturing polyacrylamide gel, showing GR repeat length variation for 20 wild rats, selected to show the range of observed alleles: lane 2, Gla14; lane 3, Fi2; lane 4, Gla41; lane 5, Sh89; lane 6, Gla8; lane 7, St1; lane 8, Fi1; lane 9, Ha9; lane 10, Wi44; lane 11, Gla1; lane 12, Gla 42; lane 13, Ox64; lane 14, Gla68; lane 16, Bk2; lane 17, Do91; lane 18, Do95; lane 19, Sh88; lane 20, Su35; lane 21, Su39; lane 22, Su41. Lanes 1, 15 and 23 contain mixed PCR products amplified from cloned alleles *Grl*$^{7,18\text{-}21}$. **b)**: frequency distribution of CAG repeat alleles from Scottish and English wild rats.

Table 4.1. shows the GR genotypes of all wild rats typed. A representative 20 samples successfully typed under optimised PCR conditions are shown in Figure 4.7.

Two novel allele lengths were identified with 22 and 23 CAG repeats (see Fig. 4.8. for entire range of GR alleles identified in inbred and wild rats).

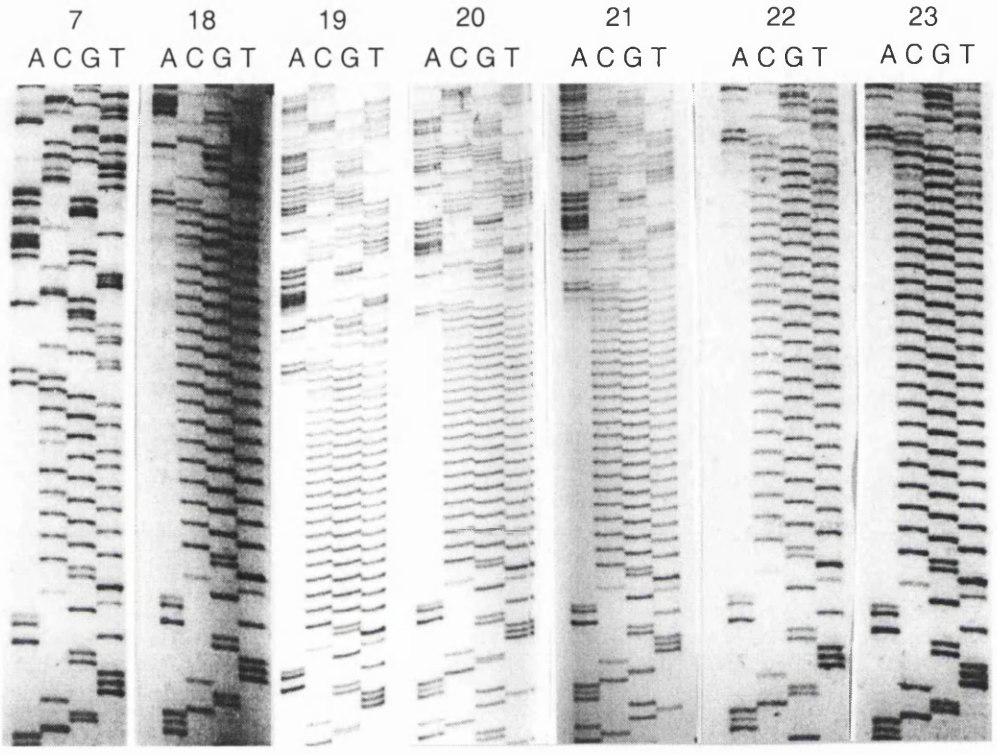


Fig. 4.8. Sequences of naturally occuring GR triplet $(CAG)_n$ repeats found in wild and inbred rats.
Repeat lengths, determined as described in Figure 3.5. chapter 3, ranged from the smallest at 7-CAG repeats (far left) to the longest at 23-CAG repeats (far right). The longest polymer tracts of 22 and 23-CAGs were found only in wild rats and not in any inbred strains tested. Sequencing was from the antisense strand using primer pG (Appendix 1). The letters A, C, G, and T above sequence lanes represent terminating nucleotides. Numbers above each sequence represent numbers of CAG repeats, respectively.

Of 155 rats, 67 (43%) were homozygous for any one of alleles $Grl^{CAG7,\ 18\text{-}23}$. Allele Grl^{CAG7} was the most common. Grl^{CAG17} was least common; its presence in only five heterozygotes explained why no homozygotes were observed. Table 4.2. shows the totals of each *Grl* allele identified in wild rats. Tables 4.3. and 4.4. show the expected and observed homozygous alleles for Glasgow and all other sample locations in the U.K. respectively, with the

exception of Stirling, from which all rats were typed as homozygous for *Grl*^{CAG7} and therefore considered to bias the data. The possible implications of these results are discussed at the end of this chapter.

Location. (town or county)	No. of Rats typed	Numbers of GR alleles GR-CAG repeat No.							
		7	17	18	19	20	21	22	23
Stirling	12	24	0	0	0	0	0	0	0
Falkirk	3	5	0	0	0	0	1	0	0
Fife	9	8	0	3	0	0	0	6	1
Glasgow	70	24	0	35	2	27	5	4	43
Berkshire	8	5	2	2	4	3	0	0	0
Hampshire	9	7	0	0	4	7	0	0	0
Shropshire	6	4	0	0	1	0	0	0	7
Sussex	8	9	2	1	0	1	1	2	0
Wiltshire	7	0	0	3	2	9	0	0	0
Oxfordshire	7	4	0	0	2	4	2	1	1
Essex	2	2	1	0	0	1	0	0	0
Yorkshire	5	1	0	0	1	3	1	1	3
Dorset	9	15	0	0	1	0	1	1	0
Total	**155**	**108**	**5**	**44**	**17**	**55**	**11**	**15**	**55**
Homozygotes		34	0	4	3	9	2	1	11

Table. 4.2. Total numbers of each GR allele found in wild rats (English and Scottish). Out of a total of 155 rats, 67 were found to be homozygous and 88, heterozygous for the corresponding GR alleles. Total homozygous animals are given for each allele.

4.3.2.) *Grl* haplotypes in wild and inbred rats

Wild rats (n=32) homozygous for CAG repeat alleles were typed by direct PCR sequencing for variation at the three silent nucleotide substitution sites (coordinates: n198, n531, n711 in published sequence of rGR cDNA). Most (n=29) rats typed as homozygotes: n198C, n531T, n711C, with CAG repeat lengths of 7, 18, 20, and 23. The remaining three rats typed homozygous for flanking markers: n198T, n531C, n711C, with CAG repeat lengths of 19, 20 and 22, respectively (see Table 4.5).

The typing results for 14 inbred strains defined 6 haplotypes, as shown in Table 4.6. The overall quality of sequencing and demonstrations of homozygosity are given in Figure 4.8. Sequence sets were run side-by-side,

bringing all nucleotide substitution sites into the same window of sequence, thus simplifying the interpretation.

Grl Allele	Number of alleles	Allele frequency	Frequency expected homozyg's	Observed homozyg's	Frequency observed homozyg's
7	24	0.17	0.029	5	0.07
18	35	0.25	0.063	6	0.09
19	2	0.01	0.0001	0	0
20	27	0.19	0.036	4	0.06
21	5	0.04	0.0016	2	0.03
22	4	0.03	0.0009	1	0.01
23	43	0.31	0.096	8	0.11
Total	**140**		**0.23**	**26**	**0.37**

Table 4.3. Frequency of *Grl* alleles in Glasgow rats.
Total alleles together with expected and observed frequency of homozygotes is given in bold at the foot of the table. Homozyg's = homozygotes.

Grl Allele	Number of alleles	Allele frequency	Frequency expected homozyg's	Observed homozyg's	Frequency observed homozyg's
7	60	0.41	0.17	17	0.23
17	5	0.034	0.0012	0	0
18	9	0.061	0.0037	1	0.014
19	15	0.1	0.01	2	0.027
20	28	0.19	0.036	4	0.055
21	6	0.041	0.0017	0	0
22	11	0.075	0.0056	0	0
23	12	0.082	0.0067	3	0.041
Total	**146**		**0.24**	**27**	**0.37**

Table 4.4. Frequency of *Grl* alleles in wild rats from other locations.
The frequency of all other *Grl* alleles are given, with the exception of those from Glasgow and Stirling. Total alleles together with expected and observed frequency of homozygotes is given in bold at the foot of the table. Homozyg's = homozygotes.

Strain/rat	CAG-repeat	Nucleotide at n198	Nucleotide at n531	Nucleotide at n711
WILD RATS				
St1-3	7	C	T	C
Fi3&6	7	C	T	C
Gla10	7	C	T	C
Gla32	7	C	T	C
Gla53	7	C	T	C
Sh29	7	C	T	C
Su32	7	C	T	C
Su36	7	C	T	C
Do92	7	C	T	C
Do100	7	C	T	C
Gla8	18	C	T	C
Gla26	18	C	T	C
Gla45	18	C	T	C
Gla65	18	C	T	C
Gla67	18	C	T	C
Gla 0	18	C	T	C
Ox59	*19*	*T*	*C*	*C*
Gla9	20	C	T	C
Gla51	20	C	T	C
Ha9	*20*	*T*	*C*	*C*
Su44	20	C	T	C
Yo84	20	C	T	C
Gla7	*22*	*T*	*C*	*C*
Gla2	23	C	T	C
Gla63	23	C	T	C
Gla64	23	C	T	C
Gla66	23	C	T	C
Sh30	23	C	T	C
Yo77	23	C	T	C
INBRED STRAINS				
BC38	**7**	**C**	**T**	**C**
BDE123	**7**	**C**	**T**	**C**
U62	**7**	**C**	**T**	**C**
LH157	**19**	**C**	**T**	**T**
OKA108	**19**	**T**	**C**	**C**
SHR (NL)	**19**	**T**	**C**	**C**
SHR (UK)	**19**	**T**	**C**	**C**
SHR-SP	**19**	**T**	**C**	**C**
MHS	**20**	**T**	**C**	**C**
WKY	**20**	**C**	**T**	**C**
MNS	**21**	**C**	**T**	**T**
Zucker ob	**21**	**C**	**T**	**T**
Zucker lean	**21**	**C**	**T**	**T**

Table 4.5. *Grl* haplotypes in a selection of wild rats and inbred strains.
Wild rats are referred to by shorthand notation. Inbred strains are shown in bold type. Haplotypes shown in italics highlight the rarest haplotypes found in wild rats.

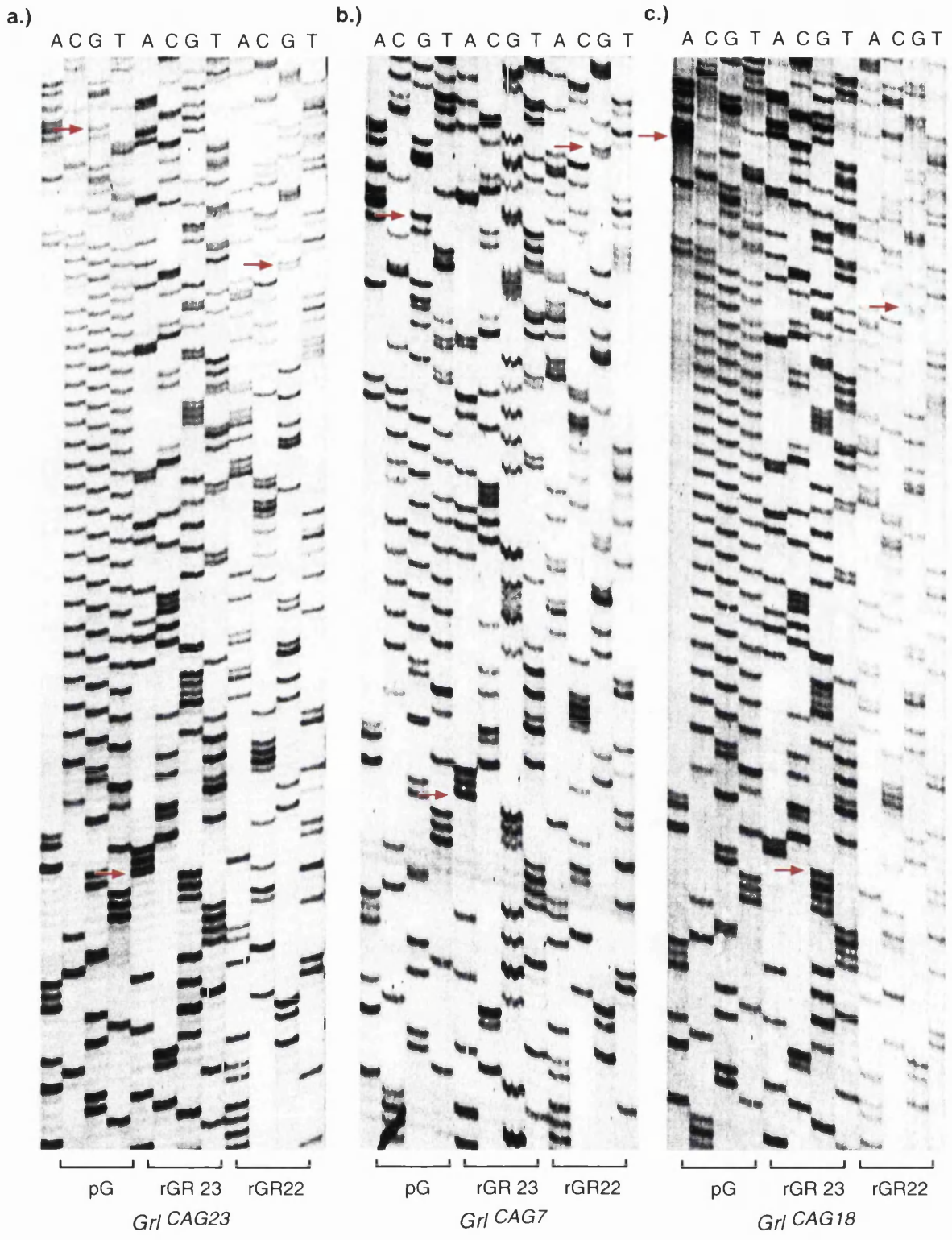


Fig. 4.9. Examples of rat *Grl* haplotypes.

Nucleotide sequences of alleles Grl^{CAG23} and Grl^{CAG7} (a. and b. respectively) show examples of n198C, n531T, n711C haplotypes for which clear homozygosity is detectable. Grl^{CAG18} (c.), shows an example of a rare n198T, n531C, n711C haplotype. Red arrows mark the positions of substituted nucleotides in the codon chages: TTC->TTT, TTT->TTC and GAT->GAC, identified using seqencing primers pG, rGR22 and rGR23 respectively (primers are listed in Appendix 1). Letters A, C, G, T at the top of each sequence lane represent terminating nucleotides. Sequences are of the antisense DNA strand.

Haplotype #	Strains	Variation at *Grl* nucleotide position:			
		198	223-288†	531*	711*
1	BC; BDE; U	C	7-CAG	T	C
2	WKY	C	20-CAG	T	C
3	LH	C	19-CAG	T	T
4	MNS; Zucker-obese; Zucker-lean	C	21-CAG	T	T
5	OKA; SHR; SHR-SP	T	19-CAG	C	C
6	MHS	T	20-CAG	C	C

Table 4.6. Haplotypes of CAG repeat length and same-sense point mutations in the coding sequence of rat glucocorticoid receptor.
*nucleotide positions based on coordinates in the allele Grl^{CAG21}. †encodes polyglutamine repeat in Grl^{CAG21}. (All nucleotide positions relative to met1).

4.4.) Discussion

In distinction to the human receptor, exon 2 of rat GR contains a polymorphic microsatellite comprising a variable number of CAG repeats. Microsatellite repeats are a widespread source of genetic variation in mammals (Rubinsztein *et al.*, 1995). They are a key tool for the mapping of phenotypic traits (see Jacob *et al.*, 1995), and some microsatellites may directly influence phenotype either through variation in protein structure (Ross *et al.*, 1993; Perutz *et al.*, 1994), or by less well defined influences over the expression of adjacent genes (Kashi *et al.*, 1997). The present finding of a discontinuous distribution of CAG repeat lengths in the GR of inbred and wild rats raises fundamental questions about the genetic origin of this discontinuity and the endocrine significance of the observed variation in the length of the polyglutamine tract.

In the sample of 155 wild rats in this study, homozygotes were identified for each allele, with the exception of Grl^{CAG17}, and indicates that the alleles are compatible with viable and competative phenotypes. The observed frequency of 45% homozygosity was high. Of the 67 homozygous rats, 12 were trapped in Stirling (Central Region, Scotland) and were homozygous

for *Grl*^{CAG7}. The frequency of homozygosity was 37% for Glasgow (70 rats trapped; Table 4.3.), and 37% for all the other sites combined (73 rats; Table 4.4.). From the observed frequency of *Grl* alleles in the Glasgow population, an expected frequency of homozygosity was calculated at 23% on the assumption of Hardy-Weinberg equilibrium. This is substantially lower than the observed frequency of 37%. This may indicate the existence of deem structures in rats with significant levels of inbreeding, similar to those reported in wild mouse populations, but further work is needed to relate capture sites to the local geography of rat populations.

There was a qualitatively similar distribution of CAG repeat lengths in the GR of inbred strains and wild rats. Neither set of animals had CAG repeat lengths of less than 7, or between 8 and 16, indicating that these alleles are either absent, or at least are significantly under-represented. The inbred strains analysed in this study originated from Europe, USA and Japan. It is therefore unlikely that the observed allelic discontinuity is due to sampling bias, although it is possible that other rat populations exhibit atypical allele lengths. Apart from genetic drift due to the effects of chance sampling, the observed rGR alleles may have a selective advantage through their function as transcription factors. The latter possibility has not been resolved.

The observed distribution of rGR alleles may reflect a non-random mechanism by which triplet repeats expand and contract. Though discontinuous distributions are relatively uncommon for non-disease related alleles, they have been demonstrated for a small number of human loci, including HUMRENA4 (renin) and markers DSX 228 and DSX 426 (properidin P; Coleman *et al.*, 1991). The myotonic dystrophy (DM) locus has an unstable $(CTG)_n$ repeat in the 3'-untranslated region (3'-UTR) of a gene encoding a protein kinase family member (DMPK; Harris *et al.*, 1996). This sequence is highly polymorphic in the normal population, presenting $(CTG)_n$ variants with between 5 and 36 copies (Brook *et al.*, 1992; Zerylnick *et al.*, 1995). In the studies of both Brook *et al.*, (1992) and Zerylnick *et al.*, (1995), significant numbers of normal individuals had a $(CTG)_5$ repeat

allele. Repeat lengths of $(CTG)_{6-9}$ were very rare and lengths of $(CTG)_{10-15}$ were also observed at high frequency. The under-representation of DM $(CTG)_{6-9}$ and *Grl* $(CAG)_{8-16}$ alleles may have a similar cause: namely that *Grl* $(CAG)_7$ and DM $(CTG)_5$ repeat alleles may be too stable to create frequent length variants, but occasionally mutate through a multi-step process to a larger allele size, from which further diversification generates a typical series of microsatellite alleles, probably by replication slippage (Sinden and Wells, 1992). In primates, there is evidence to suggest that there is a general progression towards increased microsatellite lengths with evolutionary time (Rubinsztein *et al.*, 1995).

The inbred samples analysed here included some closely related strains (Table 3.3). Amorat/Wsl and Aristorat/Wsl are congenic strains based on LOU/C (respectively: LOU/C.IgK-1b OKA and LOU/C.IgH-12b OKA; Greenhouse et al, 1990; Hedrich, 1990); all three have Grl^{CAG17}, which was uncommon in the present wild populations. BD strains E, IV, VII, IX and X all have Grl^{CAG7}, and originate from coat colour crosses of Druckrey (Greenhouse *et al.*, 1990), mainly from the founding lines BDI-III. The origin of strains BC and U (Grl^{CAG7}) are not certain, but both arose in Utrecht. Allele Grl^{CAG7} was common in the wild rat populations sampled (Fig. 4.7 and Table 4.1.). Alleles $Grl^{CAG22,23}$ were found in wild rats only, with the latter being relatively common. As expected, none of the 155 wild rats sampled exhibited very large allele sizes which might have been indicative of a trinucleotide expansion syndrome.

In addition to the expressed genetic variation within the CAG repeat, genetic variation was investigated in GR codons at nucleotide positions 198, 531, and 711 which are silent in effect and do not lead to changes in amino acid sequence (these nucleotide coordinates are based on the Grl^{CAG21} allele and extends over nucleotide positions 223-288). These four variable sites are very close together and constitute haplotypes within which recombination is expected to be very rare. For haplotypes including Grl^{CAG21}, the four sites extend over a distance of 513 bp in rGR exon 2, corresponding to a recombination interval of about 5×10^{-4} cM (Jacob *et*

al., 1995). For the range of CAG repeats from $Grl^{CAG7-23}$, this distance corresponds to 471-519 bp, respectively. Laying aside the unlikely possibility that CAG repeat lengths of 7-23 are significantly recombinogenic, GR haplotypes will therefore on average only be expected to be disrupted by recombination once per 200,000 rat generations. Six different GR haplotypes were identified from the panel of inbred strains of rat (Tables 4.5. and 4.6.). The synonymous nucleotide differences of haplotypes 2 and 6 show that the CAG repeats in these two haploypes have separate origins; this also applies to haplotypes 3 and 5 (CAG19). Haplotypes 5 (CAG19) and 6 (CAG20) do not differ at the other three nucleotide positions; since both haplotypes are Wistar-derived, the possibility that both are directly related through a mutation in the CAG repeat number cannot be excluded. Strains SHR and SHR-SP are hypertensive strains derived from outbred Wistar-Kyoto stock and OKA is believed to be a subline of SHR (Greenhouse *et al.*, 1990; Otsen, 1995). A close relationship between these strains is supported by the presence of GR haplotype 5 in all three strains (Table 4.6.). Strains MNS and MHS are Wistar-derived. They have GR haplotypes 4 and 6, respectively, which differ at all four sites (Table 4.6.). Although it is not known whether the differences in haplotypes arise as a result of recombination or from point mutation events, the results presented here suggest that haplotypes 4 and 6 were separate for thousands of generations before fixation in the Milan selection lines (Bianchi *et al.*, 1984) and it is therefore likely that MNS and MHS will show other genetic variation in the genome flanking the GR locus. In support of this proposal, Figure 4.10. shows a dendrogram of the geneologic relationships between 63 inbred laboratory rat strains and 124 of their substrains (taken from Canzian, 1997). The relatedness of strains was based on a total of 995 genetic and biochemical marker typings and implies a significant degree of genetic divergence between strains MHS and MNS.

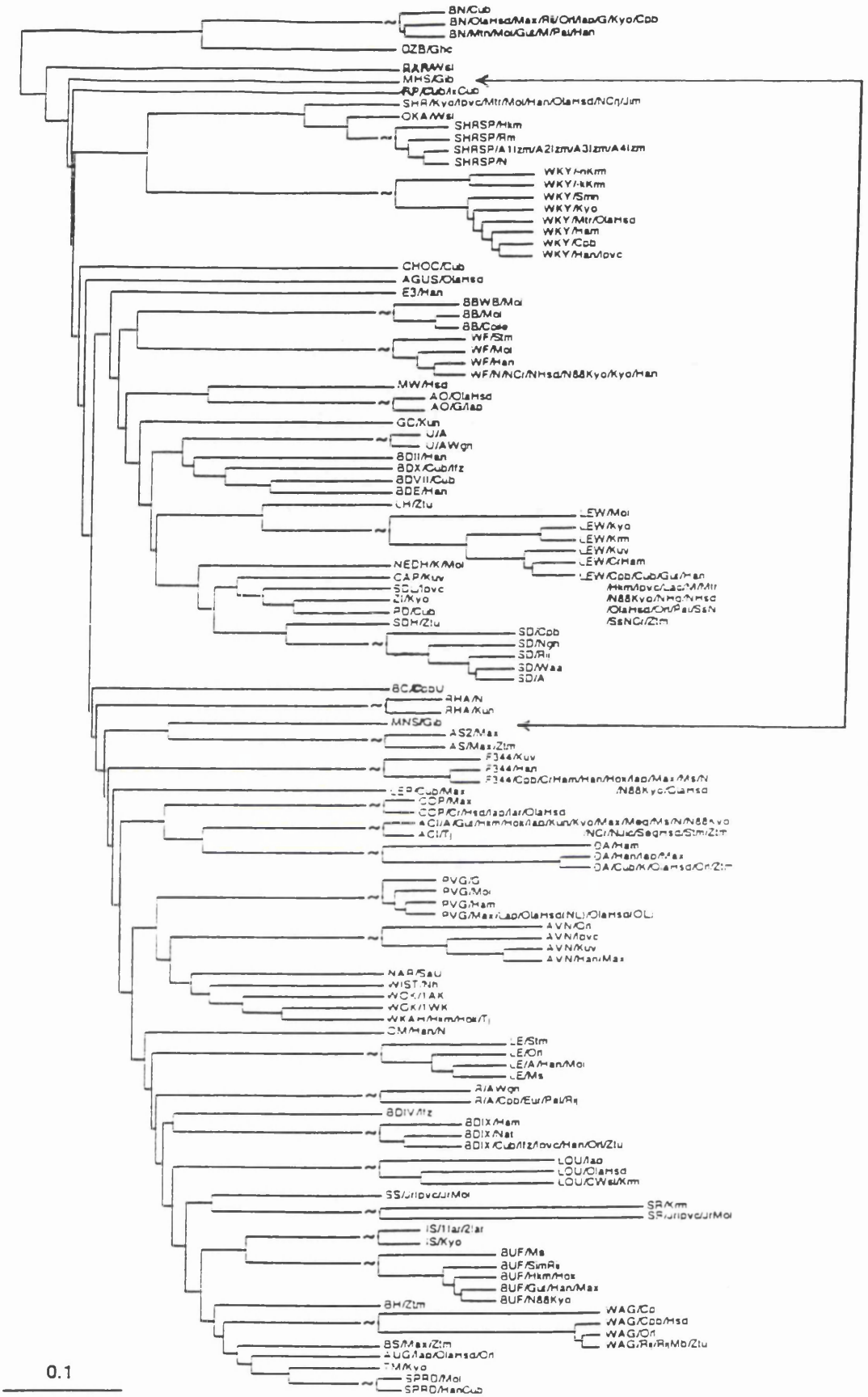


Fig 4.10. Dendrogram showing the geneologic relationship betwen 63 laboratory strains of rat and 124 of their substrains.

The scale represents percentage difference and is applicable to the left part of the figure. Strains with substrains, for which the point of branching from the main line is unknown are marked with ~. Strains MHS and MNS are indicated by arrows. (Taken from Canzian, 1997).

Chapter 5
Results 3

Glucocorticoid receptor polymorphism: possible association with glucocorticoid based phenotypes in Milan rats

The majority of data in this chapter is not my own work, but resulted from a collaborative effort between myself my supervisors and the group of G. Bianchi, Department of Nephrology, S. Raffaele Hospital, Milan, Italy. The work was initiated following the characterisation of glucocorticoid receptor steroid binding affinity differences between MHS and MNS strains of rat (C. Kenyon and M. Panarelli) and identification of the triplet $(CAG)_n$ repeat polymorphism, the result of my own work (discussed in chapter 3). This polymorphism was used to screen for association between *Grl* and glucocorticoid related phenotypes in F2 rats of an MHS x MNS cross.

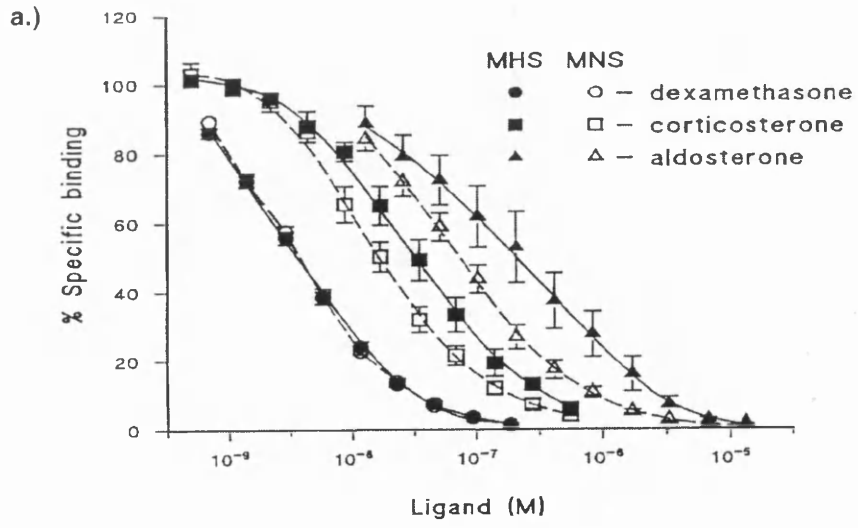
5.1.) Introduction

The Milan hypertensive strain of rat (MHS) presents altered renal function (Bianchi *et al.*, 1986) and increased adrenocortical activity (Ferrari *et al.*, 1985) when compared to its normotensive control strain (MNS). A significant proportion of the MHS hypertension is sustained by the kidney (Bianchi *et al.*, 1984) as demonstrated by a long series of observations, starting from studies of renal cross-transplantation between the two strains (Bianchi *et al.*, 1986). Recent genetic studies have demonstrated that a point mutation within the α-subunit of a heterodimeric cytoskeletal protein, adducin, increases Na-K-ATPase activity when transfected into renal epithelial cells (Tripodi *et al.*, 1996). *In vivo*, this mutation affects blood pressure, accounting for 40% of the blood pressure difference between MHS and MNS, when interacting with a mutated β-adducin subunit (Bianchi *et al.*, 1994).

Previous studies have established that MHS exhibits adrenocortical hypertrophy, together with higher steroid secretory rates and raised plasma corticosterone concentrations compared with MNS (Ferrari *et al.*, 1985; Stewart *et al.*, 1993). There are also suggestions of mineralocorticoid excess in MHS, with extracellular volume expansion, plasma renin suppression and increased body sodium content (Fraser *et al.*, 1994). Taken together, these observations might suggest an abnormality of HPA-axis

function, resulting in a reduced negative feedback regulation of corticosterone synthesis. Inefficient glucocorticoid activity may result from reduced access of hormone to the glucocortiocid receptor, or from an abnormality of the glucocorticoid mediated regulation of CRF and/or POMC gene transcription or secretion of ACTH from the hypothalamus. Any of these effects might result in raised plasma corticosterone concentrations, causing activation of mineralocorticoid receptors. Clinically, similar situations arise because of defects in steroid metabolism, or because of intrinsic abnormalities of the glucocorticoid receptor (Lamberts *et al.*, 1992; Arai and Chrousos, 1994; Bronnegard *et al.*, 1996). Steroid metabolism has been shown to be different between MHS and MNS, but not in a way which would affect steroid binding to adrenocorticosteroid receptors (Stewart *et al.*, 1993).

Recent studies (Kenyon *et al.*, 1994; Panarelli *et al.*, 1995) have suggested that the glucocorticoid receptor from strain MHS has a significantly lower affinity for steroids (cortisol, aldosterone and dexamethasone and the glucocorticoid antagonist, RU486) compared with its normotensive control strain, MNS. All ligands tested competed for specific ^{3}H-dexamethasone binding sites in the GR from both strains of rat. Ranking, in terms of affinity, was the same for either strain and followed the pattern; dexamethasome > corticosterone > RU486 > aldosterone.



b.)

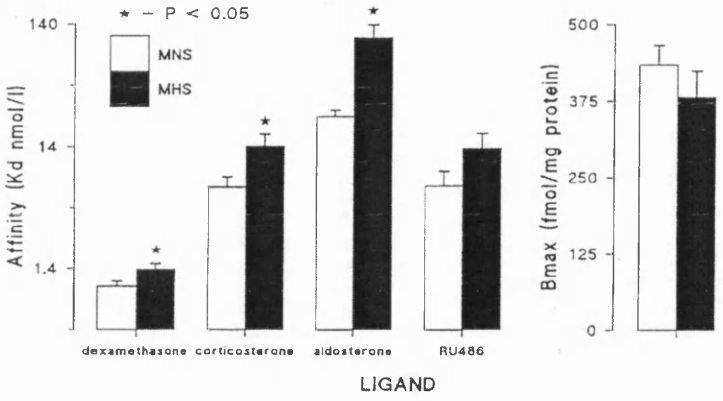


Fig. 5.1. Steroid binding in Milan rat liver cytosol extracts.
a.) Competition for ^{3}H-dexamethasone binding sites in GR by selected glucocorticoid agonists and the antagonist RU486 in MHS and MNS.
b.) Bar charts showing differences in affinity for the same glucocorticoid agonists and antagonist shown in a.). Receptor numbers (Bmax) were not different between strains. (steroid binding affinities were determined by C. J. Kenyon and M. Panarelli).

Competition binding curves for three of the ligands are shown in Figure 5.1. (for reasons of clarity, RU486 curves which overlap those of corticosterone are not shown). Aldosterone and corticosterone appeared to compete less effectively for GR binding sites in cytosol from MHS than from MNS rats. For all ligands tested, Kd values for MHS were greater than for MNS glucocorticoid receptors, indicating a reduced binding affinity (Fig. 5.1.). For RU486 the affinity difference did not achieve statistical significance. Scatchard analysis of homologous dexamethasone binding to GR indicated a one site interaction model for both MHS and MNS, with Kd values of 1.39 ± 0.15 nM (MHS) and 1.0 ±0.11 nM (MNS) respectively.

Differences in Kds between strains were greatest for the weaker ligands. Compared with MNS, Kd values for MHS GR were 1.39, 2.19, 2.06 and 4.12-fold greater for dexamethasone, corticosterone, RU486 and aldosterone, respectively. The competition curves for MNS were similar to those reported previously for other normotensive strains of rat (Panarelli *et al.*, 1995; Soro *et al.*, 1995). Bmax values (number of receptor protein molecules per unit of total protein) were not significantly different between strains; 434 ±33 (MHS) and 382 ±43 (MNS) fmol/mg protein.

5.2.) Materials and methods

Animals

MHS and MNS rat used in steroid binding analysis were obtained from the Field Station, University of Sheffield, UK and were maintained at a constant temperature on a 12 h dark, 12 h light cycle with free access to food and water. All F2 rats used for genetic analysis were bred in Milan and maintained as described by Bianchi *et al.*, (1994). F1 hybrids were produced by crossing MHS with MNS. By intercrossing F1 progeny, an F2 population was obtained, consisting of approximately equal numbers of offspring from each reciprocal cross (251 total individuals; 121 male and 130 female).

GR genotype determination

For routine genotyping of the Milan-held rats, genomic DNA was extracted from the tail according to a standard procedure (Laird *et al.*, 1991). GR sequences spanning the triplet $(CAG)_n$ repeats were amplified in 20 ml reaction volumes, by combining 250 ng of genomic DNA with 10-20 pmoles of each PCR primer (p9 and p19: see Appendix 1) and 1U *Taq* DNA-polymerase (Bioline, Glasgow, UK) in a standard reaction mixture containing 2 mM $MgCl_2$, 67 mM Tris-HCl (pH 8.0) 16 mM $(NH4)_2SO_4$, 0.01% Tween 20 and 125 μM of each dNTP. Reactions were cycled 30-times at 94°C for 50s, 53°C for 50s and 72°C for 90s with a final extension at 72°C for 5 min, using a Geneamp PCR system 9600 thermal cycler (Perkin Elmer, Norwalk, USA). DNA templates were initially denatured at 94°C for 3 minutes. PCR products were resolved as described in chapter 3 at 50 watt for 3 hours in 1x TBE buffer. Amplified GR sequences were identified after blotting onto a positively charged nylon membrane (Pall Biosupport Division, Portsmouth, UK) and hybridising with complementary PCR fragments, ^{32}P-labelled using a random primer labelling kit (Statagene, USA). Hybridisations were carried out in 10-20 ml sodium phosphate buffer (0.5 M, pH 7.2) containing 7% SDS and 10 mM EDTA for 2-6 h at 55°C. Membranes were washed in 100-125 mM sodium phosphate buffer containing 0.1% SDS at 55°C until the background was sufficiently reduced and then autoradiographed, as described in chapter 3.

Blood pressure measurements

At three months of age, under light halothane anesthesia, cannulae were inserted into the carotid arteries of F2 rats and exteriorised at the back of the neck through a subcutaneous tunnel. Animals recovered within 5 minutes and 4 h later the cannulae were connected, without restraint, to a Gould BS 3200 blood pressure recorder. For each rat, average values of systolic and diastolic blood pressure and heart rate were calculated from simultaneous measurements taken at 1 minute intervals over a 1 hour period. The body weight was recorded just before surgery (see Table 5.1.).

Urinary volume and calcium excretion

Rats were acclimatised to metabolic cages over a two day period. Urine was collected for the following 24 h and the total volume recorded. Urinary calcium concentration was measured by atomic absorption spectrophotometry (Perkin Elmer 1100B spectrophotometer), in the presence of 0.2% LiCl. Results (Table 5.1.) are expressed as mmoles calcium excreted/24h.

Statistical analysis

Values of blood pressure, body weight and urinary calcium concentration in F2 rats (Table 5.1.), are expressed as the mean ± standard error of the mean (SEM). Data from receptor binding studies for cytosols prepared on the same day with the same reagents and incubation conditions for age matched MHS and MNS rats were compared by two way analysis of variance (ANOVA); P-values of <0.05 were considered significant. Data from linkage studies were analysed by one-way or two-way ANOVA with Neumann-Keul's correction test for multiple comparisons (SPSS statistical package).

Western Blotting

Samples of rat liver proteins extracted for steroid binding analysis were analysed by Western blot as described in section 2.2.22. of this thesis.

5.3.) Results

5.3.1.) Expression levels of the 94 kd GR species in preparations of liver cytosol from MHS and MNS

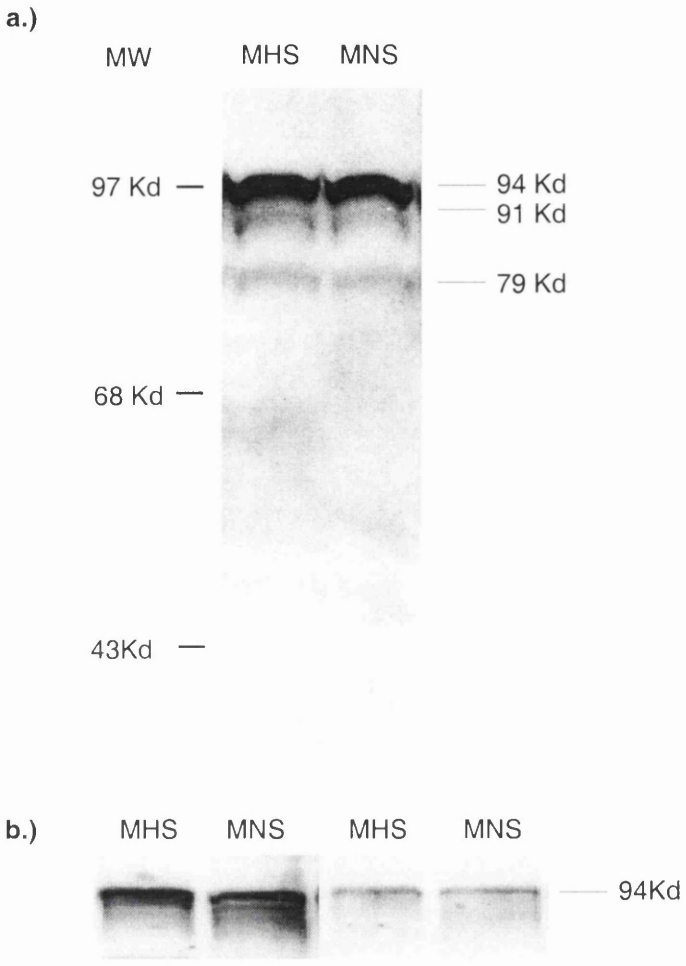


Fig. 5.2. Comparison of the levels of Milan rat liver glucocorticoid receptor protein.
a.) Hepatic GR from rat strains MHS and MNS was fractionated into three distinctive bands by SDS-PAGE. Heavy bands at 94 Kd represent major GR translations initiated from GR met1. Minor GR bands at 91 Kd and 79 Kd are most likely to result from internal translations initiated from GR met2 and met3 respectively (see text for details). MW, high molecular weight protein markers (GIBCO-BRL). For each strain, 30-40 μg of liver cytosolic extract was loaded per lane.
b.) SDS-minigels showing reduced amounts of cytosolic extract loaded per rat strain. Total protein loaded was reduced from 20-30, to 10 μg (samples on left) and finally to 2 μg (samples on right). The work presented in this figure is my own.

The size, integrity and expression level of the major GR band (94 Kd) and minor translations from met2 (91 Kd) and met3 (79 Kd) (Miesfeld *et al.*, 1986) were the same for either strain of rat (see Fig. 5.2a.). Using further

pairs of Milan rats, together with the loading of reduced quantities of protein onto SDS gels (30-40μg, down to 2 μg protein), this result was confirmed (Fig. 5.2b.).

5.3.2.) Association between GR genotype and phenotypes

The trinucleotide $(CAG)_n$ repeat difference between MHS and MNS was used to genotype F2 progeny of an MHS x MNS cross. There was no significant association between GR genotype and systolic blood pressure when the total F2 population was analysed (one-way ANOVA; Table 5.1.). When only homozygous animals were taken into account; MNS/MNS 150.1 ± 1.8 mmHg (n = 59) versus MHS/MHS 145.4 ± 1.3 mmHg (n = 54); p = 0.04. No significant association was found between GR genotype and diastolic blood pressure at any level. When the F2 population was analysed according to sex, female F2 progeny homozygous for the MNS GR allele had significantly higher systolic blood pressures than either heterozygotes, or rats homozygous for the MHS GR allele (p = 0.02: Table 5.1.).

		Glucocorticoid receptor genotype			
	Group	*MNS/MNS*	*MNS/MHS*	*MHS/MHS*	P-value
Systolic blood pressure (mmHg)	all	150.1 ±1.8 (n = 59)	147.7 ±1.0 (n = 132)	145.4 ±1.3 (n = 55)	p = 0.04
	male	147.9 ±2.7 (n = 22)	149.1 ±1.6 (n = 68)	146.2 ±1.9 (n = 29)	NS
	female	151.4 ±2.4 (n = 37)	146.3 ±1.2 (n = 64)	144.4 ±1.7 (n = 26)	p = 0.02
Body weight (g)	male	439 ±11 (n = 21)	420 ±5 (n = 65)	406 ±7 (n = 29)	p <0.01
	female	276 ±4 (n = 36)	276 ±3 (n = 63)	279 ±5 (n = 26)	NS
Urinary calcium (mmol/ day)	male	29.6 ±4.8 (n = 21)	24.0 ±2.2 (n = 59)	31.4 ±3.8 (n = 25)	NS
	female	55.6 ±7.6 (n = 35)	63.8 ±6.0 (n = 63)	89.7 ±8.9 (n = 24)	p <0.005

Table 5.1. Association between glucocorticoid receptor genotypes and blood pressure, body weight and urinary calcium excretion in F2 Milan rats.
P-values for each variable are derived from F2 rats of MHS/MHS verses MNS/MNS genotype. NS = non-significant. The raw data from which the values in this table were determined was generated by L. Torielli, G. Casari, L. Zagato and G. Bianchi, Milan Italy.

The body weight of male, but not female F2 rats was associated with GR genotype; male rats homozygous for the MNS GR allele were 11% heavier than male MHS GR homozygotes ($P < 0.01$). In parental MHS and MNS rats of the same age, body weights were also significantly different (MHS, 413 ± 8; MNS, 387 ± 8; $P = 0.02$, $n = 10$).

In female but not male F2 progeny, MHS GR homozygotes excreted significantly higher levels of calcium than MNS GR homozygotes ($p<0.005$); strain differences in calcium metabolism in Milan rats have been described elsewhere (Cirillo *et al.*, 1989). There was no indication that heart rate and urine volume were different between strains (data not shown).

5.4.) Discussion

Previous studies of phenotypic differences between MHS and MNS have implicated renal function in the control of blood pressure (Persson *et al.*, 1985). The genes coding for the epithelial membrane protein adducin, were found to contain mutations giving rise to 40% of the blood pressure increase in MHS (Bainchi *et al.*, 1994). One hypothesis was that an abnormality of GR function might account for part of the remaining difference in blood pressure between MHS and MNS. This was the subject of the investigation presented in this chapter.

Compared with MNS, the MHS strain of rat shows hypertrophy of the adrenal cortex with increased adrenocortical activity. The result is an increased cortical secretion rate and raised plasma corticosterone concentrations (Mantero *et al.*, 1983; Ferrari *et al.*, 1985; Stewart *et al.*, 1993; Fraser *et al.*, 1994). Negative feedback control of pituitary ACTH secretion by GR in conditions of glucocorticoid excess would be expected to suppress overactivity of the adrenal cortex. Any deviations from normal GR function are therefore likely to be associated with abnormal patterns of hormone secretion (Mantero *et al.*, 1983; Brandon *et al.*, 1991; Hurley *et al.*, 1991; Cole *et al.*, 1993; Stewart *et al.*, 1993; Keightley and Fuller, 1994). With reduced

affinity of GR for its hormonal ligand, higher than normal concentrations of corticosterone, together with a possible up-regulation of GR expression would be required to maintain homeostasis. On the other hand, conditions of glucocorticoid excess which do not result from abnormalities of GR function, would be expected to result in down regulation of the GR message in order to compensate for the overproduction of glucocorticoids (see section 1.3. and references therein). A reduced receptor affinity (either as a primary or secondary mechanism) may therefore be a real phenomenon in MHS *in vivo*, since no evidence of hepatic GR down regulation was identified (Fig 5.2.).

Comparisons of steroid binding in hepatic cytosol from MHS and MNS rats have suggested that affinities for dexamethasone, corticosterone and aldosterone are lower in MHS. Differences in receptor affinity were least for the strongest ligand (dexamethasone) and greatest for the weakest ligand (aldosterone). Binding capacities (Bmax) were not significantly different between strains (see Figure 5.2.). This observation is supported by previous reports of non-significant differences in the levels of GR mRNA in both liver and kidney from these rat strains (Stewart *et al.*, 1993). The apparent lack of GR down-regulation in MHS might be explained by a complementary reduction in steroid binding affinity accommodating the increase in plasma corticostreone levels. In patients with primary cortisol resistance (Arai and Chrousos, 1994; Bronnegard *et al.*, 1996), a lowering of GR binding affinity is predicted to cause an increase in blood pressure due to an increase in cortisol concentration acting on type I mineralocorticoid receptors. The resulting pathophysiology is similar to that found in MHS rats: increases in body sodium, plasma volume and plasma renin suppression.

The specificity and sensitivity of glucocorticoid hormone actions are controlled by a variety of factors including: 1.) access of hormone to the receptor which is regulated by enzymes and by cell membrane steroid transporters; 2.) a number of accessory proteins which, when complexed with the receptor, maintain a high affinity binding state and facilitate

translocation to the nucleus and interaction with glucocorticoid response elements in the DNA; 3.) tissue-specific receptor expression; 4.) the primary structure of the receptor protein. Most of these factors can be excluded as likely explanations of the suggested differences in GR binding affinity. Lower hepatic 11b-hydroxysteroid dehydrogenase (11β-HSD) activity has been observed in MHS compared with MNS (Stewart *et al.*, 1993). The liver isoform of 11β-HSD favours the reduction of 11-dehydrocorticosterone (biologically inactive) to produce corticosterone (Jamieson *et al.*, 1992). Reduced levels of 11β-HSD in MHS liver might therefore limit access of corticosterone to GR *in vivo*. However, reduced 11β-HSD activity is unlikely to be a cause of reduced receptor binding affinity in MHS *in vitro* for two reasons. Firstly, 11β-HSD is a microsomal enzyme which would be absent from the cytosolic extracts used in these experiments. Without added co-factors, even residual activity would be negligible. Secondly, the ranking of the differences in affinity between MHS and MNS for the steroids tested (aldosterone > corticosterone > RU486 > dexamethasone) does not match substrate specificity for 11β-HSD (Panarelli, 1994). The involvement of plasma membrane steroid hormone transporters in the regulation of receptor binding in cytosol is unlikely.

In clinical cases of steroid resistance, reduced binding affinity often results from receptor thermolability (Werner *et al.*, 1992). Characteristically, for these receptors, steroid binding affinity is markedly decreased by prolonged incubation at elevated temperatures (up to 37^{o}C) which is most likely the result of the dissociation of heat shock proteins (hsp) from the receptor. High affinity binding of GR is only observed when the receptor is complexed with other proteins including hsp90 and hsp70 (Pratt, 1993). Panarelli *et al.*, (1995) were able to show that GR from the SHR strain of rat (see chapter 3), which has a higher affinity for dexamethasone than the WKY strain, also shows a greater thermostability. No such difference between MHS and MNS receptors was found (results not shown).

Comparison of GR coding sequences from MHS and MNS strains of rat revealed no structural mutations which might explain the apparent differences in receptor steroid binding affinity. The only sequence difference identified was a polymorphism of the trinucleotide $(CAG)_n$ repeat (described in chapter 3). The MHS GR coding sequence has one CAG repeat less than that of MNS. While it is unlikely that the $(CAG)_n$ repeat polymorphism would account for the suggested differences in steroid binding between strains ($(CAG)_n$ repeat expansions in the human androgen receptor have no measurable effect on steroid binding; Chamberlain *et al.*, 1994) possible effects on the transcriptional regulatory properties of GR remain to be determined (see chapter 7). The lack of down regulation of GR by elevated plasma corticosterone levels (Stewart *et al.*, 1993 and pp 134-135 of this chapter) is suggestive of reduced GR activity in MHS.

The level at which glucocorticoids affect body weight is difficult to interpret. Low doses of glucocorticoid are required to maintain normal growth (Kenyon *et al.*, 1986) whereas supraphysiological doses are catabolic (Tonolo *et al.*, 1988). Similar effects are seen in children whereas adult patients with Cushing's disease tend to develop central obesity. Transgenic mice expressing an antisense GR message (displaying partial and tissue specific depletion of GR) develop obesity (Pepin *et al.*, 1992). Parental MHS rats are generally heavier than MNS, which is associated with hyperlipidaemia in the hypertensive strain. However, in F2 rats the MHS GR allele is associated with reduced body weight in male rats. It is not known whether this is due to changes in lipid metabolism or a difference in growth rate. *Grl* may represent a QTL which is in close linkage with another gene which affects weight gain. It is of interest that a gap junction protein locus on chromosome 18 is also found associated with body weight (Katsuya *et al.*, 1995).

Adrenal hypertrophy has been described in MHS, which appears to be caused by hyperplasia and hypertrophy of zona glomerulosa cells, rather than zona fasciculata cells (Mantero *et al.*, 1983). Whether reduced negative feedback of ACTH secretion due to abnormal GR properties can account for adrenal hypertrophy in MHS is unclear.

A significant association ($p < 0.005$) was found between the MHS *Grl* genotype and urinary calcium content in female F2 homozygotes. Hypercalcuria in the Milan hypertensive strain has been reported previously (Cirillo *et al.*, 1989). Despite normal serum calcium concentrations, in fasting conditions, urinary calcium is increased, while bone calcium content is significantly reduced. These findings strongly suggest that hypercalciuria in MHS may be explained by altered renal calcium handling. The incidence of hypercalcuria in F2 rats homozygous for the MHS GR allele suggests the possibility of a mutation, either in *Grl* (in GR itself or more likely in a closely linked gene involved in calcium metabolism) or at another locus in close linkage with *Grl*. Why this phenotype is restricted to female rats is unclear. This difference may reflect the mechanistic complications of calcium metabolism, which is diversified still further by gender. As described in chapter 4, the *Grl* haplotypes between MHS and MNS show a high degree of genetic divergence which, on the basis of their geneologic relatedness, is likely to exist elsewhere in the Milan rat genomes, including *Grl*.

One way to show whether or not *Grl* has an effect in determining phenotype in MHS could be the development of congenic strains of rat, in which *Grl* is essentially expressed *in vivo* on a normal or 'control' genetic background (Weil *et al.*, 1997). The development of such strains in Milan is currently in progress, in which the MHS *Grl* locus will be recombined into the normotensive (MNS) genetic background by a process of cross-breeding. The development of MHS *Grl*-linked phenotypes in the resulting hybrid strains might be indicative of a role for *Grl* in contributing to MHS phenotypes.

Chapter 6
Results 4

Steroid binding by glucocorticoid receptor proteins expressed in tissue culture

6.1.) Introduction

Functionally, triplet repeat expansions in the coding sequence of hormonally regulated transcription factors such as GR may be important at either of two levels: effects on steroid binding affinity and the ability of the molecule to function as a transcription factor.

Presented in this chapter is a detailed examination of the ability of rat GR proteins with variable length polyglutamine tracts to bind steroid ligands. Binding experiments for two steroid ligands, dexamethasone and corticosterone, were carried out for both natural and constructed GR alleles following expression in a selected cell line. Tissue culture cells provide a uniform system in which all cellular components, including proteins of the GR heterocomplex and concentrations of endogenous ligand, are standardised for each allele tested. This allows an unbiased functional assessment of GR variants.

Part 1

6.2.) Analysis of natural GR alleles expressed in COS-7 cells

6.2.1.) Methods for cloning of natural GR alleles

The expression clones pcDNA1Neo-rGR*21*, *20*, and *7* were constructed from the following plasmids: pRBal117 (see chapter 3, Fig. 3.2.); pSL301 (Invitrogen, Abingdon, Oxon, U.K.), a 3.2 kb bacterial cloning vector containing a superliker housing 46 novel restriction sites. The plasmid also carries a ColE1 replicon (promoting high copy number) and an ampicillin resistance gene; pcDNA1Neo (Invitrogen), a 7.0 kb eukaryotic expression vector carrying both colE1 and SV40 polyoma replicons (promoting high copy numbers in bacterial and eukaryotic cells, respectively). Nine novel

restiction sites are located in a short polylinker, preceded by a CMV promoter. A kanamcyin resistance gene is included for selection of recombinants in *E. coli*. All methodology for DNA restriction and cloning steps were as described in sections 2.2.6. and 2.2.10. Resulting clones were verified either by restriction analysis or by sequencing across ligation junctions.

Step 1: Deletion of single NcoI site from pSL301

A convenient method of constructing natural rat GR cDNAs with differing triplet repeat lengths, was to substitute the 5' 317 bp from an existing rGR cDNA (e.g. from pRBal117) with similar sequence, PCR-amplified from specific GR alleles containing triplet $(CAG)_n$ repeats of unique length. This was possible using a novel *Nco*I site in the GR coding sequence starting at n317 (relative to met1) and similarly required a vector, either without an *Nco*I site, or a single *Nco*I site which could be deleted such as pSL301. *Nco*I site deletion from pSL301 was achieved by *Nco*I-restriction, followed by Klenow fill-in of 3' recessed ends (described in section 2.2.10.) and re-ligation using T4 DNA ligase producing the vector, pSL301.1 (Fig. 6.1c.).

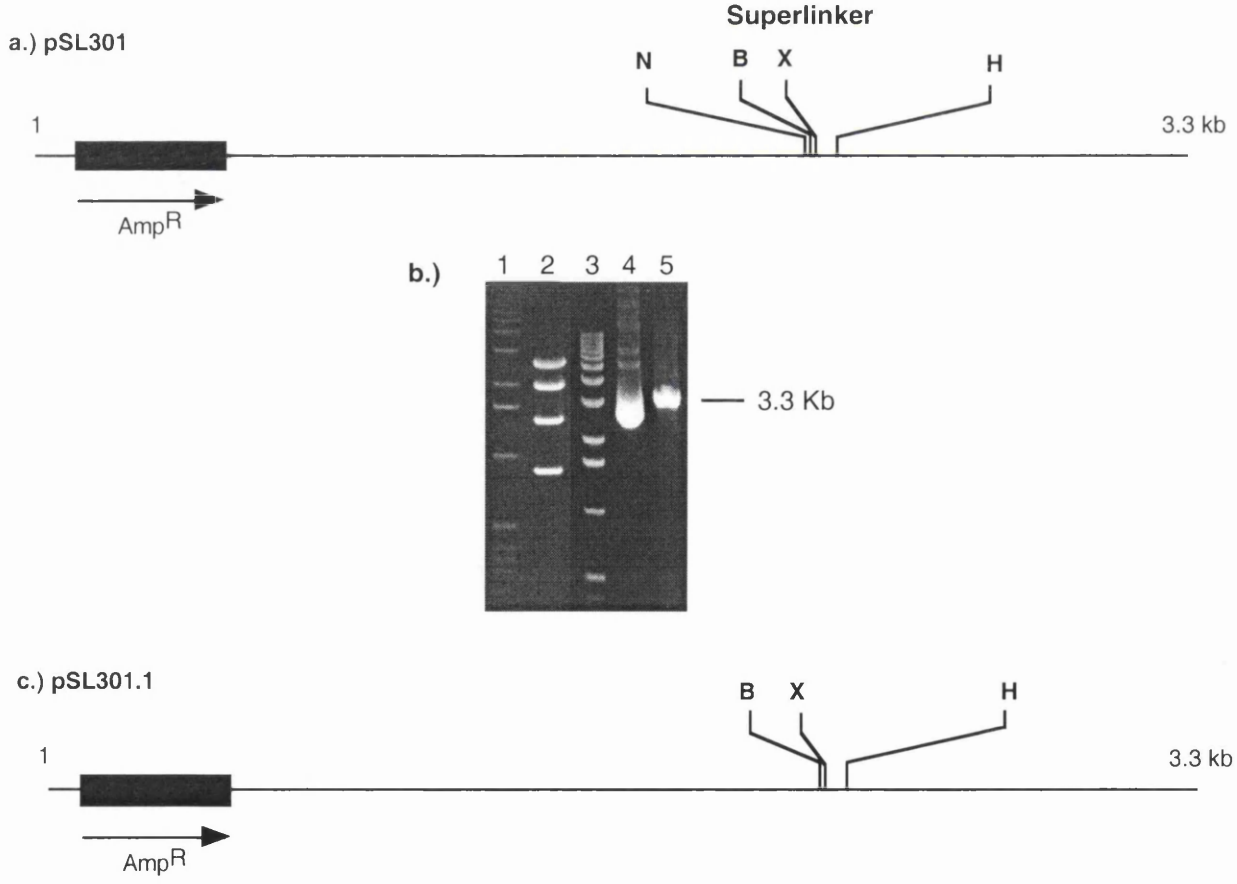


Fig. 6.1. Removal of *Nco*1 site from pSL301, producing pSL301.1.
a.) pSL301 native vector.
b.) *Nco*1 restriction of eukaryotic expression vector pcDNA1Neo and pSL301. pcDNA1Neo contains four *Nco*1sites, making it unsuitable for rGR *Nco*1 manipulations. Lanes 1 and 3, 1 Kb DNA ladder; lane 2, pcDNA1Neo cut with *Nco*1; lane 4, pSL301, uncut; lane 5, pSL301 *Nco*1 cut. Linear vector migrates at an expected 3.3 Kb.
c.) Minimal restriction map of pSL301.1 (superlinker *Nco*1 site deleted).

Step 2: PCR amplification of GR triplet repeat DNA, Cloning and sequencing

Amino-terminal GR coding sequences of genotypes *20* (from rat strain MHS) and *7* (from rat strain BC: chapter 3, Table 3.3.), including the first ATG start codon and triplet $(CAG)_n$ repeat, were amplified from rat genomic DNA as described in section 2.2.3. using Vent^{TM} DNA polymerase in conjunction with PCR primers rGR19 (carrying a *Bam*HI restriction site) and pG (Appendix 1). Vent^{TM} DNA polymerase was necessary to produce mutation-free PCR products. Amplified DNA was digested using *Bam*HI and *Nco*I, column purified to remove excess PCR primers and digested DNA ends (section 2.2.10.) and then ligated into the *Bam*HI/*Nco*I cut pSL301.1. Following transformation of DS941 competent cells, a selection of colonies from each rat strain DNA-specific ligation were picked into 5 ml LB medium supplemented with ampicillin (50μg/ml) and grown overnight at 37^{o}C as described in sections 2.2.11. and 2.2.12.

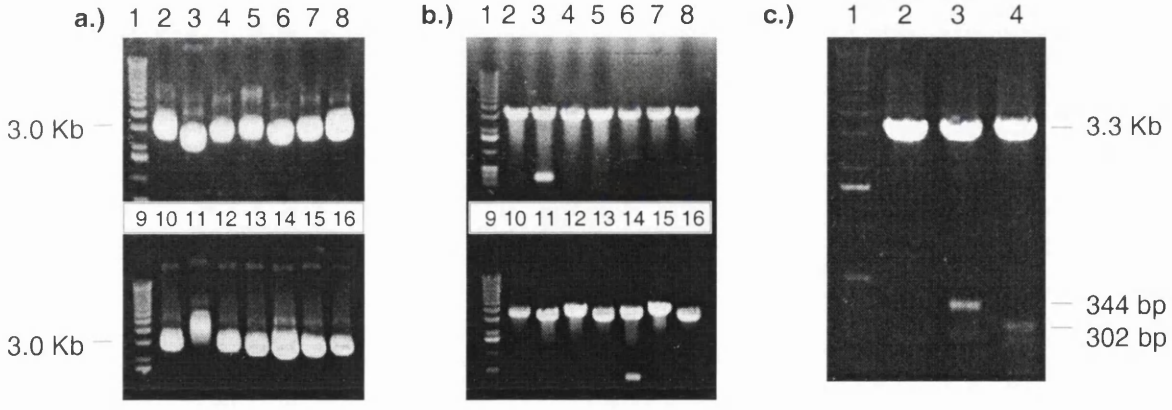


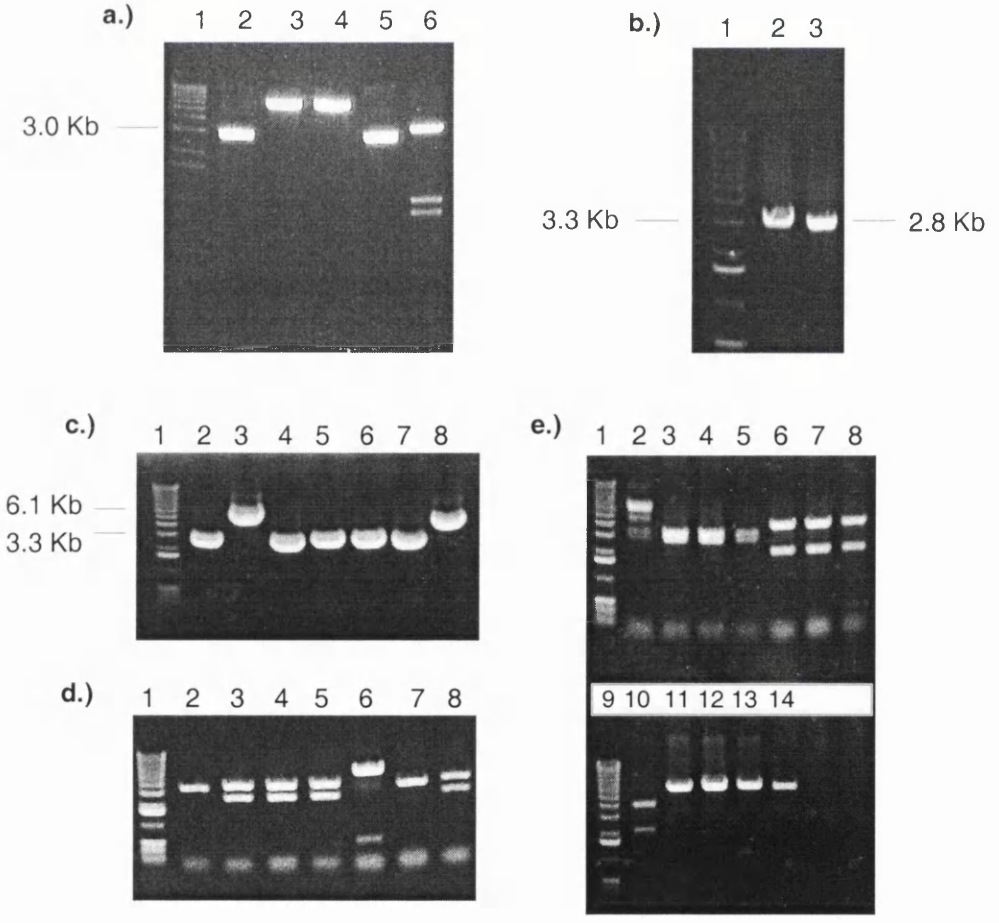
Fig 6.2. Identification of pSL301.1 clones containing rGR triplet repeats.
a.) Examples of miniprepped DS941 transformed using pSL301.1-rGR triplet repeats PCR product ligations (see text for details). Lanes 2-8, potential MHS (20-repeat) transformants; lanes 10-16, potential BC (7-repeat) transformants; lanes 1 and 9, 1 Kb DNA ladder.
b.) Clones from panel a.) digested using *Bam*HI and *Nco*I. Lanes 3 and 14 identify potential recombinants; lanes 1 and 9, 1 Kb DNA ladder.
c.) Following *Bam*HI and *Nco*I digestion, strain BC and MHS specific clones were identified by fractionation on 2% gels. Lane 1, I Kb DNA ladder; lane 2, pSL301.1 with no insert; lane 3, MHS triplet repeat positive clone; lane 4, BC triplet repeat positive clone.

Figure 6.2a. shows examples of plasmid miniprep DNA from each set of clones. A second round of *Bam*HI/*Nco*I digestion was able to identify those

plasmids containing a PCR fragment of the expected size. Faithful amplifications from each GR allele were identified by sequencing 5-10 μg of plasmid DNA from sense and antisense primers p9 and p19 respectively (Appendix 1), using sequenase version 2.0 for plasmid sequencing (section 2.2.13.). Clones carrying mutation-free inserts were preserved by streaking onto LB-ampicillin plates. A clone of each GR genotype was grown as a midiculture to provide DNA for further subcloning.

Step 3: Subcloning of rat GR cDNA from pRBal117 into pSL301.1

Manipulation of the rat GR coding sequence required an initial subcloning of the entire cDNA from pRBal117 (containing one *Nco*I site) into the vector pSL301.1. The rGR cDNA (2.8 kb; comprised of the receptor open reading frame, plus 24 nucleotides of the 5' UT and the first 360 nucleotides of the 3'UT) was lifted from pRBal117 by *Bam*HI restriction and ligated bi-directionally into pSL301.1 at a unique *Bam*HI site. Linearised vector DNA (Fig. 6.3b) was de-phosphorylated using calf intestinal phosphatase (CIP: see section 2.2.10.) to prevent vector re-annealing. This resulted in the clone pSL301.1-rGR*21* (Fig. 6.3f.).



f.) pSL301.1-rGR *21*

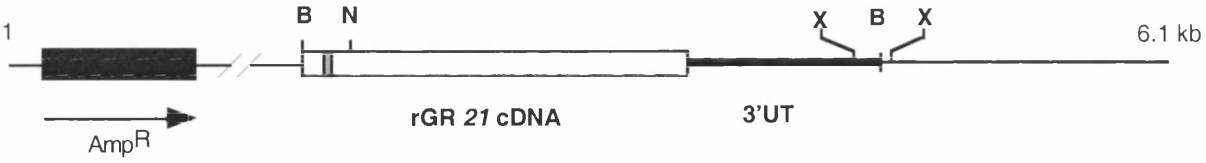


Fig. 6.3. Subcloning of the entire rat GR cDNA into pSL301.1.
a.) Restriction analysis of pRBal117 confirmed authenticity. Restriction enzymes used were: lane 2, *BamH*I; lane3, *Bgl*II; lane4, *Nco*I; lane5, *Xba*I; lane 6 *Hin*dIII. Lane 1, 1Kb DNA ladder.
b.) Band purified *Bam*HI-cut pSL301.1 vector (lane 2) and rGR cDNA insert (lane 3). Each lane shows a loading of approximately 50 ng of DNA. Fragments were ligated at a ratio of 1:1.
c.) Clonal selection showing examples of potentially positive recombinants (lanes 3 and 8).
d.) and **e.)** Restriction analysis of recombinants to check orientation of possible rGR inserts, using d.) *Eco*R1, lanes 2-8. Lane 1, 1 Kb DNA ladder and e.) *Bam*HI, lanes 2-5, *Bgl*I, lanes 6-8 and 10 and *Nco*I, lanes 11-14.
f.) Linear map of clone pSL301.1-rGR*21*.

Step 4: Removal of 3'-BamHI site from pSL301.1-rGR21

The *Bam*HI/*Nco*I subcloning required to produce GR triplet $(CAG)_n$ repeat length variants would be compromised by the presence of both 5' and 3' *Bam*HI sites. The 3' site was therefore removed by *Xba*I restriction at sites closely flanking the 3' *Bam*HI site, one in the rGR 3'UT (at position n2815: nucleotide coordinate taken from the published sequence) and the other at the 3' end of the pSL301.1 superlinker (Fig. 6.3f.). pSL301.1-rGR*21* was digested with *Xba*I, the 87bp *Xba*I/*Xba*I fragment removed (isolation of larger clone fragment by gel electrophoresis followed by band excision and dialysis: section 2.2.10.) and the clone religated through common *Xba*I cohesive ends. The resulting clone was named pSL301.1-rGR*21Xba* (Fig. 6.4.).

pSL301.1-rGR *21 Xba*

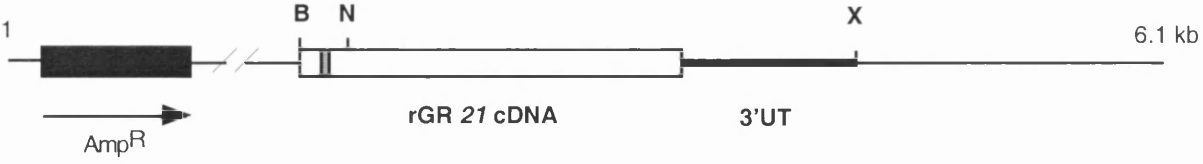
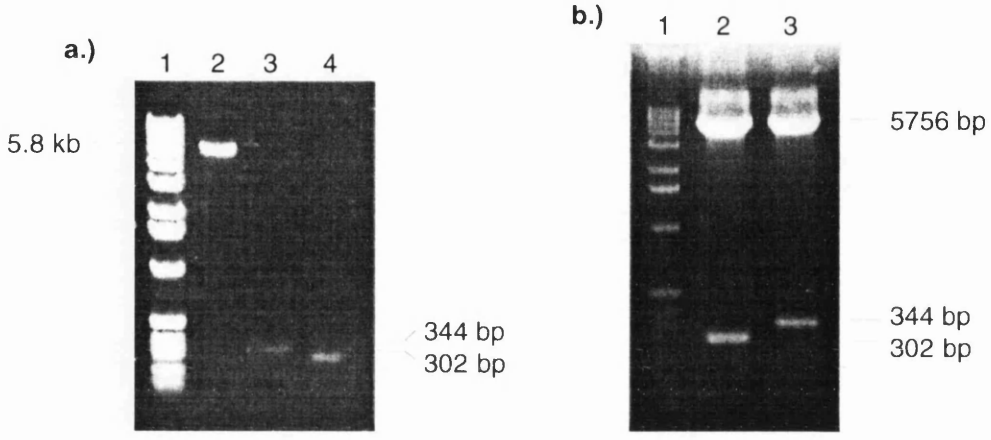


Fig. 6.4. Rat GR clone pSL301.1-rGR*21Xba*.
Removal of the 3' *Bam*HI site from clone pSL301.1-rGR*21* gave rise to a clone with novel *Bam*HI and *Xba*I sites, thus allowing simple directional subcloning into the mammalian expression vector pcDNA1Neo.

Step 5: Amino terminal BamHI/NcoI fragment replacement

The clone pSL301.1-rGR*21Xba* was digested with *Bam*HI and *Nco*I and the larger vector fragment isolated and purified as described in section 2.2.10.

pSL301.1-rGR clones containing mutation-free PCR amplified GR inserts of differing polyglutamine tract length (from step 1) were also digested (from midiprepped DNA) with *Bam*HI and *Nco*I. Insert DNA fragments were band fractionated on 1.5% agarose gels, purified by dialysis (Fig. 6.5a.) and substituted directionally into the linearised clone pSL301.1-rGR*21Xba* (Fig. 6.5c.).



c.) pSL301.1 rGR *20* and *7* *Xba*

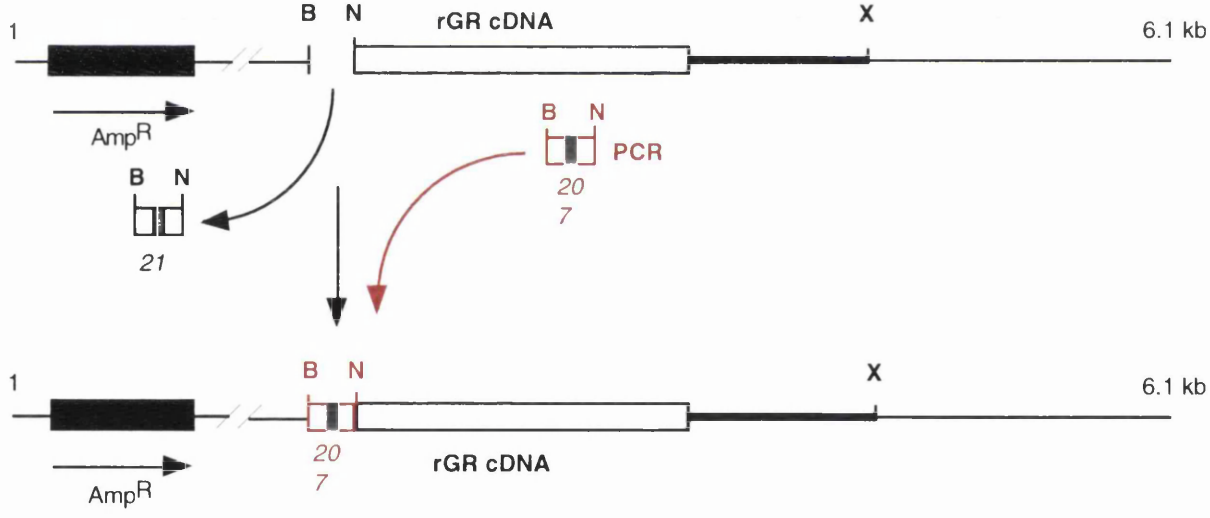


Fig. 6.5. Replacement of triplet $(CAG)_n$ repeat containing amino terminal fragment of rGR cDNA.
a.) Purified DNA fragments used in triplet repeat subcloning. Lane 2, 5.8 Kb *Bam*HI/*Nco*I cut pSL301.1-rGR*21 Xba* ; lane 3, 344 bp strain MHS triplet repeat specific PCR product insert; lane 4, 320 bp strain BC triplet repeat specific PCR product insert. Lane 1, 1 Kb DNA ladder.
b.) Verification of positive triplet repeat variant clones. Lane 2, BC specific; lane 3, MHS specific; lane 1, 1 Kb DNA ladder.
c.) Strategy for *Bam*HI/*Nco*I fragment replacement, producing 20 and 7 CAG repeat variant clones.

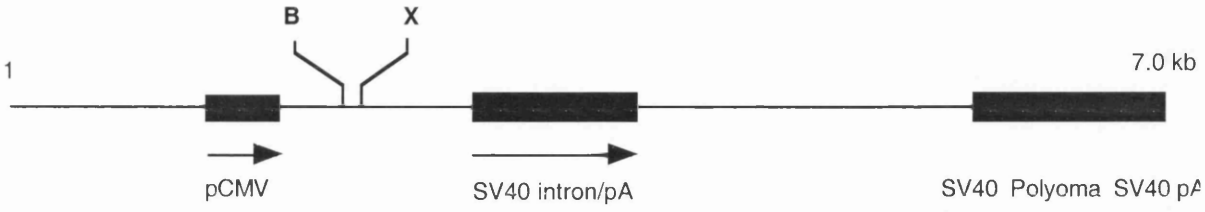
Potential recombinants were selected as described in Figure 6.3 and verified by *Bam*HI/*Nco*I restriction (Fig. 6.5b.) which also demonstrates

maintenance of the *Nco*I site in the coding sequence of rGR. Because no other manipulations were made, *Bam*HI/*Nco*I digestion was considered a sufficient check for these clones.

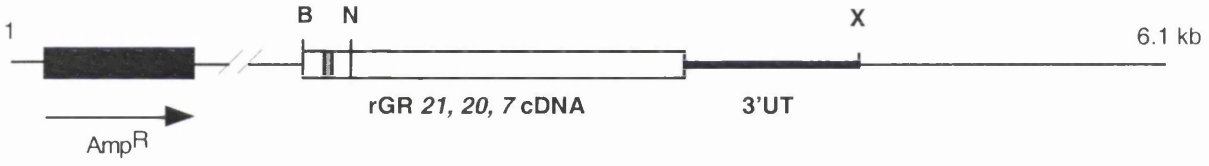
Step 6: Subcloning of full length rat GR cDNAs into pcDNA1Neo

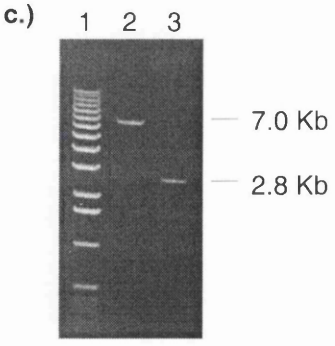
Clones pSL301.1-rGR*21, 20 and 7 Xba,* together with pcDNA1Neo (polylinker) were digested with *Bam*HI and *Xba*I. rGR cDNAs and linearised pcDNA1Neo were purified (Fig. 6.6c.) and ligated as described previously (Fig. 6.5.).

a.) pcDNA1Neo



b.) pSL301.1-rGR *21, 20, 7 Xba*





d.) pcDNA1Neo-rGR *21, 20, 7*

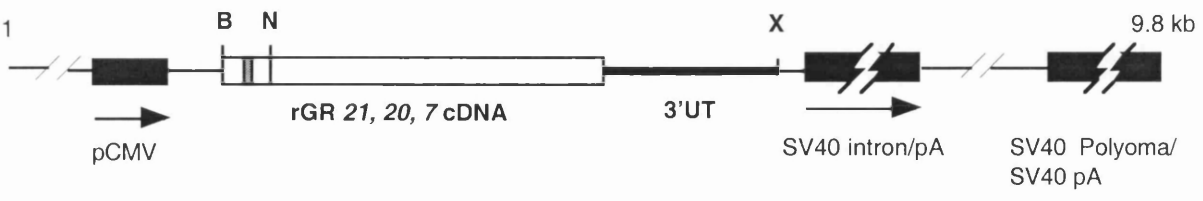


Fig. 6.6. Subcloning of rGR cDNA from pSL301.1 into mammalian expression vector pcDNA1Neo.
a.) Liner map of vector pcDNA1Neo.
b.) General liner map of pSL301.1-rGR*Xba* clones.
c.) Band purified *Bam*HI/*Xba*I-cut pcDNA1Neo (lane2) and example of rGR cDNA (21 repeat: lane 3). Lane 1, 1 Kb DNA ladder.
d.) General liner map of pcDNA1Neo-rGR*Xba* clones.

The orientation of rGR met1 ATG was confirmed for each pcDNA1Neo-rGR expression clone. Figure. 6.7c. shows part of the polylinker and ATG met1 of clone pcDNA1Neo-rGR*21* as an example. Clone pcDNA1Neo-rGR*18* was isolated following selection for pcDNA1Neo-rGR*20* clones in DS941. The authenticity of this clone was verified by sequencing of the triplet repeat and immediate flanking sequences, and by restriction analysis (see Fig. 6.7.). Prior to COS-7 cell transfection, all rGR cDNA expression clones were purified by cesium chloride density gradient centrifugation (Fig. 6.7d.).

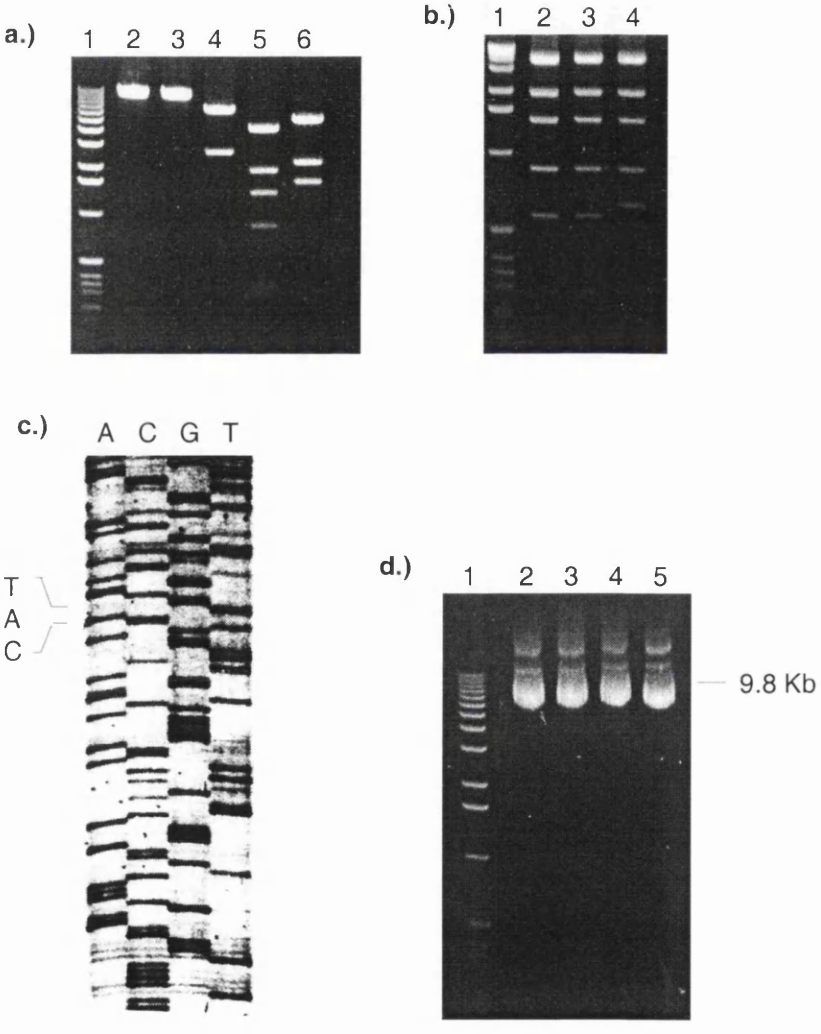


Fig. 6.7. Analysis of pcDNA1Neo-rGR clones.
a.) Restriction analysis of pcDNA1Neo-rGR*21* is shown as an example. Plasmid DNA was digested with *Bam*HI, lane2; *Xba*I, lane3; *Bam*HI/*Xba*I, lane4; *Bam*HI/*Nco*I, lane5 and *Bam*HI/*Eco*RI, lane6. Lane 1, 1 Kb DNA ladder.
b.) *Nco*I restriction analysis of clones pcDNA1Neo-rGR*21*, *20* and *7* (lanes 2-4, respectively). Lane 1, 1 Kb DNA ladder.
c.) Sequencing over met1 ATG confirming orientation of rGR cDNA in pcDNA1Neo clones. Clones were sequenced from the antisense primer, rGR29 (Appendix 1). The letters A, C, G and T above sequence tracks represent terminating nucleotides.
d.) pcDNA1Neo-rGR*21*, *20*, *18* and *7* clones, lanes 2-5, respectively. 1 µg of plasmid DNA was loaded per lane. Lane 1, 1 Kb DNA ladder.

Construction of triplet repeat negative clone, pcDNA1Neo-rGRµ (-)

A GR clone in which the triplet $(CAG)_n$ repeat and first two start codons (met1 and met2) were deleted, was constructed using the pSL301.1-rGRcDNA clone diagrammed in Figure 6.5c. In place of the subcloned rat strain specific PCR product, the *Bam*HI/*Nco*I gap was closed using a *Bam*HI/*Nco*I-ended double stranded oligo, constructed from oligo half sites rGR32 and rGR33 (Appendix 1).

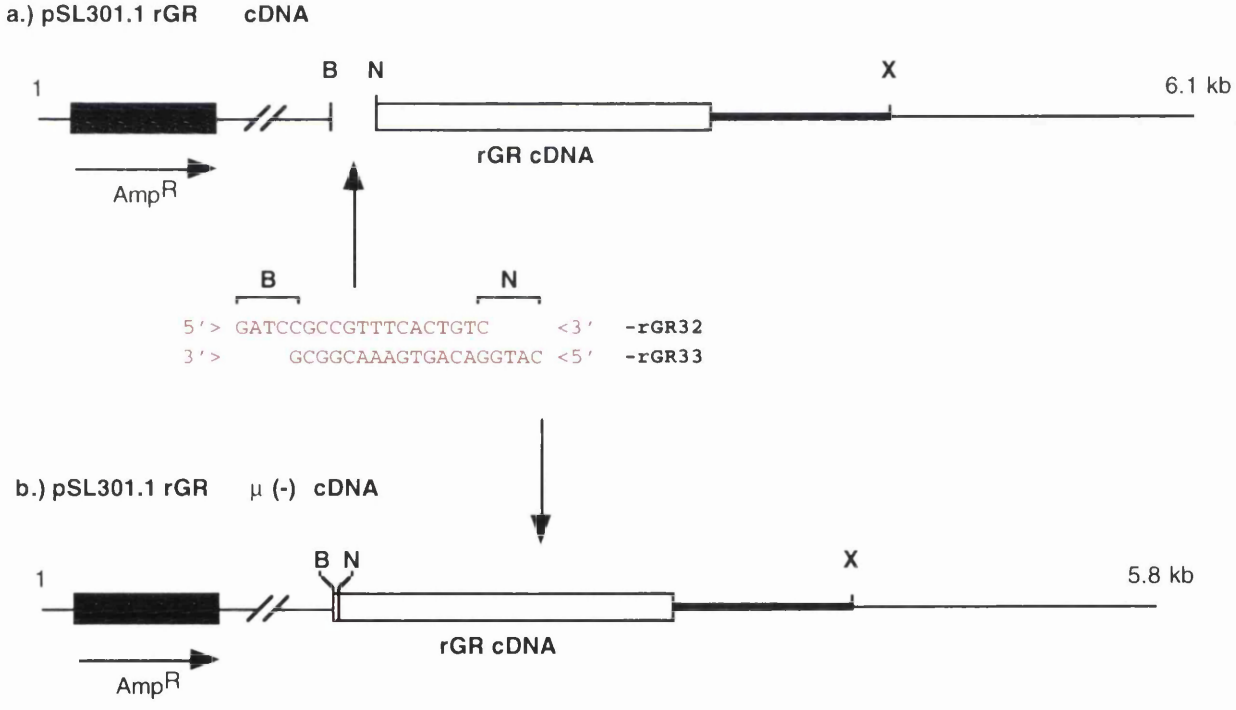


Fig. 6.8. Removal of triplet $(CAG)_n$ repeat and first two start ATG codons from rGR cDNA.
a.) pSL301.1 *Bam*HI/*Nco*I-cut. The insert oligo fragment is shown in red. Oligo half sites are detailed in Appendix 1.
b.) Resulting triplet repeat negative clone pSL301.1-rGR µ(-) cDNA. B, *Bam*HI; N, *Nco*I; X, *Xba*I.

The resulting rGR cDNA was then subcloned into the expression vector pcDNA1Neo as previously described.

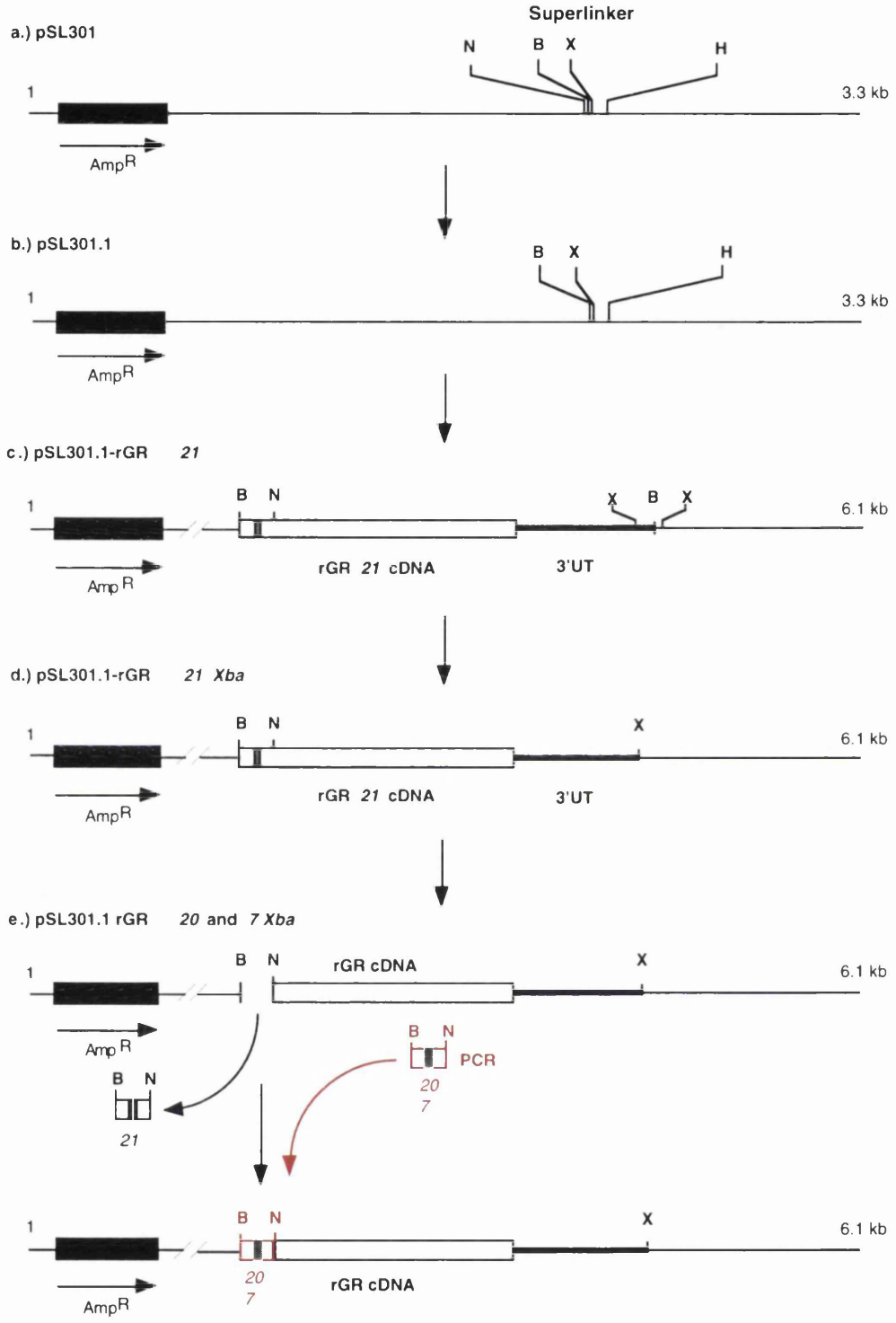


Fig. 6.9. Summary of the subcloning of rat GR cDNA from clone pRBal 117 into pSL301.1.

a.) Manipulation vector pSL301, showing superlinker with positions of unique *Nco*I, *Bam*HI and *Xba*I restriction sites.

b.) Deletion of *Nco*I site from pSL301, producing vector pS301.1.

c.) *Bam*HI ligation of 2.8 kb rat GR cDNA from clone pRBal117 into pSL301.1.

d.) Removal of 3' *Bam*HI site from cDNA cloning site.

e.) Replacement of 5' *BamHI/Nco*I restriction fragment (genotype *21*), with similar PCR amplified fragments of genotypes *20* and *7* (see text for details).

Restriction sites are as follows: N, *Nco*I; B, *Bam*HI; X, *Xba*I; H, *Hind*III. Amp^R, Ampicillin resistance gene. 3'UT, GR untranslated sequence. ORF, open reading frame. PCR, GR amplified PCR product DNA. kb, kilobase pair.

6.2.2.) Culture of COS-7 cells and transfection with pSVβ-gal and pcDNA1Neo-rGR clones

The initial cell line chosen for transfection and GR steroid binding experiments was COS-7, which contains trace levels of endogenous GR. COS-7 cells are derived from CV-1 cells but, unlike CV-1 cells, contain the SV40 T-antigen, promoting high copy number of transfected plasmids carrying the SV40 origin of replication (Gluzman, 1981).

Pilot experiments of transient transfection into COS-7 cells using DOTAP were carried out with the constitutive β-galactosidase expression clone pSVβ-gal transfected at varying concentrations. β-galactosidase expressing cells were identified 48 h post transfection by X-gal staining (section 2.2.18.), which showed the relative numbers of transfected cells. For preliminary analysis of GR expression and subsequent GR expression for steroid binding, cells at 80% confluence, were transfected with varying quantities of pcDNA1Neo-rGR expression clones (10 μg for steroid binding assay) DNA using DOTAP (section 2.2.17.). COS-7 cells were grown on 10 cm tissue culture dishes (Nunclon, GIBCO-BRL) in DMEM prior to and throughout the 'transfected' growth period.

6.2.3.) Western blotting using an rGR-specific monoclonal antibody

Prior to the use of GR monoclonal antibody Mab250, an optimal working concentration was determined by dot-blot analysis (section 2.2.22.). Antibody at dilutions of 1:500 and 1:1000 were incubated with strips of PVDF membrane spotted with rat liver cytosol extract, prepared as described in section 2.2.21.. Cytosol was spotted at concentrations of: 0.01-0.02, 0.1-0.2, 2 and 10 μg total protein.

GR protein from alleles, *Grl*$^{CAG\ 7,\ 18,\ 20,\ and\ 21}$ expressed in COS-7 cells was analysed on 15x20 cm 8% SDS-polyacrylamide gels (section 2.2.22.). Aliquots of transfected cells were lysed directly in SDS-gel loading buffer prior to gel loading to try and avoid unnecessary disruption of the GR protein. Samples were band fractionated against prestained high molecular weight protein standards and Sprague Dawley rat liver cytosol extract.

Proteins were blotted onto PVDF membrane and probed using the rat GR N-terminal specific monoclonal antibody Mab250 at a dilution of 1:750. Specific antibody binding to rat GR was detected autoradiographically using the ECL Western blotting kit (see section 2.2.22.).

6.3.) Results

6.3.1.) Expression of β-galactosidase in COS-7 cells

Cells successfully transfected with pSVβ-gal and therefore expressing β-galactosidase appeared blue following staining with X-gal. Figure 6.10. shows the proportional increase in numbers of blue cells with increasing concentrations of transfected plasmid DNA (Fig. 6.10. panels a.) to c.)). The transfection of a completely unrelated vector (pcDNA1Neo) produced no blue cells (Fig 6.10 panel d.)). Under optimum conditions, it is recommended that DOTAP can promote transfection efficiencies of up to 50% or more, depending on the cell type and the quality of transfected DNA. This level of transfection was considered attainable with around 5-10 μg of pSVβ-gal DNA (Fig 6.10. panels b.) to c.)). A quantity of 5 μg was therefore chosen as the starting concentration of GR clone DNA in testing for the expression of GR proteins in COS-7 cells.

6.3.2.) Expression of rat GR proteins in COS-7 cells

Detection of overexpressed GR proteins

Following 48h growth, COS-7 cells transfected with GR expression clones were harvested, disaggregated and counted using a haemocytometer. Cells were pelleted and washed twice in ice cold PBS (pH 7.4). Routinely, 1-2x10^6 cells were harvested per 10cm plate. Figure 6.11b. shows the level of GR expression in approximately 1x10^6 cells per GR clone.

All clones were expressed, producing similar levels of GR protein. No obvious differences in protein stability were found. A low level of endogenous GR was also detected in untransfected COS-7 cells (Fig 6.11., lane

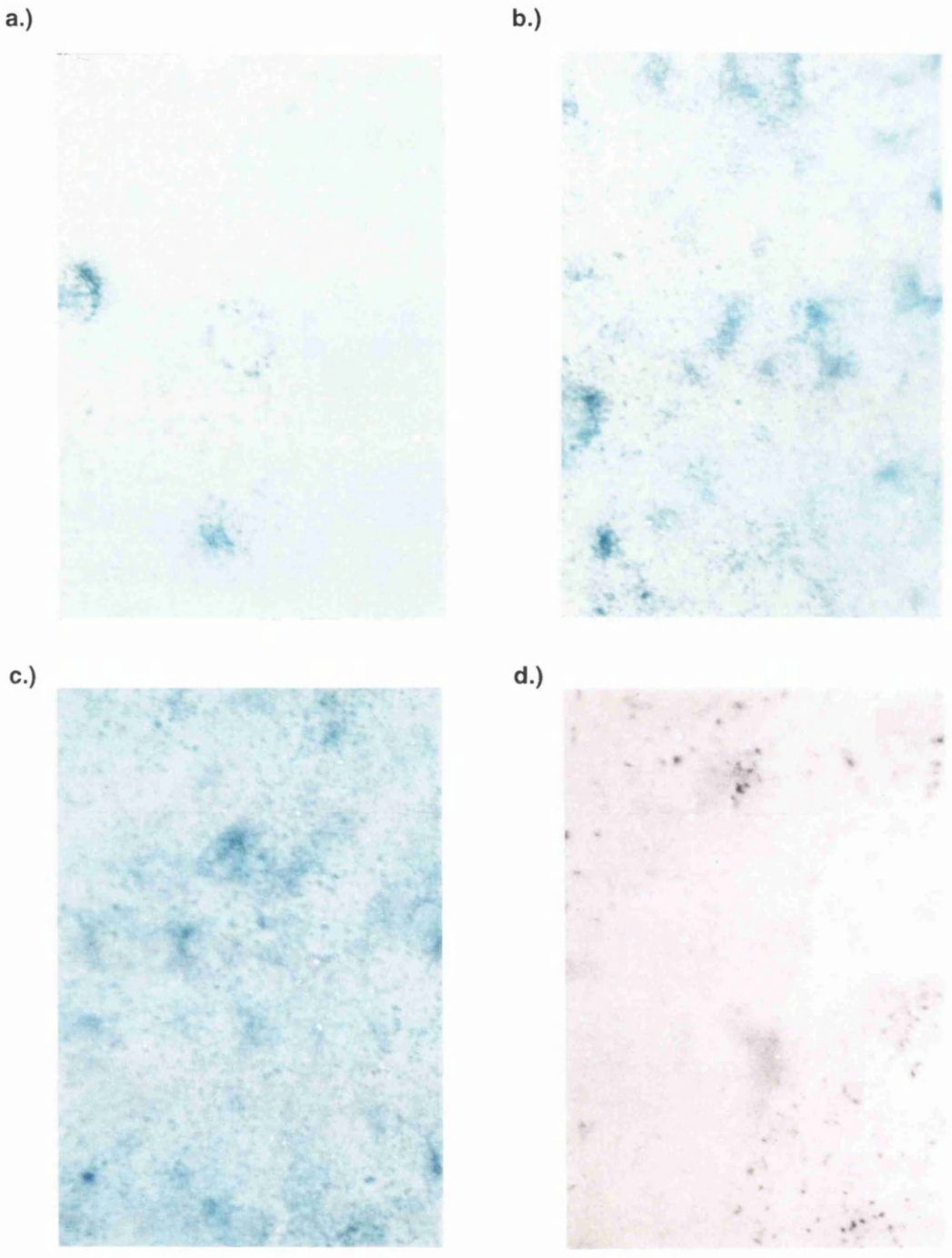


Fig. 6.10. COS-7 cells transfected with the constitutive β-galactosidase expression clone pSVβ-gal.
Cells were transfected with: **a.)** 2 μg, **b.)** 5 μg or **c.)** 10 μg pSVβ-gal. Positively transfected cells (expressing β-galactosidase) appeared blue following treatment with X-gal/IPTG. Cells transfected with native pcDNA1Neo were unable to breakdown X-gal and remained unstained (panel **d.)**). All plasmid DNA was transfected using DOTAP (see text for details).

6). Transfection with the vector pcDNA1Neo alone (Fig. 6.11., lane 2) produced no noticeable increases in the level of GR.

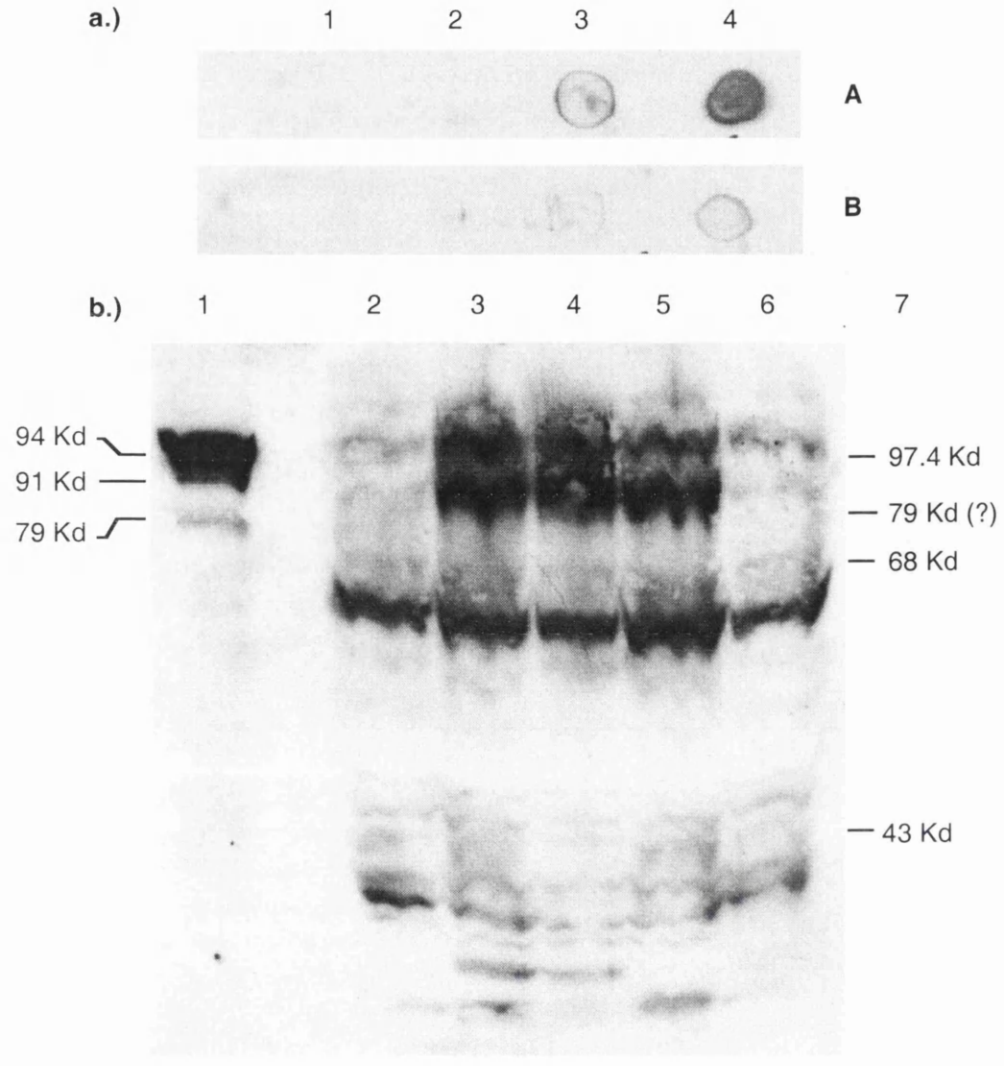


Fig. 6.11. Specific binding of rat glucocorticoid receptor proteins by rat GR monoclonal antibody Mab250.
a.) Dot blot of rat liver cytosol extract: dot 1, 0.1-0.2; dot 2, 1-2, dot 3, 10-20 and dot 4, 100 μg total protein, probed with differing concentrations of Mab250; panel A, 1:500 dilution. Panel B, 1:1000 dilution.
b.) Western blot of rat GR proteins expressed from different GR alleles in COS-7 cells and separated on an 8% SDS polyacrylamide midi-gel. Lane 1, 50 μg rat liver cytosol extract (Sprague Dawley: size standard). Lanes 2-5, COS-7 cells transfected with 5 μg each of: native pcDNA1Neo; lane 2 and pcDNA1Neo-rGR*21*; *20* and *7*, lanes 3-5, respectively. Lane 6, untransfected COS-7. Lane 7, Prestained high molecular weight size markers (GIBCO). Relative marker positions are shown. Each loading in lanes 2-6 represents the proceeds of $1x10^6$ cells (see text for details).

Evidence of additional GR molecules

The size of the highest molecular weight GR band (Fig 6.11., lanes 1-6; 94 kd) was verified using molecular weight size standards (GIBCO, U.K.) and

Sprague Dawley rat strain hepatic GR run in parallel (Fig. 6.11, lanes 7 and 1 respectively). Expression of rat GR proteins from the pcDNA1Neo vector produced evidence of an additional GR molecule which was not expressed to a significant level in rat liver (Fig. 6.11, lane 1). In COS-7 cells, this additional GR species migrated faster than full length (94 Kd) rGR, suggesting it might have arisen from an internal translation event. Expression of pcDNA1Neo-cloned rat GR alleles in CV-1 cells, (which is included at this point to help identify the problem with pcDNA1Neo clones and will be discussed in greater detail in part 2 of this chapter) also produced an additional GR band. This migrated more slowly than the 94 Kd GR species (Fig. 6.12.), suggesting a translation initiation from within the pcDNA1Neo vector.

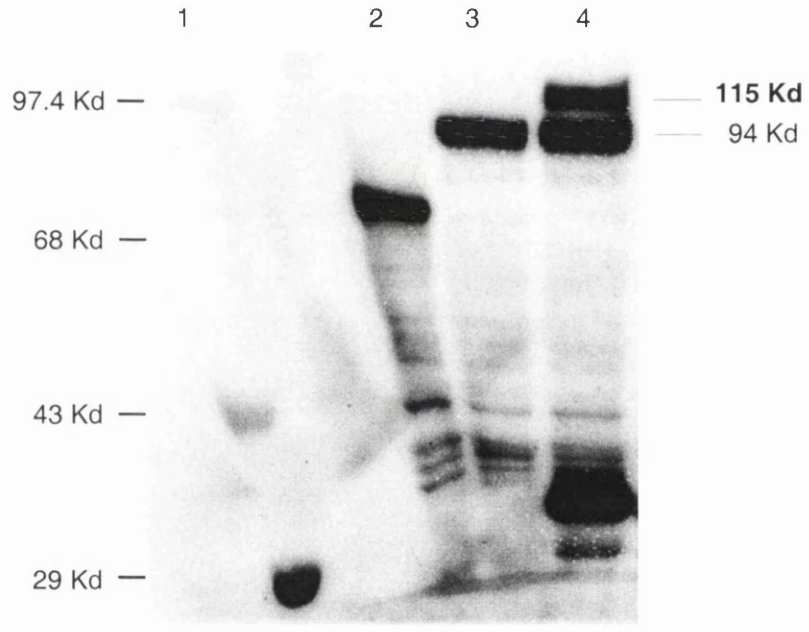


Fig. 6.12. Comparison between pcDNA1Neo and pSTC-vector directed expression of the GR *21* allele in CV-1 cells.
Lane 1, high molecular weight markers (GIBCO). Lanes 2-4; GR protein expressed from clones; pSTCrGR3-556 (LBD (-) clone, described in part 2 of this chapter) (lane 2); pSTCrGR*21* (lane3) and pcDNA1Neo-rGR*21* (lane 4). Lanes 2-4 were loaded with 20-30 μg of total cellular protein extracted by sonication (detailed in section 2.2.21.)

This additional GR form was absent from rat liver cytosol preps and was undetectable in untransfected CV-1 cells (e.g. Fig. 6.20.), or the rat hepatoma cell line 2S-Fasa (e.g. Fig. 6.23.).

Steroid binding by GR expressed in COS-7 cells

Pilot assays of dexamethasone binding by COS-7 cells transfected with and expressing GR allele *21* (from clone pcDNA1Neo-rGR*21*) were performed using a whole cell assay based on the general method outlined in section 2.2.21. $0.5x10^6$ cells were assayed at each concentration of unlabelled steroid. Untransfected COS-7 cells were used as a control to monitor for binding by endogenously expressed GR. Binding data, represented as a Scatchard plot in Figure 6.13., identified low levels of a high affinity binding site (Kd, 2.78 nM) in untransfected COS-7, characteristic of rat GR (see introduction to chapter 3 and chapter 5).

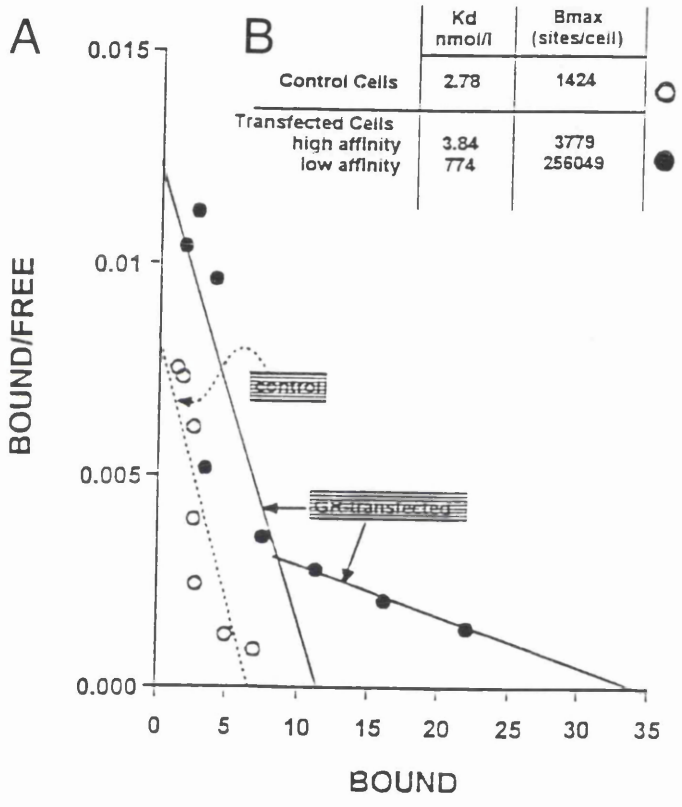


Fig. 6.13. Dexamethasone binding characteristics of control and GR-transfected COS-7 cells.

A; Scatchard plots of ^{3}H-dexamethasone binding by rat GR proteins expressed in COS-7 cells. Plot through open symbols; binding to control cells. Plot through solid symbols; binding to GR-transfected cells.

B; Measurements of Kd (nM) and Bmax (sites/cell) for control and GR-transfected COS-7 cells.

In cells transfected with GR allele *21*, there was evidence of two binding sites, one with similar binding characteristics to that of untransfected cells (low capacity, high affinity; Kd, 3.84 nM) and a second site of low affinity,

high capacity (Kd, 774 nM). Consistent with the observations from untransfected cells and Western blots (Fig. 6.11.), the most likely explanation for two apparent binding sites in transfected cells is that the high affinity site represents full length GR, partly endogenous receptor, partly expressed from GR allele *21* (Bmax of the high affinity species more than doubles in transfected, compared with untransfected cells), while the low affinity site may represent the lower molecular weight (79 Kd) species (see Fig. 6.11.).

6.4.) Discussion

The expression of rGR from pcDNA1Neo clones in COS-7 cells presented a number of fundamental problems in the analysis of steroid binding by GR: 1.) Because of the high transfection efficiency and level of expressed GR protein required for an optimal steroid binding assay, the use of DOTAP was adopted as the initial method of DNA delivery into COS-7. At the time this work was carried out in Glasgow, transfection with DOTAP was considered to be perhaps the most efficient method of transfection into this cell line. However, the efficiency of transfection was found to be inconsistent. The number of unsuccessful transfections (monitiored by β-gal staining for pSVβ-gal, or Mab250 detection of transfected and expressed GR protein following fractionation by Western blot) was high, at around 60-70% (results not shown).

2.) COS-7 cells express endogenous basal levels of GR. Although relatively low, this level of expression becomes significant in the analysis of transfected GR (Fig. 6.11b. and Fig. 6.13). Large numbers of transfected COS-7 cells would introduce significant levels of COS-7 GR into steroid binding assays which would contribute a significant proportion of the high affinity binding sites. This was found to hinder the interpretation of data generated from GR proteins expressed from transfected alleles (see Fig. 6.13).

3.) With the pcDNA1Neo expression system, there was the added complication of additional forms of GR. In COS-7 cells, rGR expressed from the pcDNA1Neo CMV promoter produced the expected 94 Kd species and an abundance of a lower molecular weight species of around 79 Kd (Fig. 6.11.). The obvious problems incurred by this molecule (generated from all pcDNA1Neo cloned rGR cDNAs) in steroid binding assays is shown in Figure 6.13. The presence of a form of GR with a completely different steroid binding characteristic to the full length GR molecule would present problems in the final data analysis.

In CV-1 cells, expression of GR from pcDNA1Neo clones also generated an additional GR band on Western blots (Fig. 6.12.). In this case however, the extra GR molecule was of a higher molecular weight than the 94 Kd species, estimated at 115 Kd and consistent with a translation initiation starting from within the pcDNA1Neo CMV promoter (e.g. from ATG n1688: coordinate taken from the pcDNA1Neo vector). Although vector promoter initiations should be prevented by numerous in-frame stop codons, which occur before met1 of the cloned cDNA, the additional GR bands expressed from pcDNA1Neo clones in both COS-7 and CV-1 cell lines are difficult to explain. To date, there have been no reports in the literature of GR glycosylation, a common source of post-translational modification producing differential bands for membrane proteins or proteins targeted to subcellular compartments. The other possibility, that of phosphorylation, which is known to create larger molecular weight species in the case of the progesterone receptor (S. Rusconi, personal communication) can be ruled out in this case, since the transfer of the GR cDNAs to another expression vector (see end of this discussion and Fig. 6.12.) produces a single 94 Kd GR band from the same coding sequence.

The expression of the triplet repeat negative rGR clone pcDNA1Neo-rGRμ(-) (diagrammed in Fig. 6.14.) in CV-1 cells also produced evidence of two GR molecules. One migrated at around 79 Kd, which is most likely to result from translation initiation from GR met3 which was relocated to the start of this truncated cDNA.

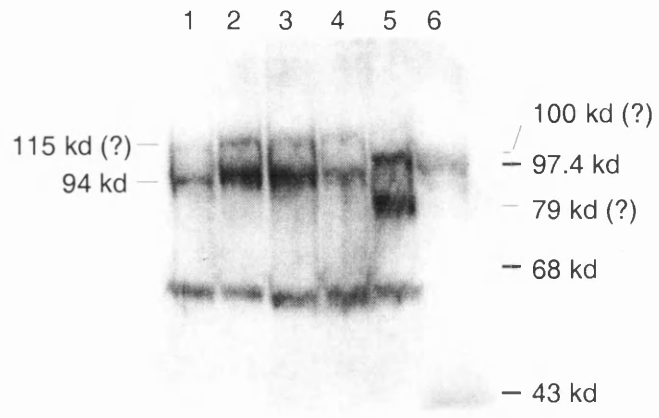


Fig. 6.14. Comparison of rGR and rGR μ(-) alleles expressed in COS-7 cells.
Lanes 1-4, expressed pcDNA1Neo-rGR *7*, *18*, *20* and *21* alleles respectively showing extra band at around 115 Kd. Lane 5, clone pcDNA1Neo-rGR μ(-) expressed to produce GR bands of around 100 and 79 Kd (see text for details). Lane 5, High Molecular Weight Markers.

The larger molecule migrated at around 100 Kd and was consistent with the expected size of a GR product generated from the removal of the first 106 amino acids from the initially suggested 115 Kd species (i.e., 687aa [size of rGR μ(-)]/795aa [size of full length rGR] x 115 Kd = 100 Kd (Fig 6.12.).

It was therefore proposed to find an alternative GR negative cell line expressing no, or only trace amounts of GR (e.g. CV-1, Gluzman, 1981; rat hepatoma cell lines EDR3, Cook *et al.*, 1994, or 6.10.2., Miesfeld *et al.*, 1986) and secondly, to alter the expression system to provide a more specific GR product. Figure 6.12., (lane 3) shows the specificity of GR produced from an alternative mammalian expression vector, pSTC. The rational for using this vector will be described at the beginning of part two of this chapter.

GR seems to be expressed naturally *in vivo* as a heterogeneous population of protein molecules, initiated from different methionine codons along the GR transcript (Miesfeld *et al.*, 1986 and results presented in this thesis; Fig. 6.11.). The main factor which determines choice of start codon in a protein coding sequence, is the arrangement of nucleotides around each ATG start site, referred to as the kozac sequence. This is particularly relevant to those ATGs at or close to the 5' end of the coding sequence and an optimal Kozak sequence has been described (Kozak, 1986). When the Kozak sequence around met1 matches this 'optimum' ((CC)ACC*ATG*G) then maximum

translation initiation can be expected, reducing the probability of initiations at other methionine codons further downstream. Because met1 of rat GR is preceded by a relatively poor Kozak sequence (S. Rusconi, personal communication), this may explain the additional initiations at met2 and met3, which have Kozaks closer to the optimum (met3 being the closest). Table 6.1. below, compares the first 5 methionine Kozak sequences of GR in the rat.

Kozak sequence **('optimum' = (CC)ACCATGG)**				
met1	met2	met3	met4	met5
CCAATGG	GTAATGG	*TCCATGG*	TATATGG	GTGATGG
(1)	(28)	(107)	(111)	(119)

Table 6.1. Comaparison of Kozak sequences in and around the first 5 methionine codons of the rat GR coding sequence.
The Kozak sequence closest to 'optimum' (Kozak, 1986) for translational initiation, is shown in italics. Numbers in parentheses below ATG start codons give amino acid positions relative to met1. A comparison of initiation codons at each of these sites is compared for the GR of different species in the general discussion (Table 7.1).

The use of multiple possible start codons in the rat GR message might explain the different translation patterns seen in COS-7 and CV-1 cells. However, the mechanisms which control the choice or extent to which individual start codons are used remains unclear.

Part 2

6.5.) Expression of GR alleles in CV-1 cells

6.6.) Cloning of natural and construct GR alleles in vector pSTC

The following plasmids were used to construct further GR alleles based on the mammalian expression vector pSTC:

pSTCrGR*21* (a gift from S. Rusconi, University of Fribourg, Switzerland): a 7.3 kb rat GR cDNA eukaryotic expression clone. The rGR expression is driven by a CMV promoter linked to a thymidine kinase leader and AUG start codon (leading directly into the rGR coding sequence). The core plasmid sequence is based on a modified version of pSP64 and carries an ampicillin resistance gene. Replication in eukaryotic cells is driven by an SV40 replicon.

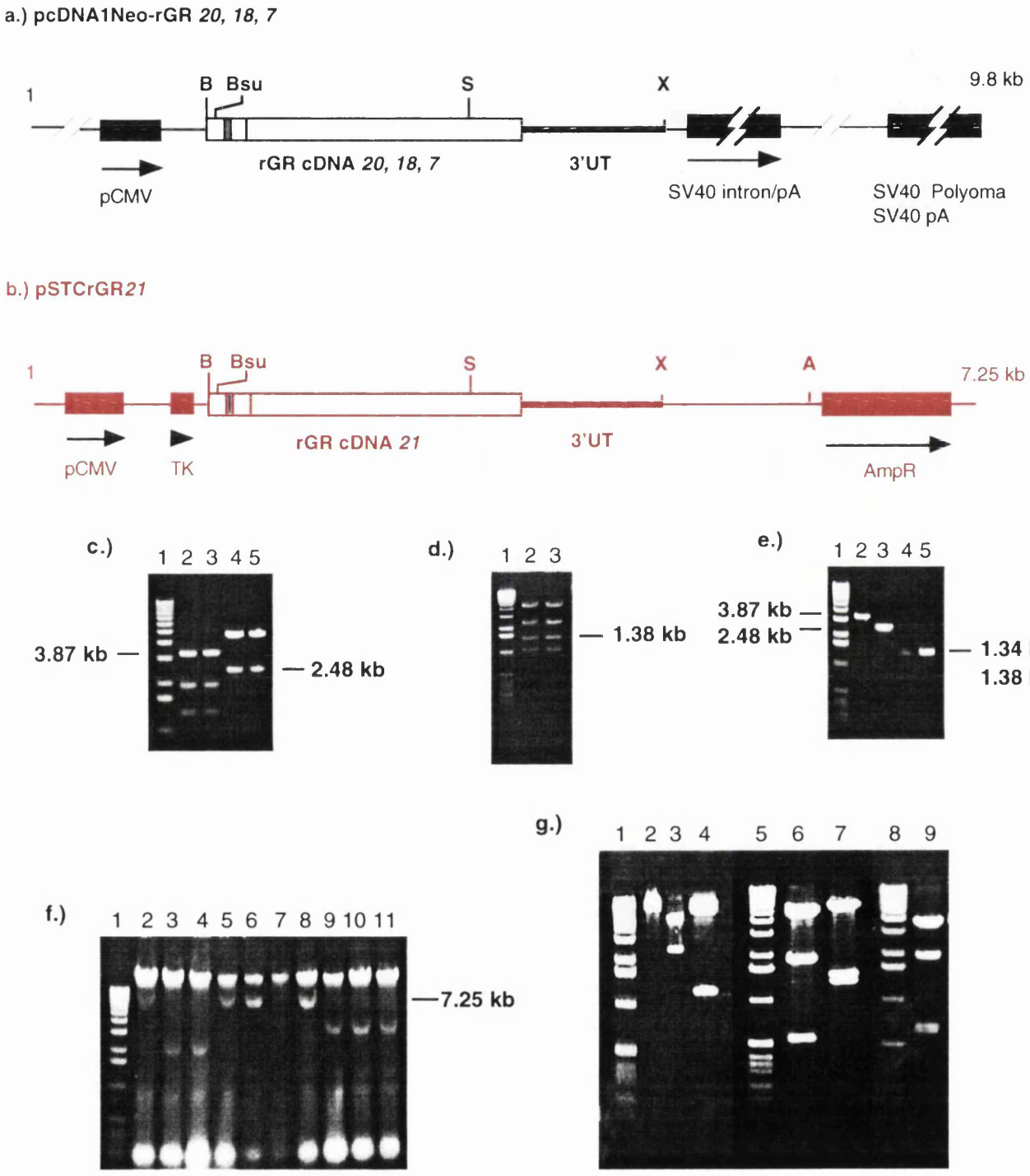
pSP64-4, 6 and 8-CAG series (a gift from S. Rusconi): pSP64 vectors containing polymers of 4, 6 and 8-CAG repeats.

Subcloning of natural rGR cDNAs from pcDNA1Neo clones into the vector pSCT.
The expression of rGR cDNAs from pcDNA1Neo clones (part 1 of this chapter) generated significant levels of additional GR isoforms. Preliminary assays indicated that these would have presented problems in the interpretation of steroid binding assays, which for GR assume a one-site interaction model (Panarelli, 1995).

The solution to this problem was to clone the rGR cDNAs into a suitable expression vector at a site which would allow modification of transcription and translation initiation from GR. This was achieved by linking the start of the GR coding sequence to a thymidine kinase leader and ATG start codon in the vector pSTC.

The clone pSTCrGR*21*, already containing a full length GR cDNA provided a convenient base for subcloning. Using appropriate restriction sites, a 1381

bp rGR *Bsu*36I/*Sph*I cDNA fragment from previously generated pcDNA1Neo plasmids (part 1, Fig. 6.6) was subcloned into the pSTC vector as described in Figure 6.15. below.



h.) pSTCrGR *20, 18, 7*

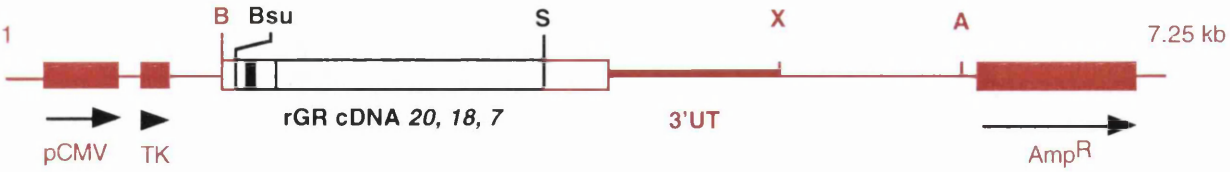
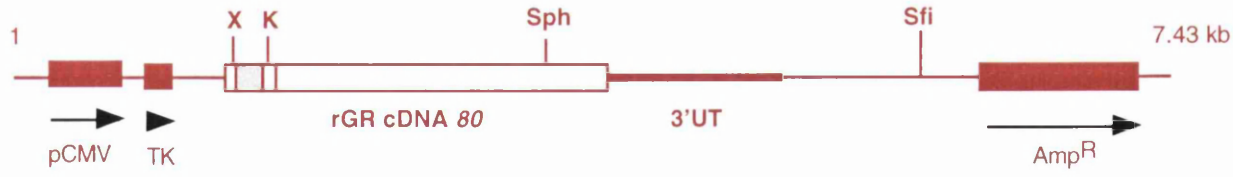


Fig 6.15. Subcloning of rGR cDNA variants from pcDNA1Neo into pSTC.
a.) Minimal restriction map of pcDNA1Neo, showing restriction sites used in cDNA subcloning.
b.) Minimal restriction map of expressioin vector pSTC.
c.) Digestion of clone pSTCrGR*21* using *Bsu*36I/*Avr*II (lanes 2 and 3) and *Sph*I/*Avr*II (lanes 4 and 5). Lane 1, 1 kb DNA ladder.
d.) Digestion of clone pcDNA1Neo-rGR*21Xba* using *Bsu*36I/*Sph*I (lanes 3 and 4). Lane 1, 1 kb DNA ladder.
e.) Size verification of purified DNA fragments used in subcloning. Lane 2, 3.87 kb pSTCrGR*21 Bsu*36I/*Avr*II vector fragment; lane 3, 2.48 kb pSTCrGR*21 Sph*I/*Avr*II vector fragment; lane 4, 1.34-1.39 kb pcDNA1Neo-rGR*21Xba Bsu*36I/*Sph*I rGR cDNA fragment. Lane 1, 1 kb DNA ladder.
f.) SCOP analysis of colonies carrying potential recombinants and selection of clones of around 7.0 kb.
g.) Typical restriction analysis of potentail recombinant clones. Lane2, *Sph*I; lane 3, *Apa*I; lane 4, *Xba*I; lane6, *Sph*I/*Apa*I ; lane7, *Sph*I/*Xba*I ; lane 9 *Bam*HI/*Bsu*36I. Lanes 1, 5 and 8, 1 kb DNA ladder.
h.) Minimal restriction map of recombinant pSTCrGR clones. B, *Bam*HI; Bsu, *Bsu*36I; S, *Sph*I; X, *Xba*I; A, *Avr*II.

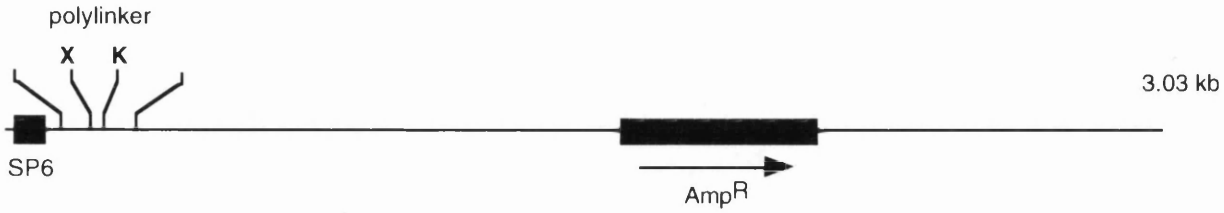
Construction of rGR cDNAs containing novel triplet $(CAG)_n$ repeat sequences.

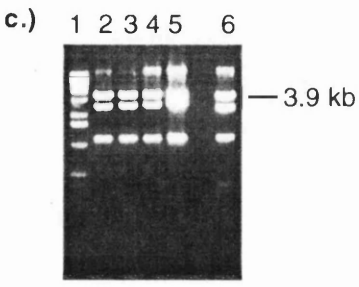
pSTCrGR*10, 20, 40* and *80* and pSP64 clones carrying triplet $(CAG)_n$ repeats of 4, 6 and 8 were kindly provided by S. Rusconi. GR clones with triplet repeats of 4, 6 and 8 were constructed in a standard way. Using unique restriction sites, pSTCrGR*80* (Fig. 6.16a.) was digested into three fragments comprising the entire pSTC vector plus the majority of the rGR cDNA, but without the triplet $(CAG)_n$ repeat. Variable length triplet repeats were subcloned separately from the corresponding pSP64 plasmids as described in Figure 6.16..

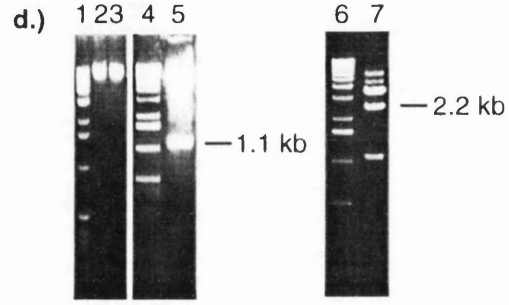
a.) pSTCrGR*80*

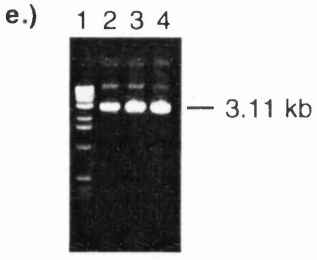


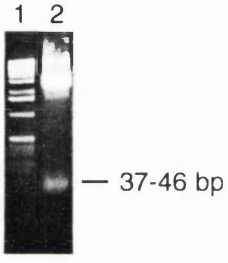
b.) pSP64-CAG 4, 6, 8

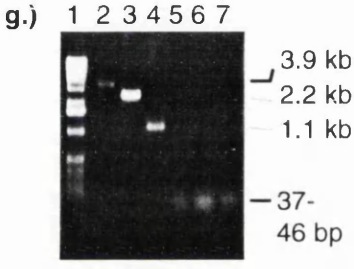


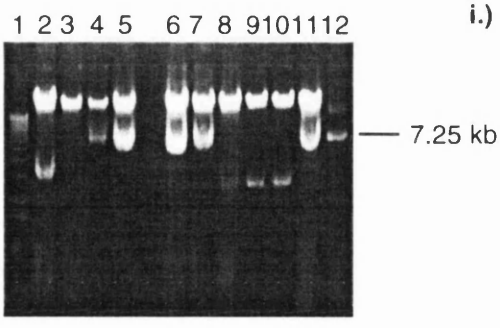


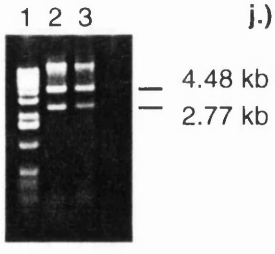


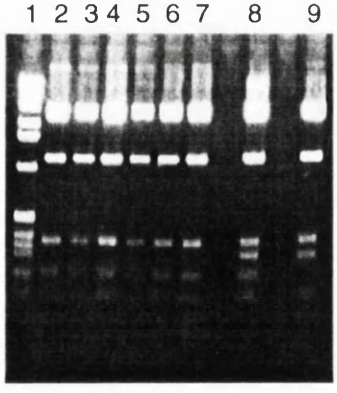












k.) pSTCrGR*4, 6, 8*

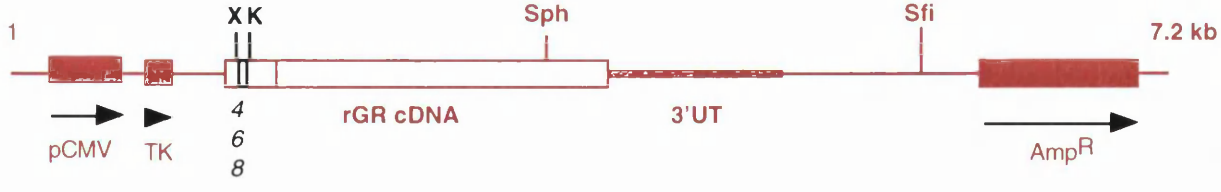


Fig. 6.16. Subcloning of novel $(CAG)_n$ repeat lengths into pSTC cloned rGR cDNAs.
a.) Minimal restriction map of pSTCrGR*80* showing positions of restriction sites used in subcloning.
b.) pSP64 clones carrying $(CAG)_n$ repeat lengths of 4, 6 and 8.
c.) pSTCrGR clones *10, 20, 40* and *80* cut with *Xho*I, (lanes 2-5, respectively). Lane 6, pSTCrGR*80* Sfi-cut, libertaing 3.9 kb vector band.
d.) pSTCrGR*80* clone cut with *Kpn*I, lane 2; *Sph*I, lane 3; *Kpn*I/*Sph*I, lane 5 and *Sph*I/*Sfi*I, lane 7. Lanes 4 and 6, 1 Kb DNA ladder.
e.) pSP64 clones 4-8 CAG cut with *Xho*I. Lane 1, 1 Kb DNA ladder.
f.) Example of pSP64 clone (8 CAG) cut with *Xho*I/*Kpn*I. Lane 1, 1 Kb DNA ladder.
g.) Gel purified cloning fragments for pSTCrGR *4, 6* and *8* construction: 3.9 kb vector band, lane 2; 2.2 kb vector band, lane 3; 1.1 kb rGR cDNA band, lane 4; 37-46 bp triplet $(CAG)_n$ repeat bands, lanes 5-7. Lane 1, 1 Kb DNA ladder.
h.) Potential positive recombinant pSTCrGR *4* and *6* clones. Lanes 2-5, 4 CAG variants. Lanes 6-11, 6 CAG variants. Lane 12, pSTCrGR*10* size control. Lane 1, 1 Kb DNA ladder.
i.) Initial *Bam*HI restriction analysis to check potential 4 CAG and 6 CAG recombinant pSTCrGR clones. Lane 1, 1 Kb DNA ladder.
j.) *Bam*HI/*Nco*I restriction analysis of potential recombinant pSTCrGR*4* (lanes 2-4) and *6* (lanes 5-7) clones. Lane 8, pSTCrGR80. Lane 9, pSTCrGR21. Lane 1, 1 Kb DNA ladder.
k.) Minimal restrictrion map of pSTCrGR *4, 6* and *8* recombinants.

Coding sequence differences between natural and construct rGR alleles

It must be clarified at this point that the production of novel triplet $(CAG)_n$ repeat lengths in the rGR coding sequence (Fig. 6.18.) was only possible in a realistic time frame in a way which resulted in amino acid differences in the encoded GR proteins. A small number of residues flanking the triplet repeats (part of the pSP64 cloning fragments) in construct GRs were therefore different to wild type. The pSTCrGR clones could therefore be subdivided into two groups based on CAG repeat length and the status of the encoded rGR cDNA. The following list describes rGR variants on the basis of triplet $(CAG)_n$ repeat length: 7, 18, 20 and 21 (natural alleles) and 4, 8, 10, 20 and 80 (construct alleles). Figure 6.17. below shows the difference in amino acids between each set of clones.

20Q (natural allele):

66 67 68 69 70 71 72 73 74 75-76 77 78-96 97 98 99 100 101 102 103 104 105 106 107

NH_2>**F S** K G S T S N V $(Q)_2$ R $(Q)_{18}$ P G L S K V S L S **M G**<COOH

20Q (construct allele):

66 67 68 69 70 71 72 73 74 75 76 77 78-98 99 100 101 102 103 104 105

NH_2>**F S** T L A C G S L E E D $(Q)_{20}$ G V R Y G **M G**<COOH

Fig. 6.17. Amino acid differences between natural and construct rat GR alleles.
A comparison is given between natural and construct 20-CAG repeat GR alleles in the region of the polyglutamine sequence. Amino acids are shown in the single letter code. Residues common to both cDNAs are shown in bold type. Numbers above each residue indicate position relative to met1.

6.7.) PCR amplification of CAG repeats and Sequencing

Trinucleotide $(CAG)_n$ repeat lengths of pSTCrGR cDNA clones carrying constructed GR alleles were verified by direct sequencing of PCR products.

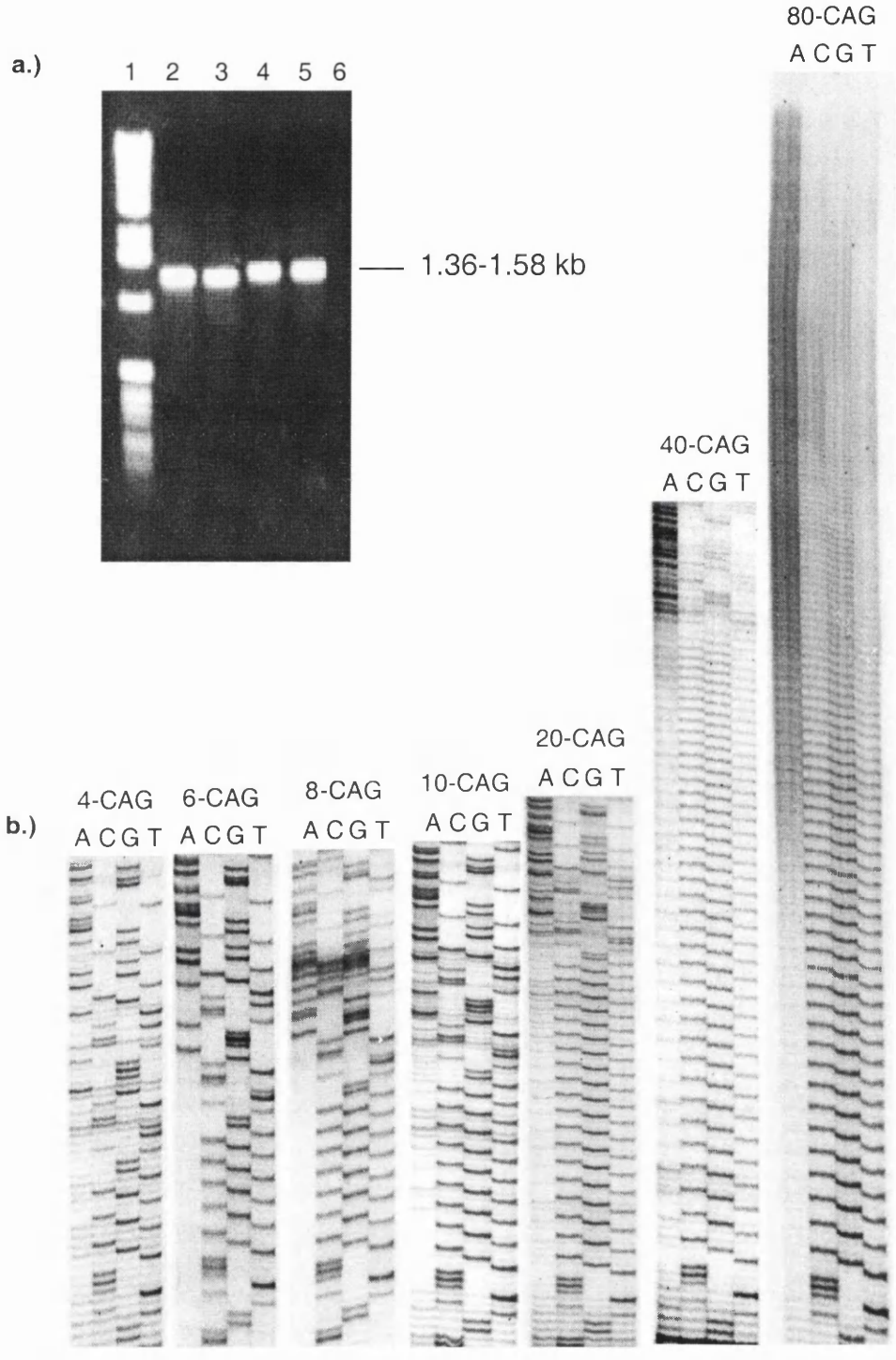


Fig. 6.18. Sequence verification of construct GR allele triplet $(CAG)_n$ repeat lengths.
a.) PCR amplification of triplet repeat sequences from construct rGR alleles. Lanes 2 and 3 duplicate amplifications from pSTCrGR*4*; lanes 4 and 5 duplicate amplifications from pSTCrGR*80*; lane 6, no DNA negative control. Lane 1, 1 kb DNA ladder.
b.) Comparison of triplet $(CAG)_n$ repeat sequences from construct rGR alleles. Sequences are of the antisense DNA strand. A, C, G, T, represent terminating nucleotides.

Target sequences were amplified by combining 1-5 ng of clone DNA with 10-20 pmoles of PCR primers: rGR34-BIO (pSTC vector specific) and rGR22 (Appendix 1) in a standard reaction mixture containing 1.5 mM Mg^{2+} (see section 2.2.3.). Reactions were performed for 26 cycles with an annealing temperature of 56°C. Figure 6.18. shows the specificity of primer annealing under these reaction conditions. PCR products from each rGR clone were purified using Hybaid PCR purification columns and 250-500 ng immobilised onto Dynabeads™ for sequencing using primer pG.

6.8.) Functional assays in CV-1 cells

Plasmids

In addition to pSTCrGR clones, a number of other plasmids were used in the analysis of rat GR function in tissue culture cells.

pSTC-MMTV-lacZ: Contains the *lac Z* coding sequence linked to the mouse mammary tumour virus (MMTV) long terminal repeat, which contains at least two known functional GREs (Payvar *et al.*, 1983; Yamamoto, 1985 and references therein). This vector contains an inducible GR-responsive promoter, and was used in pilot studies of GR function.

pSTC-CMV-CAT: This plasmid played no role in GR functional assays and was used as a 'fill-up' plasmid in the composition of transfection mixes (see Table 6.2.).

pSTC-CMV-TAG: Contains the SV40 large T-antigen. Expression is driven by a CMV promoter. The core plasmid sequence is based on a modified version of pSP64 and carries an ampicillin resistance gene. Replication in eukaryotic cells is driven by an SV40 replicon. This plasmid was used to complement the growth of plasmid clones in CV-1 cells carrying an SV40 origin of replication since this cell line does not carry an integrated SV40 large T-antigen as does COS-7 (see part 1 of this chapter).

pSTCrGR-3-556: A rat GR cDNA clone in which the ligand binding domain from amino acid position 556 to the end of the coding sequence has been deleted. This clone was used as a negative control in transactivation assays.

All of these clones were gifts from S. Rusconi, University of Fribourg, Switzerland.

Densitometry of plasmid clones

The DNA concentration of each pSTCrGR clone, in addition to routine spectrophotometry was confirmed by densitometry (see section 2.2.17.). Figure 6.19a. shows a gel loading of 200 ng of each GR clone.

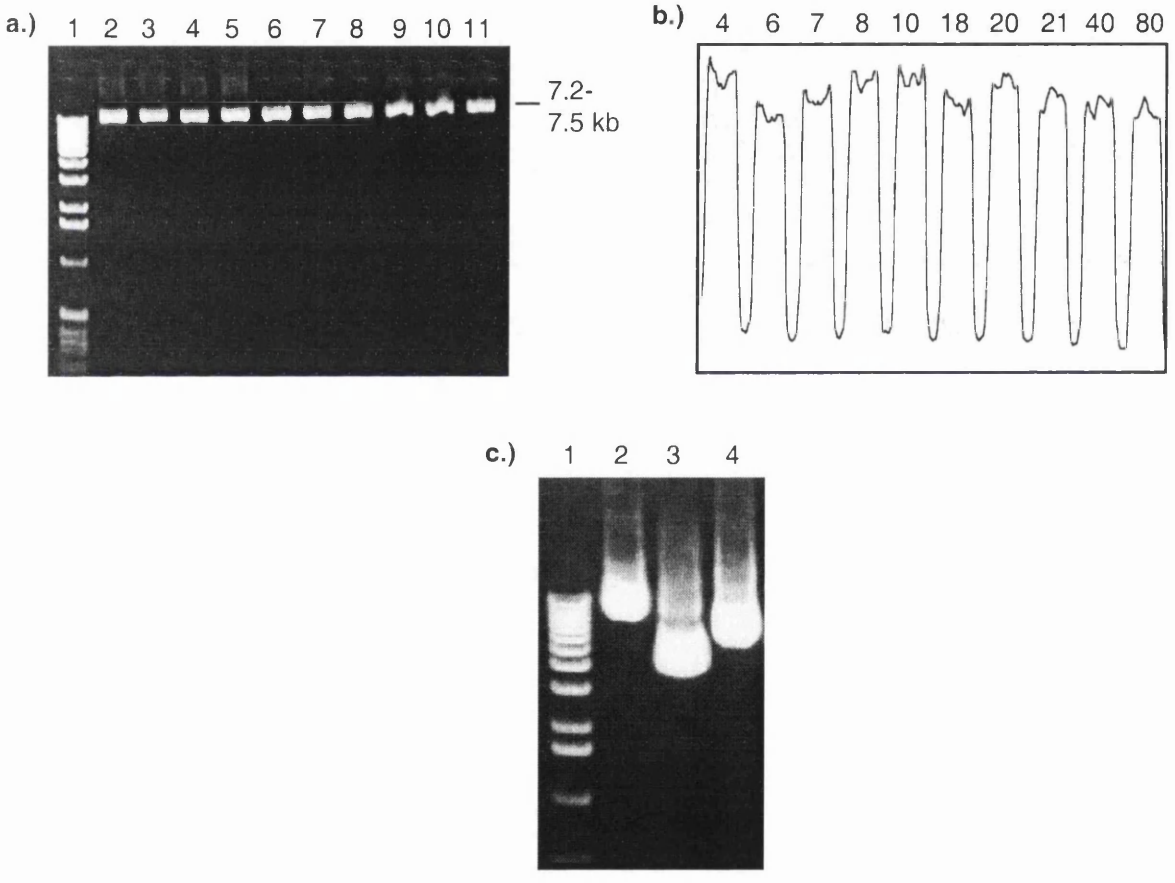


Fig. 6.19. Plasmid clones used for GR functional assays in CV-1 cells.
a.) Side-by-side comparison of pSTCrGR clones on 1% agarose gel. 200 ng of DNA was loaded per lane in order of GR allele CAG repeat length: 4, 6, 7, 8, 10, 18 20, 21, 40 and 80 CAG repeats, lanes 2-11 respectively. Lane 1, 1 kb DNA ladder.
b.) Densitometric comparison of the amount of DNA in each gel lane from panel a.).
c.) Check of the quality of other plasmid clones used in GR functional assays. Lane 2, pSTC-MMTV lacZ; lane 3, pSTC-CMVCAT; lane 4, pSTC-CMVTAG. Lane 1, 1 kb DNA ladder. 1 mg of DNA was loaded per lane.

A total of 10 μg (6 cm dish) or 20 μg (10 cm dish) of DNA was used in each transfection experiment. Table 6.2. below summarises the components of the different functional assays. The high integrity of the supercoiled plasmid DNA required for efficient transfection is shown in Figure 6.19a. and b.

DNA component:		Quantity (mg): 10 cm dish	6 cm dish
For *lac Z* transactivation			
MMTV-lac Z	(reporter gene)	6	3
CMV-CAT	(fill-up plasmid)	2-2.5	2.5-2.9
pSTC-GR clone	(transactivator)	0.5-1	0.1-0.5
Calf thymus (CT) DNA	(carrier DNA)	10	4
For rGR steroid binding assays			
pSTC-GR clone	(expression clone)	10	-
pSTCCMV-TAG	(translational enhancer)	1	-
Calf thymus (CT) DNA	(carrier DNA)	9	-

Table 6.2. Typical components of transfection mixes used in rGR functional assays.

Cell culture and transfection

CV-1, 2S-Fasa, EDR3 and HEK293 cells were routinely maintained as described in section 2.2.16. Transient transfections into CV-1, EDR3 and HEK293 cells were carried out at a confluence of 80% using the $CaPO_4$ coprecipitation method. Prior to transfection, medium serum supplements were reduced to 3% FCS which was maintained throughout the 'transfected' growth period (48h).

β-galactosidase assay

Each of the pSTC cloned GR alleles (natural and construct) were tested for their ability to function as transcription factors in a crude transactivation assay. CV-1 cells in 6 cm tissue culture dishes were co-transfected with the *lacZ* gene (cloned in pMMTV-lacZ) together with identical quantities (0.5 μg and then 0.1 μg) of each GR allele (see Table 6.2. above). The transactivation function of GR was stimulated by the addition of 5 nM dexamethasone to the cell medium, both in the 12-16 h

incubation period with DNA-$CaPO_4$ precipitates and in the following 'transfected' growth period. Transfected cells expressing β-galactosidase were identified in one of two ways: either directly, where cells were stained blue following breakdown of the chromagenic β-galactosidase substrate X-gal (section 2.2.18.), or photometrically following the breakdown of ONPG to p-nitrophenol by β-galactosidase containing cell lysates (section 2.2.19.). For both types of experiment, transfections of each allele were performed in triplicate.

β-galactosidase assays were also used to assess the transfectability of different cell lines which were potentially useful as host cells for the assay of GR steroid binding. CV-1 cells, originally derived from African green monkey kidney have been maintained in culture to the point where they contain no detectable levels of steroid receptors, by Western blot or by steroid binding assay (Giguere *et al.*, 1986; Arriza *et al.*, 1987). This property makes them ideally suited to experiments assaying for functional properties of GR. However, because the efficiency of transfection into CV-1 cells by the calcium phosphate coprecipitation method is generally not very high (of the order, 30-40%), other cell types were also investigated. To be considered a suitable alternative, other cell types have to be transfected at least as efficiently as CV-1 using a suitable transfection technique and have negligible levels of endogenous GR. The rat hepatoma cell line EDR3 (Cook *et al.*, 1994) which is also a GR negative cell line may be better suited to assays of rat GR because of species compatibility. The human embryonic kidney cell line HEK293 might be considered useful because of its very high transfectabilty (up to 80-90%) and insensitivity to corticosterone. Both EDR3 and HEK293 lines were therefore compared with CV-1 for efficiency of transfection. Each cell type grown on 6 cm tissue culture dishes was transfected with the GR clone pSTCrGR*21* by calcium phosphate coprecipitation and assayed for β-galactosidase activity following staining with X-gal.

6.8.1.) Western blotting of CV-1 expressed GR proteins

Prior to functional assays, GR proteins from natural and construct GR alleles expressed in CV-1 cells were analysed on 8% SDS-polyacrylamide minigels for levels of expression and intactness and compared with GR protein from rat liver or 2S-Fasa cells. In each case, protein samples (4-5 mg total protein extract) were band fractionated, blotted onto PVDF membrane and probed using the rat GR N-terminal specific monoclonal antibody Mab250 (section 2.2.22.).

6.8.2) Steroid binding assays

Cytosol extracts from rat liver tissue and from tissue culture cells (CV-1, 2s-Fasa and HEK293) were prepared as described in section 2.2.21.

Pilot experiments of GR steroid binding in cytosol extracts from rat liver and from 2s-Fasa, a constitutive GR expressing rat hepatoma cell line (Garland, 1986), were used to establish an optimal experimental strategy for the assay of GR proteins expressed in CV-1 cells. Binding assays were performed as described in section 2.2.21. A total of 50 μg of cellular protein extract per assay point was used in all experiments.

Kd (Dex), Kd (B) and binding capacities (R1) were derived from Scatchard plots using the curve fitting program LIGAND, performed as described in section 2.2.21.

6.9.) Results

6.9.1.) Expression of GR alleles in CV-1 cells

Prior to steroid binding assays, GR proteins expressed in CV-1 cells were compared on SDS-minigels (Fig. 6.20.). In terms of coding sequence, alleles could be subdivided into natural or construct, on the basis of sequence differences flanking the triplet CAG repeat (see Fig. 6.18., p165). Within subgroups, the only difference between alleles was in the length of their CAG repeats. In transfected CV-1 a major GR band was detected for all alleles, which migrated at around 94kd. No significant difference was detected between alleles in the intensity of this GR band (Figs. 6.20 and 6.21.). Quantitatively minor bands were observed at around 91 and 79 kd. These are likely to be polypeptides for which translation initiated at the second or third methionine codons, met2 or met3 in the GR transcript (Fig. 6.20.; Miesfeld *et al.*, 1986). No GR bands were detected in untransfected CV-1 cells.

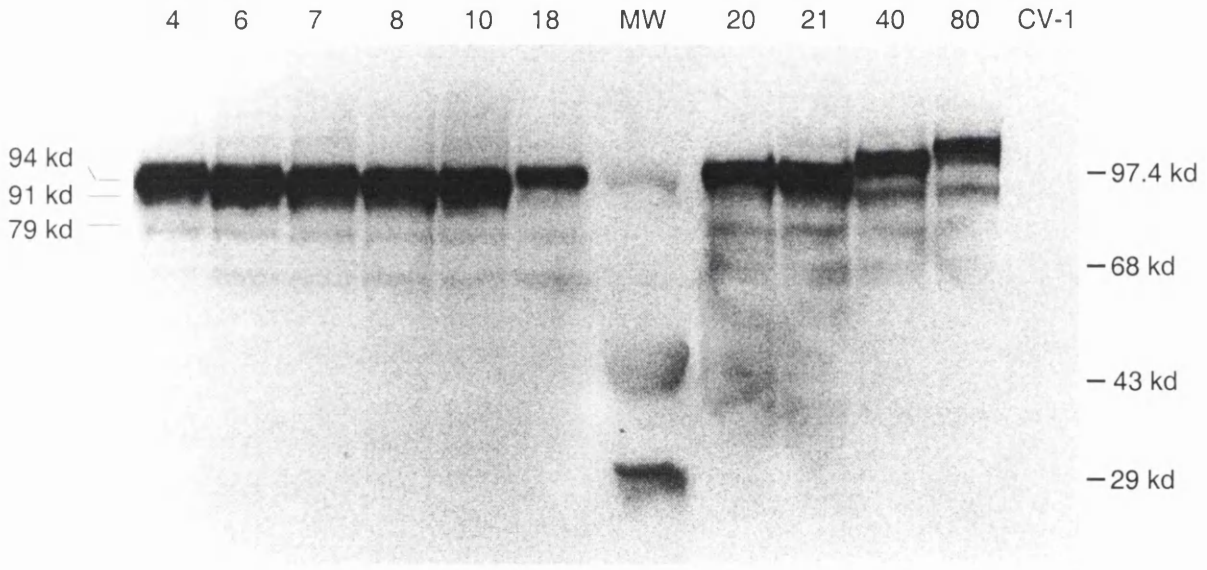


Fig. 6.20. GR proteins expressed from natural and construct GR alleles in CV-1 cells. Numbers above each lane represent the numbers of glutamine codons in translated GR proteins. The size difference of 11 kd between the largest (80-repeat) and smallest (4-repeat) alleles is clearly visible. MW, High Molecular Weight protein markers; CV-1, untransfected CV-1. Sizes in kd on the right mark the positions of proteins of the size marker. Sizes in kd on the left indicate the positions of the three major GR products. Each lane represents a loading of $2x10^4$ cells lysed directly in gel loading buffer.

Western blots of GR-transfected CV-1 cell lysates derived by NP-40 lysis showed GR to be present exclusively in nuclear exctracts (see general

discussion). This information was useful in the design of large scale GR steroid binding assays (see section 6.9.2.).

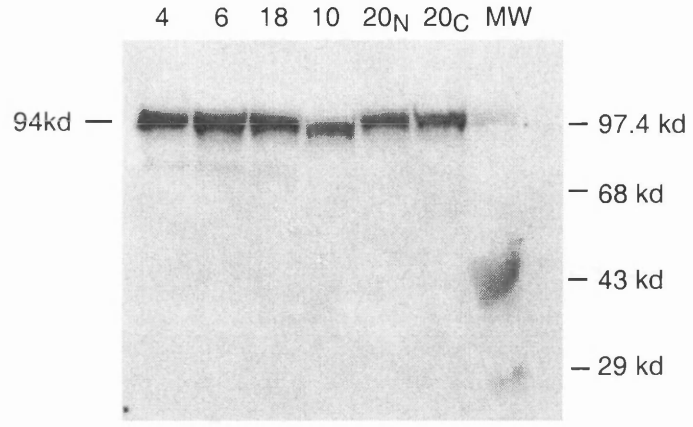


Fig. 6.21. Demonstration of the consistency of GR allele expression in CV-1 cells.
A selection of GR alleles including 20-CAG natural (N) and 20-CAG construct (C) were expressed in CV-1 cells (see text for details). $2x10^4$ cells from each transfection was analysed as in Fig. 6.20. A consistency of transfection was observed, which extended to both 20-CAG natural (N) and 20-CAG construct (C) alleles. Numbers above each lane represent GR allele polyglutamine repeat length. MW, High Molecular Weight markers.

6.9.2.) Functional assays of GR alleles expressed in CV-1 cells

β-galactosidase assays

X-gal staining of CV-1 cells cotransfected with pSTC-cloned GR alleles and *lacZ* demonstrated that the expressed GR proteins were functional in the presence of 5 nM dexamethasone. Comparison of the number of blue cells generated (average of 10 fields of view of the microscope: magnification, 4/0.1) per allele revealed no gross functional differences. Figure 6.22. shows, as a representative example, the level of staining and hence β-galactosidase activity following expression from 0.5 μg of allele GR*21* in various cell lines. In the absence of steroid, no blue cells were identified, implying that GR was not activated (Fig. 6.22c., compared with a. and b.). Calcium phosphate transfection of the GR negative rat hepatoma cell line EDR3 under exactly the same conditions proved unsuccessful with 0.5 μg of transfected DNA (efficiency estimated from β-galactosidase activity: Fig. 6.22d.). In contrast, the human embryonic kidney cell line HEK293 transfected with an efficiency of 80-90% (Fig. 6.22e.).

Analysis of GR function by ONPG assay also indicated comparable ability of each allele to activate *lacZ*. Table 6.4. shows the extent of ONPG conversion to yellow p-nitrophenol by CV-1 cell extracts expressing β-galactosidase.

GR Allele:	4Q	6Q	7Q	8Q	10Q	18Q
OD_{420}:	0.88	1.07	1.10	0.79	1.02	0.90

GR Allele:	20Q	21Q	40Q	80Q	21Q (H-)	GR (3-556)
OD_{420}:	0.95	1.03	1.04	1.03	0.20	0.15

Table 6.3. Indirect measurement of GR induced β-galactosidase activity by ONPG conversion.
ONPG conversion to yellow p-nitrophenol was measured by absorbance at 420 nm. Values represent the average from three separate transfection experiments (using 0.5 μg GR clone DNA/6cm dish). For each allele, GR activity was stimulated using 5 nM dexamethasone. The negative control 21Q (H-) received no dexamethasone. Clone pSTCrGR 3-556 has no hormone binding domain, but recieved 5 nM dexamethasone. The letter Q following each allele length refers to the single letter amino acid code for glutamine.

Steroid Binding Assays

Pilot assays of steroid binding to GR were performed on cytosol extracts from rat liver and the cell line 2S-Fasa, to establish a reliable experimental strategy.

a.)

	Concentration of unlabelled ligand (nM)						
	0	0.5	1.58	5.0	15.8	50.0	158.0
	cpm bound at each concentration of unlabelled ligand						
Dex	218.2	245.2	207.6	146.1	92.6	56.5	45.5
Dex	242.1	218.1	174.1	144.1	95.6	66.5	40.0
B	239.6	234.1	210.1	205.6	141.1	107.1	61
B	241.2	222.7	231.7	229.6	151.6	114.1	60.5

b.)

Dex	48.5	42.0	40.0	54.5	42.0	44.5	39.5
Dex	39.5	40.5	47.0	54.5	47.5	44.5	47.0
B	49.0	45.5	48.0	50.5	62.0	58.5	51.5
B	42.5	54.5	44.0	53.5	59.0	69.0	58.0

Table 6.4. Raw cpm for a.) 2S-Fasa and b.) untransfected CV-1 cell extracts bound with ^{3}H-dexamethasone at differing concentrations of unlabelled competing ligand.
Curves were prepared in duplicate for each competing ligand. *Dex*, dexamethasone; *B*, corticosterone.

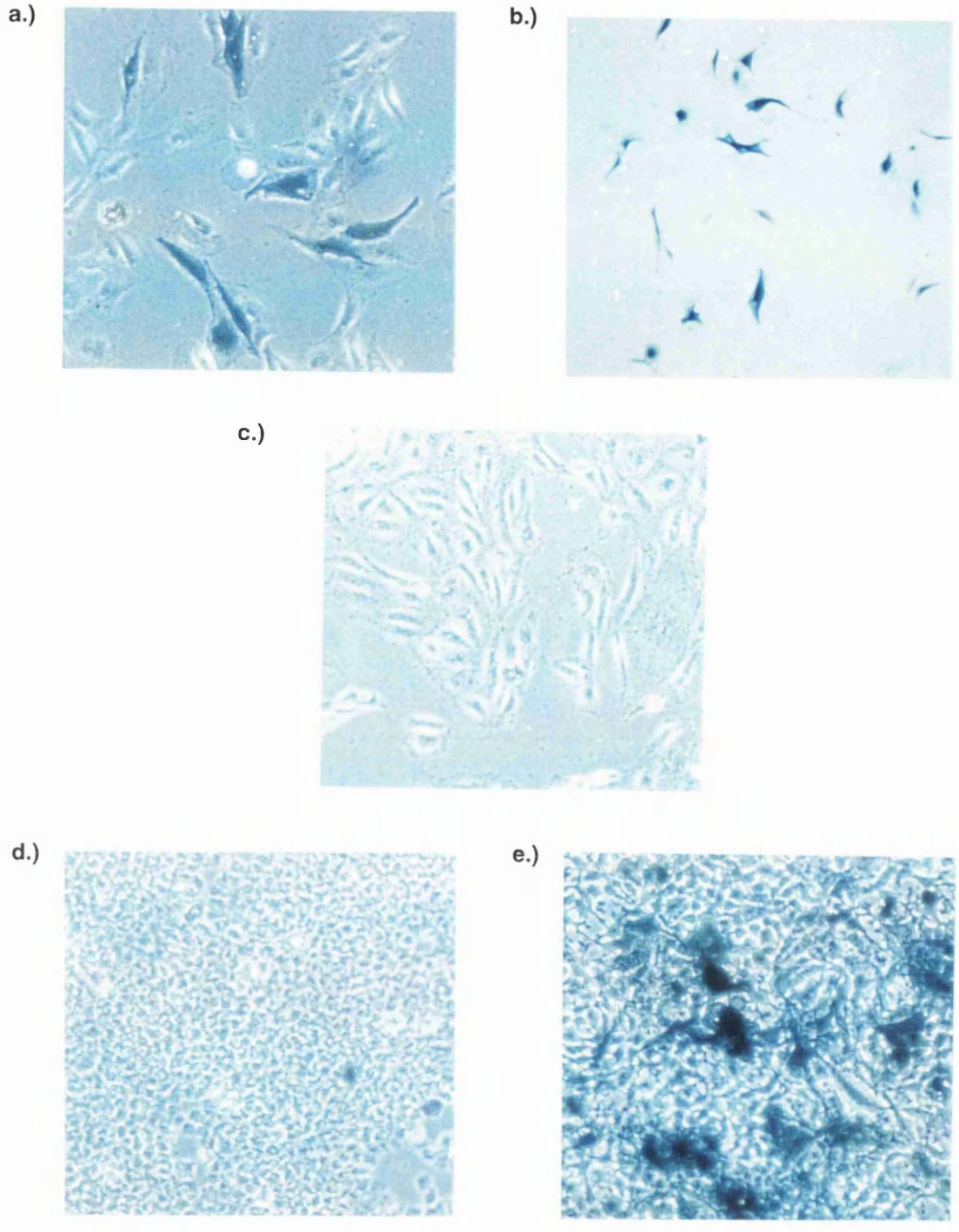
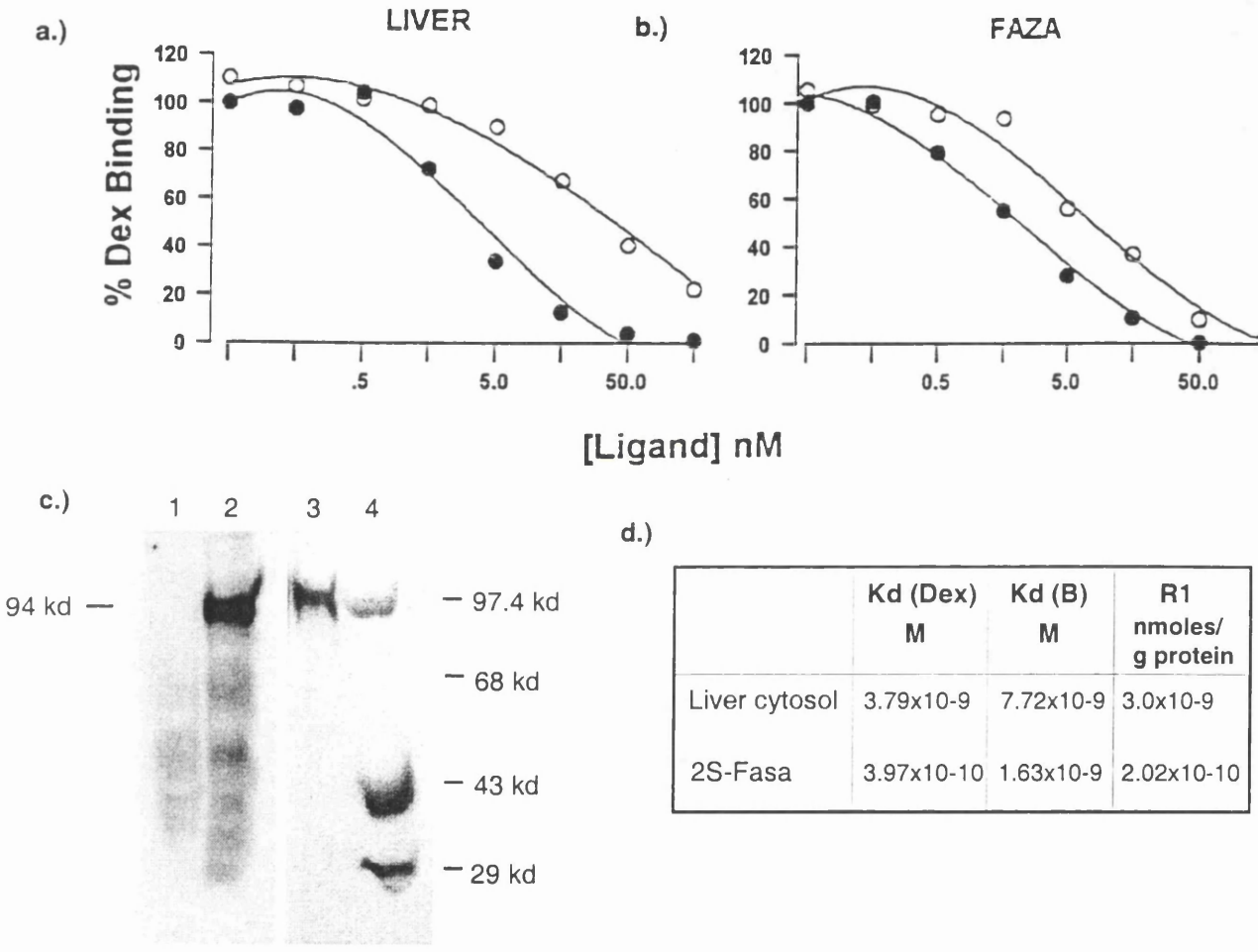


Fig. 6.22. Expression of β-galactosidase in tissue culture cells transfected with cloned rat GR and lacZ.

a.)-c.) CV-1 cells transfected with: a.), 0.5 µg pSTCrGR21 + 5 nM dexamethasone (dex); c.), 0.5 µg pSTCrGR21 with no added dex. b.) is the same as a.), but at higher magnification to show consistency of transfection.

d.)-e.) EDR3 showing one transfected cell (d.) and HEK293 showing around 80% of cells transfected (e.) with 0.5 µg pSTCrGR21 + 5 nM dex.

Competition assays for ^{3}H-dexamethasone binding to GR proteins were performed using unlabelled dexamethasone or corticosterone as competing ligand. As an example Table 6.4. shows the levels of counts obtained at each concentration of unlabelled ligand for 2S-Fasa cells compared with untransfected CV-1 cells. Plots of % specific bound counts against ligand concentration (Fig. 6.23.), together with calculated Kd and R1 values produced data similar to that reported elsewhere for rat GR (Panarelli, 1994). Untransfected CV-1 cells produced no measurable specific binding (Table 6.4.).



d.)

	Kd (Dex) M	Kd (B) M	R1 nmoles/ g protein
Liver cytosol	3.79x10-9	7.72x10-9	3.0x10-9
2S-Fasa	3.97x10-10	1.63x10-9	2.02x10-10

Fig. 6.23. Pilot assays of GR steroid binding in cytosol extracts from rat liver and rat liver cell line 2S-Fasa.
a.) and **b.)** Competition binding curves for rat liver cytosol and 2S-Fasa cell extracts, respectively.
c.) Western blot showing samples of 20 μg each of untransfected CV-1 (lane 1), rat liver cytosol (lane 2) and 2S-Fasa cell extracts. Lane 4, High Molecular Weight protein markers.
d.) Kd (Dex), Kd (B) and R1 values for GR from rat liver and 2S-Fasa cells.

Competition assays for ^{3}H-dexamethasone binding by cloned GR alleles were performed on protein preparations from transfected CV-1 cells using the methodology optimised in pilot experiments. Figure 6.24. shows the

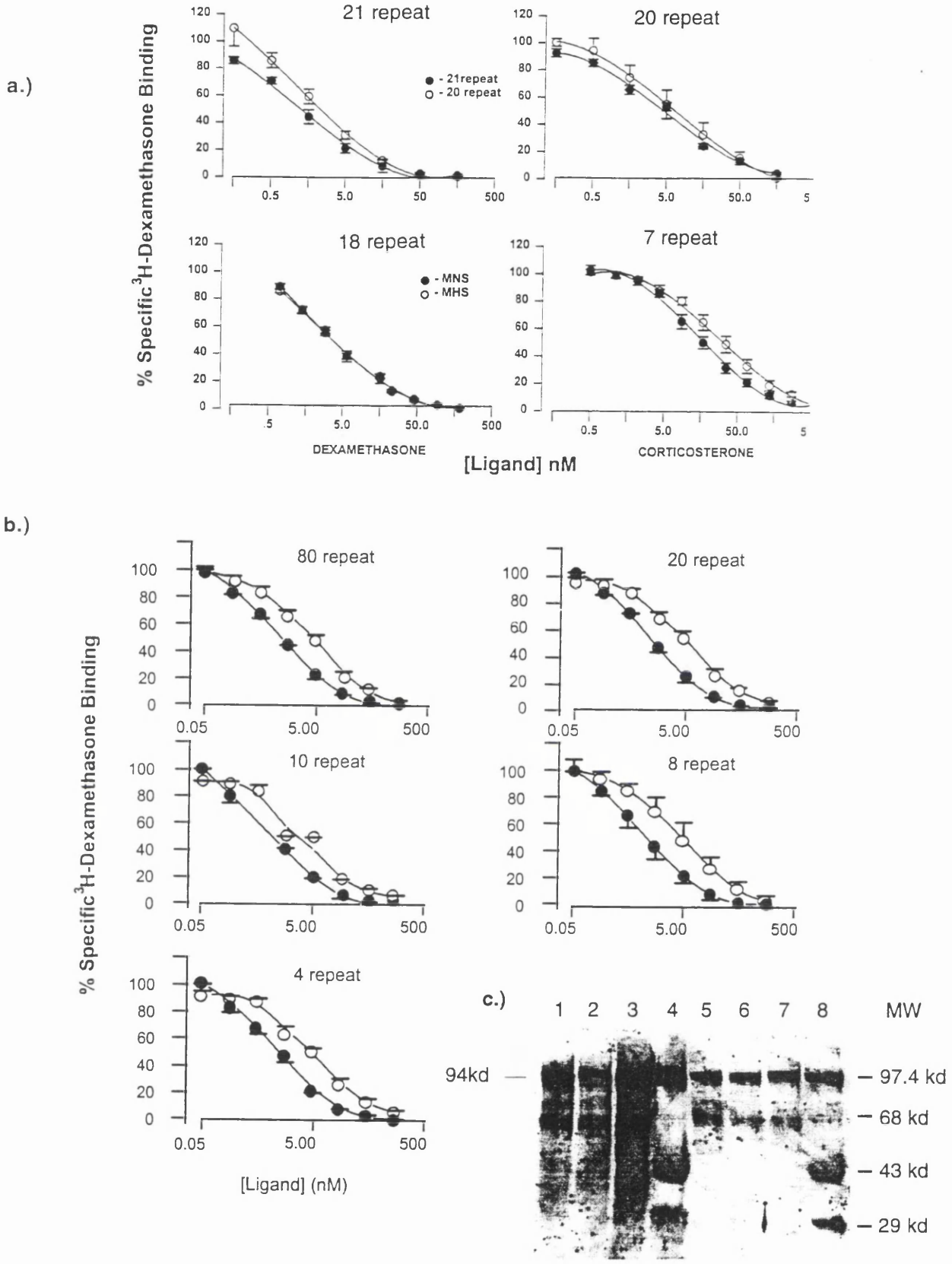


Fig. 6. 24. Competitive steroid binding by CV-1 expressed natural and construct GR alleles.
a.) and **b.)** Combined data for each natural and construct GR allele respectively. Plots are of % specific bound counts against ligand concentration (nM). Open circles, dexamethasone; closed circles, corticosterone.
c.) Comparison of GR proteins from whole cell lysates and sonicates of the same CV-1 transfections. The gel was divided into two halves. Samples on the left represent whole cell loadings. Those on the right, cell sonicates. Lanes 1 and 2, the proceeds of duplicate 10Q and lane 3, 20Q construct allele transfections (2x104 cells loaded per lane). Lanes 5-7 were loaded with the same alleles as in lanes 1-3, but using 20 mg of sonicated cell extract in place of whole cells. Lanes 4 and 8, High Molecular Weight protein markers.

effect of ligand concentration on its binding to GR for the combined data for a.) natural alleles; *Grl*$^{CAG7,\ 18,\ 20,\ 21}$ and b.) for constructed alleles; *Grl*$^{CAG4,\ 8,\ 10,\ 20,\ 80}$. Table 6.5. shows the means and standard errors for Kd

a.)

rGR $(Q)_n$ allele	Kd (Dex) nM	Kd (B) nM	R1 pmole/mg total protein
Natural			
21Q a	1.45	11.9	1.37
21Q b	1.91	11.0	1.0
21Q c	2.54	11.1	0.6
21Q d	0.43	1.31	0.37
21Q e	0.73	3.7	0.92
21Q f	0.76	5.25	0.9
21Q g	1.08	10.9	0.29
21Q h	0.56	-	0.61
21Q i	0.31	1.66	0.67
21Q j	0.54	2.91	o.77
21Q k	0.96	2.91	0.73
mean (n=11)	**1.0**	**6.27**	**0.75**
SE	**0.21**	**1.39**	**0.27**
20Q a	0.92	9.54	0.82
20Q b	0.8	5.37	1.49
20Q c	1.91	9.72	0.83
20Q d	0.78	6.8	0.97
20Q e	0.9	-	0.95
20Q f	0.79	-	0.8
20Q g	1.35	-	0.91
20Q h	1.1	-	0.88
mean (n=8)	**1.01**	**7.8**	**0.96**
SE	**0.14**	**1.51**	**0.79**
18Q a	1.58	13.9	0.92
18Q b	1.92	11.1	1.11
18Q c	3.12	12.1	0.44
18Q d	0.84	-	0.67
18Q e	0.89	-	0.75
mean (n=5)	**1.67**	**12.37**	**0.78**
SE	**0.42**	**0.82**	**0.11**
7Q a	0.93	11.1	0.7
7Q b	1.56	10.5	1.28
7Q c	1.05	10.1	0.51
7Q d	1.34	8.25	0.8
7Q e	0.88	15.1	0.42
7Q f	1.27	3.77	1.3
7Q g	0.62	6.15	0.83
mean (n=7)	**1.1**	**9.28**	**0.83**
SE	**0.12**	**1.39**	**0.13**

b.)

rGR $(Q)_n$ allele	Kd (Dex) nM	Kd (B) nM	R1 pmole/mg total protein
Construct			
80Q a	0.30	1.48	0.57
80Q b	0.24	0.97	0.46
80Q c	0.59	2.94	0.47
80Q d	0.62	2.4	0.55
80Q e	0.53	1.66	0.45
mean (n=5)	**0.46**	**1.89**	**0.50**
SE	**0.08**	**0.35**	**0.03**
20Q a	0.18	0.77	0.28
20Q b	0.25	0.79	0.40
20Q e	0.17	0.63	0.28
20Q f	0.50	2.83	0.53
20Q g	0.53	3.29	0.57
20Q h	0.72	4.01	0.44
20Q i	0.74	3.65	0.59
mean (n=7)	**0.44**	**2.28**	**0.44**
SE	**0.09**	**0.57**	**0.05**
10Q a	0.24	1.08	0.27
10Q b	0.52	1.53	0.35
10Q c	0.15	0.46	0.51
mean (n=3)	**0.30**	**1.02**	**0.37**
SE	**0.11**	**0.31**	**0.07**
8Q a	0.15	0.78	0.32
8Q b	0.45	2.01	0.34
8Q c	0.26	1.40	-
8Q d	0.46	2.51	-
8Q e	0.61	2.50	-
mean (n=5)	**0.30**	**1.40**	**0.33**
SE	**0.15**	**0.62**	**0.01**
4Q a	0.28	1.58	0.34
4Q b	0.60	1.74	0.44
4Q c	0.58	3.66	0.52
4Q d	0.71	2.36	0.51
mean (n=4)	**0.54**	**2.34**	**0.45**
SE	**0.09**	**0.47**	**0.04**

Table 6.5. The effect of polyglutamine tract length on steroid binding activity of natural (a.) and constructed (b.) glucocorticoid receptor variants.
Binding affinities (Kd) and receptor capacity (R1) for dexamethasone (Dex) and corticosterone (B) are given for each allele. Results are expressed as the mean ± SEM; n = number of separate Scatchard determinations.

(dex), Kd (B) and Bmax for each natural (a.) and construct (b.) allele. Examples of plots of raw data, which demonstrate the reproducibility of these experiments is shown in Appendix 2. To Check that sonication had no qualitative effect on GR stability, sonicates of transfected cells were analysed against samples of whole cell lysates from the same transfection (Fig. 6.24c.). No noticeable difference in the integrity of GR proteins was observed.

Polyglutamine repeat length was found to have no significant effect on Kd for either dexamethasone or corticosterone, or the receptor capacity (R1) within each group of natural or construct alleles (Fig. 6.24. and Table 6.5.). Natural 20 and 21 repeat Milan rat alleles $Gr1^{CAG20,\ 21}$, also showed no difference in affinity for dexamethasone or corticosterone, implying that any difference in steriod binding which may have existed between these alleles *in vivo* was no longer apparent (see general discussion and Fig. 6.24a.). In support of this observation, values of R1 from GR alleles expressed in tissue culture were comparable with those measured *in vivo* for rat hepatic cytosol preparations (Panarelli, 1995).

In contrast, the direct comparison of the entire competition data for the natural versus the construct GR alleles, revealed a significant increase in receptor affinity (reduction in Kd) for both dexamethasone and corticosterone (Table 6.6.). This result is supported by a leftward displacement of steroid binding curves for the natural alleles for each ligand (Fig. 6.25a.). Taking the 20-repeat alleles as the closest comparison between natural and construct alleles, the displacements in competition binding and difference between Kds (for dex and B) were maintained between alleles (Fig. 6.25b.). Presenting the 20-repeat data for dexamethasone competition in the form of a Scatchard plot, in which the individual points for each curve represent the mean data from all experiments, provided confirmatory indication of a significant difference in kds (dex) between natural and construct alleles (Fig. 6.26).

Statistical analysis

The significances of difference in Kds and R1 values between chosen allelic comparisons were determined by parametric (two-tailed t-test) and non-parametric statistics (Mann Whitney U-test) where appropriate (Table 6.6.). Values of Kd (dex) Kd (B) and receptor capacity R1 were significantly

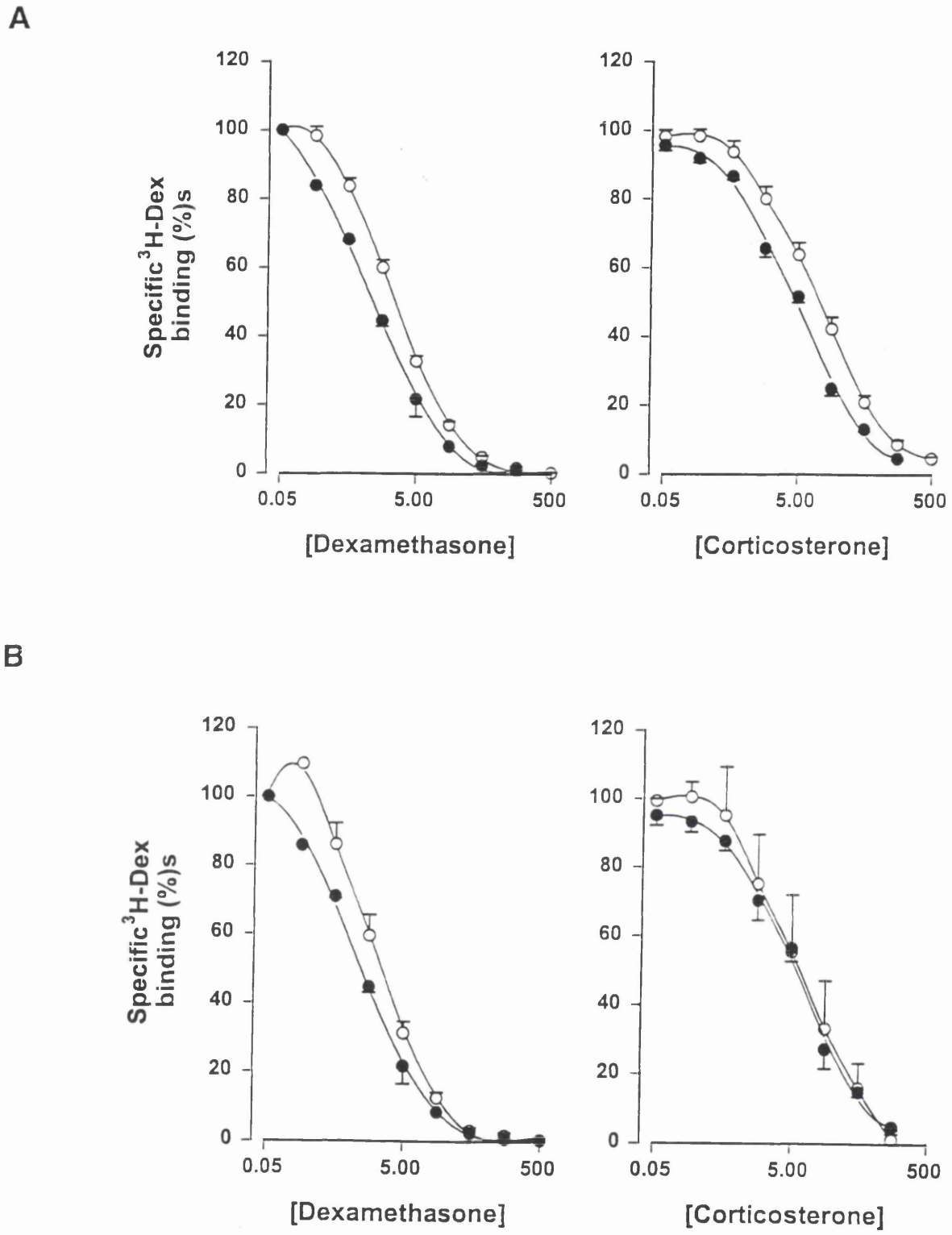


Fig. 6.25. Competition curves for natural and construct GR alleles.
A; Competition curves for pooled natural (open circles) and construct (solid circles) GR alleles.
B; Competition curves for pooled natural 20 repeat (open circles) and construct 20 repeat (solid circles) GR alleles.
In both cases, each point plotted represents the mean of the pooled binding data collected from all of the alleles at each concentration of unlabelled competing ligand.

	Kd (dexamethasone) nM	Kd (corticosterone) nM	Binding capacity pmol/mg protein
20-repeat natural allele	1.01 ± 0.14	7.8 ± 1.51	0.96 ± 0.79
(n)	(8)	(4)	(8)
21-repeat natural allele	1.0 ± 0.21	6.27 ± 1.39	0.75 ± 0.27
(n)	(11)	(10)	(11)
P-value	NS	NS	NS

	Kd (dexamethasone) nM	Kd (corticosterone) nM	Binding capacity pmol/mg protein
All natural allele	1.16± 0.11	8.17 ± 0.82	0.83 ± 0.05
(n)	(31)	(24)	(31)
All Construct allele	0.43 ± 0.04	1.96 ± 0.21	0.44 ± 0.023
(n)	(24)	(24)	(21)
P-value	< 0.001	< 0.001	< 0.001

	Kd (dexamethasone) nM	Kd (corticosterone) nM	Binding capacity pmol/mg protein
20-repeat natural allele	1.01 ± 0.14	7.8 ± 1.51	0.96 ± 0.79
(n)	(8)	(4)	(8)
20-repeat construct allele	0.44 ± 0.09	2.28 ± 0.57	0.44 ± 0.05
(n)	(7)	(7)	(7)
P-value	< 0.001	< 0.01	< 0.001

Table 6.6 Statistical comparison between chosen groups of alleles.
Kd values were compared by Mann Whitney tests, binding capacities were compared by two tailed t-tests.

different between natural and construct allelic groups, but not within groups, which included the Milan rat alleles, *Grl*$^{CAG20, 21}$.

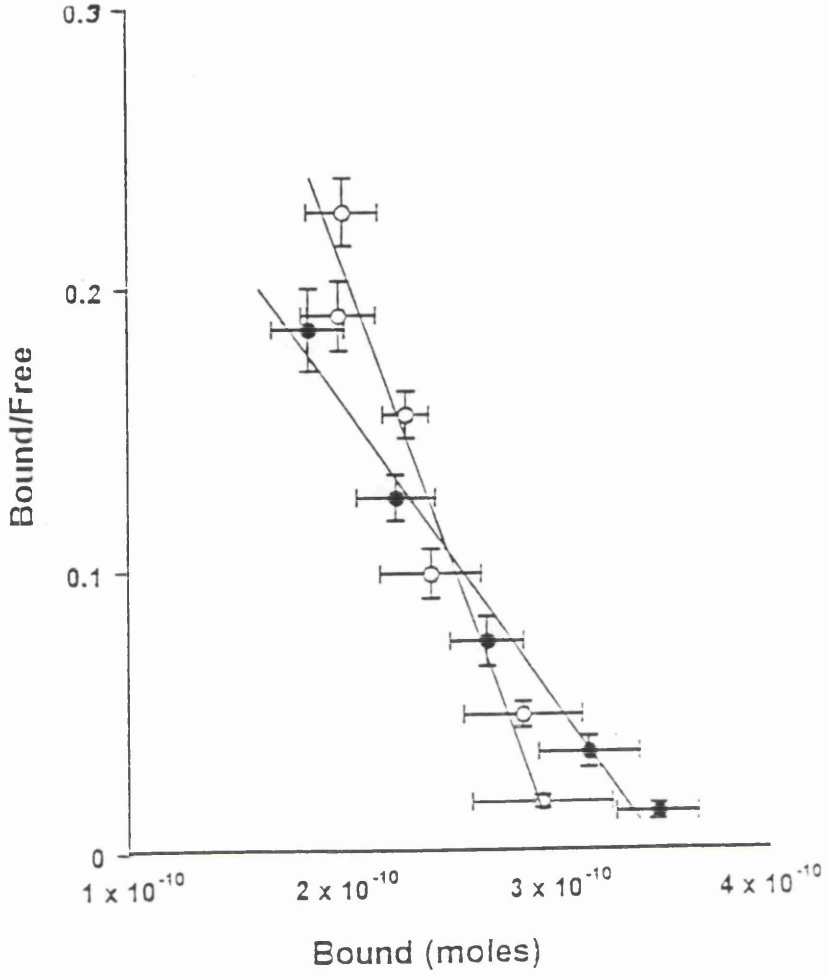


Fig. 6.26. Scatchard plots of dexamethasone binding data for 20 repeat natural and 20 repeat construct alleles.
Competition binding data used in the calculation of Kds (dex) for 20 repeat allele were pooled at each concentration of cold competing ligand. Plots are therefore of mean data from 20 repeat natural (solid circles) and 20 repeat construct (open circles) alleles.

6.10.) Comment

Prior to the use of the different GR alleles in steroid binding assays, it was important to establish their gross functional competence. The ability of each of the expressed GR proteins to induce similar levels of activation of the *lacZ* gene cloned in pSTC-MMTVlacZ (Table 6.3.) demonstrated that all proteins were functional. Induction of GR activity in the presence of 5 nM dexamethasone implied that in each case, GR was folded correctly and complexed with HSP90 in a way which was compatible with endogenous GR function. These results also suggested that all GR variants were equally

competent as transcriptional activators, able to interact with a GRE with a similar level of efficiency.

Statistical comparison of Kd values between natural and construct alleles (Table 6.5.) suggests significant differences in affinity for both dexamethasone and corticosterone (summarised in Table 6.6.). Most importantly, a comparison of affinity differences between natural and construct 20-repeat alleles, supports this observation. The possible implications of these differences are discussed in chapter 7.

Measurements of receptor capacity (R1), were also found to be significantly different between natural and construct alleles. The reasons for this difference are unclear. It could be that, compared with natural alleles, the construct alleles are inherrently less stable, although Western blots (Fig. 6.20. and 6.21., pp 175-176 and Fig. 6.24.) do not support this. Alternatively, these alleles may have been less able to bind stabilising proteins or molybdate under steroid binding assay conditions, making them more prone to degradation over time. There was sufficient overlap in steroid binding assays to rule out the possibility of experimental drift.

Conceptually, the assay of receptor function in cell lines such as CV-1 is useful in that possible secondary effects on receptor function which might arise *in vivo*, are eliminated, providing a consistent assay system. Such effects might result from differences in circulating endogenous hormone levels or from the action of a host of different modulators of receptor function, reviewed in chapter 7 (pp 191-197).

Chapter 7
General Discussion

7.1.) Coding sequence differences and distribution of GR alleles in inbred and wild rats

The primary aim of the work described in this thesis was to seek variation in the coding sequence of rGR and to determine what effect this may have on the function of GR in different inbred strains of rat. A further objective was to determine the extent to which polymorphisms of GR might account for previous observations of differences in steroid binding affinity and the role of glucocorticoids in rat models of essential hypertension (Kenyon *et al.*, 1994; Panarelli *et al.*, 1995, chapter 3, p 87) and obesity (White and Martin, 1990).

Initial findings were positive, in that sequence variation was found in the triplet $(CAG)_n$ repeat sequence in the GR modulatory domain. In addition, silent substitutions in GR codons at nucleotide positions 198, 531, and 711 were also identified which do not lead to changes in amino acid sequence (see Fig. 7.1.). The four sites of genetic variation lie in close proximity in GR, constituting distinct haplotypes with an exceedingly low frequency of recombination, which were used to investigate genetic origins of different rat strains (discussed in detail in chapter 4).

The distribution of CAG repeat lengths in GR was investigated for both inbred strains (Chapter 3, part 1) and wild rats (Chapter 4) of the species *R. norvegicus* and was found to be similar. Neither set of animals had CAG repeat lengths of less than 7, or between 7 and 17, producing a clear discontinuity in the allelic distribution. In the sample populations analysed in this thesis, it was considered unlikely that the 'missing' subset of rGR alleles arose due to sampling bias. The similarity in the distribution of alleles found in sufficiently separated populations of wild rats (sampled from Scotland and England) would argue against this. The possibility of genetic drift is also unlikely. The same distribution of rGR alleles was found in wild rat populations from all capture sites and in the inbred rats

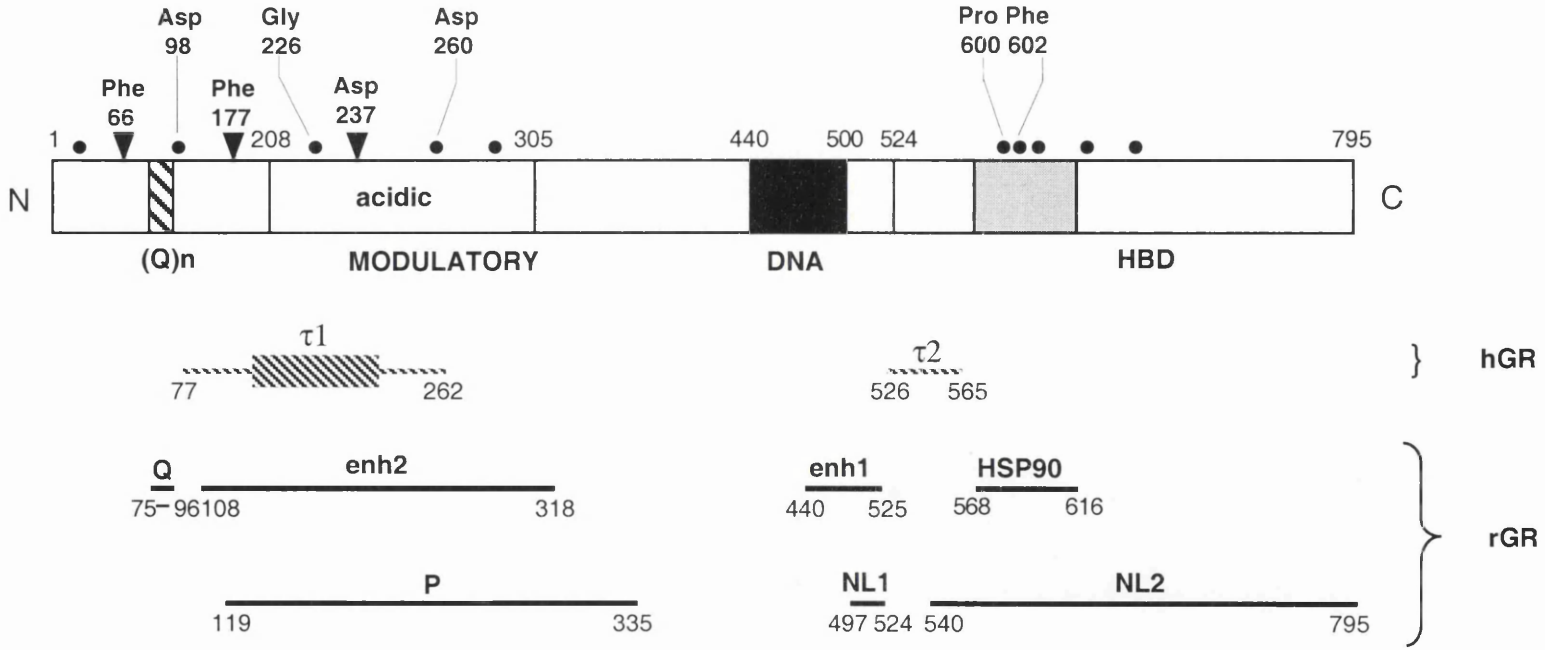


Fig. 7.1. Functional domains of the rat glucocorticoid receptor.
Estimated positions of domains are shown, with numbered residues for rat and human receptors, respectively (see text for references). The domains are: modulatory, modulatory domain; DNA, DNA binding domain; HBD, hormone binding domain; HSP90; HSP90 binding site; NL1 and NL2, nuclear localisation signals 1 and 2; P, phosphorylation domain; D, dimerisation domain enh1 and enh2, transcriptional activation/enhancer domains 1 and 2; $(Q)_n$, polyglutamine tract. For comparison, the equivalent transactivation regions of human GR, τ1 (hGR, 77-262) and τ2 (hGR, 526-556) are shown as hatched lines. Hatched box in the τ1 domain localises the τ1-core polypeptide (hGR 187-244). Amino terminal transactivation domains of GR from different species overlap a highly acidic region required for maximum receptor activity. This region spans residues 208-305 (rat), 196-293 (mouse) and 187-285 (human) and is termed 'acidic'. The grey box marks the position (residues 595-614) of the HSP90 binding site, conserved in all members of the steroid receptor superfamily. Polyglutamine tracts (heavy hatched box: rGR residues 75-96, for *Grl*^{CAG21}) were polymorphic. Heavy dots mark the positions of required alterations to the published sequence of rGR cDNA (Miesfeld *et al.*, 1986), five of which (marked with the correction above the dot) predict a change in the encoded amino acid (see text for details). Silent nucleotide substitutions in the GR coding sequence are located by solid arrows. These were identified at positions: n 198 $(Phe)_{66}$, n 531 $(Phe)_{177}$ and n 711 $(Asp)_{237}$ flanking the polyglutamine tract.

analysed. Work is currently in progress to determine whether the GR alleles identified at chosen locations were fully representative of the resident rat population(s).

Complementary to the distribution of GR alleles found in *R. norvegicus,* it would be interesting to see whether this distribution pattern is typical of other rat species or is species specific. The answer to this question would require the analysis of GR alleles, both in other populations of *R. norvegicus* and in other species of rat, such as *R. rattus.* An interesting extension of this work might also include other readily accessible rodent species such as hamster, or squirrel to determine the variability and distribution of GR alleles with respect to CAG repeat length in other distinct rodent populations.

The absence or significant under-representation of alleles may have resulted from the mechanism by which these alleles diversified (discussed in chapter 4, pp 128-131). Alternatively, the observed rGR alleles may have a selective advantage through their function as transcription factors. This latter possibility is currently being addressed. Studies are in progress in collaboration with Dr. Bernd Groner and co-workers, Institute for Experimental Cancer Research, Freiburg, Germany, to determine the functional effect of individual rGR repeats in the context of GR modulated transcription and will be discussed in greater detail in section 7.2.2.

7.2.) Expression of natural and construct GR alleles in CV-1 cells and assays of GR function

7.2.1.) Steroid binding

The main focus of this thesis was the analysis of structure-function relationships of rGR. To determine whether the polyglutamine tract length of the receptor had an effect on affinity for steroid, different GR triplet repeat alleles, with lengths of less than 7, between 7 and 17 and greater than 23 CAG repeats were constructed and expressed in CV-1 cells (pp 164-183). The binding affinities of natural or constructed rGR variants for the glucocorticoids, dexamethasone and corticosterone were not different between alleles in either natural or construct group. This implies that the

intra-allelic differences in the length of the polyglutamine tract on its own is unlikely to have immediate effect in determining the affinity of rat GR for steroid, although tissue specific effects on steroid binding or promoter specific effects on transcription should not be ruled out, particularly *in vivo*. More noticeable from these studies, however, was the significant difference in affinity between natural and construct GR alleles for glucocorticoids ($P<0.001$ for dex and B; see Table 6.5). In the comparison of alleles through the natural and construct 20 repeat variants, having the closest possible sequence similarities, the significance of difference in Kds was maintained (for dex, $p < 0.001$-0.0015; for B, $p < 0.01$-0.08). This difference in affinity was *not* the result of inter-allelic differnces in the length of the long homopolymeric repeats in the different alleles. The increase in affinity of the constructed alleles may have resulted from the codon substitutions introduced into the sequences flanking the polyglutamine repeat as part of the construction process. Possible effects of the arginine residue, located at the amino-terminal end of natural GR polyglutamine tracts and missing from construct GR alleles can not be ruled out. The difference in sequence between the natural and construct alleles is shown on p168.

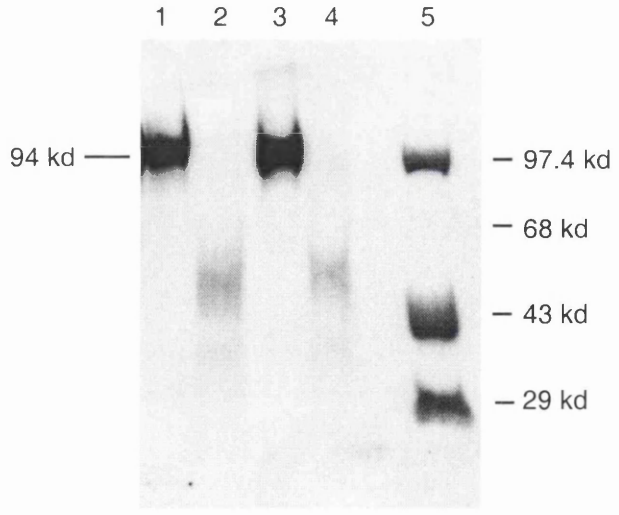


Fig. 7.2. The cellular location of rGR proteins overexpressed in CV-1 cells.
Rat GR proteins expressed from all rGR CAG repeat alleles in CV-1 cells translocate completely to the nucleus in the absence of hormone. Lanes 1 and 3, nuclear extracted proteins from cells transfected with 21 and 4 CAG repeat alleles; lanes 2 and 4, cytosol extracted proteins from cells transfected with 21 and 4 CAG repeat alleles, respectively. Sample loadings were of the order, 20 μg/lane. Lane 5, high molecular weight protein markers.

The mechanism by which coding sequence variation in the amino terminus of GR might affect affinity for steroid at the C-terminal end, or

even the performance of GR as a transcriptional regulator remains unclear. Gross molecular effects may be ruled out, given that all of the GR variants overexpressed in CV-1 cells behaved as expected, showing translocation to the nucleus, consistent with Martins *et al.*, (1991), which by Western blotting appeared to be complete. Figure 7.2., above compares representative samples of cytosolic and nuclear protein extracts from CV-1 cells transfected with natural allele Grl^{CAG21} and construct allele Grl^{CAG4}.

7.2.2.) Transcriptional activity

In contrast to effects on steroid binding, the construct GR alleles showed a similar ability to activate MMTV-lacZ following hormonal induction as the natural alleles, indicating that none of the structural differences between GR molecules had any major effect on their ability to bind a GRE and activate transcription (Table 6.3. p 177). This contrasts with reports in the literature which describe possible regulatory roles for polyglutamine tracts in the control of transcription (see chapter 1, section 1.9.4.). In most cases, the molecular mechanisms for such effects are unclear. It is possible that the polyglutamine tract of rGR has an undefined role in transcriptional regulation, which is simply not identified following GRE activation in the MMTV-lacZ system. It remains possible that certain transcriptional regulatory effects are limited to specific promoter contexts, implying a level of tissue specificity in GR mediated gene regulation.

An interesting extension of the work of this thesis would therefore be to consider the transactivation properties of both natural and construct GR alleles in their ability to function as modulators of transcription, which appears to depend on the promiscuity of protein-protein intractions attributed to most gene regulators, rather than their ability to bind DNA. In the studies of Stocklin *et al.*, (1996) discussed in chapter 1 (pp 24-25), the glucocorticoid receptor has been shown to act as a synergistic coactivator for the transcription factor STAT5, which mediates responses in mammary epithelial cells to the lactogenic hormone, prolactin. Following transient expression in COS cells, GR modulates STAT5-mediated transcription, independently of a GRE. Compared with untreated cells, prolactin induces β-casein promoter activity ~10-fold, an effect which is either absent or minimal following glucocorticoid treatment. Simultaneous induction of β-casein activity with both prolactin and glucocorticoids on the other hand

significantly increases promoter activity ~40-fold. Conversely, in the presence of a GRE-containing promoter, STAT5 represses GR-mediated transactivation. The level of glucocorticoid-dependent promoter induction mediated by STAT5, not only provides sufficient scope for identifying possible GR functional variants, but does so by capitalising on the ability of different transcription factors to co-regulate one another through interaction at, so far, unrecognised transcriptionally active surfaces.

Thus, while simple promoters give a preliminary index of glucocorticoid signalling or GR activity, extrapolation to endogenous genes should be exercised with caution. The regulation of the β-casein promoter through the interactions between STAT5 and GR may introduce a level of complexity which more closely parallels the *in vivo* situation in which subtle alteration in the primary structure of GR may be of greater functional consequence.

7.3.) Effects of amino terminal substitutions on GR function

Although the steroid binding difference between natural and construct alleles is not great, it is interesting to see that the probable effect of the engineered flanking substitutions around the polyglutamine tract is to increase steroid binding affinity. This could mean that the mutant structure of the rGR amino terminus is able to modulate the activity of the steroid binding pocket. This raises the more general question of whether the amino and carboxyl termini are able to affect each other's activity, either by contact, or through a mediator such as HSP90. Unfortunately, the complete three dimensional structure of rGR and proximity of its interactive surfaces is not available. However, a model describing the influences of GR carboxyl terminal structure on amino terminal function has been described. In this model, changes in structure at the carboxyl terminus are predicted to affect the flow of information within GR, resulting in effects on transcriptionally active surfaces (Guido *et al.*, 1996).

The molecular mechanisms of GR are complex, requiring the concerted effect of a number of interactive elements which are induced by the binding of ligand. Since GR is expressed at similar levels in almost all tissues and cell types it follows that additional systems must be in place to help regulate activity in a tissue or promoter specific fashion. In the studies of Guido *et al.*, (1996), different steroidal ligands, even those with the same essential properties, agonistic or antagonistic, have been shown to be capable of stimulating promoter specific receptor function, most likely by inducing unique receptor conformations (Agarwal, 1994; Chen *et al.*, 1994). Agonists and antagonists differ in substituents, stereochemistry and the degree of saturation at various positions around the steroid nucleus. For example, GR antagonists possess an 11-β aryl substitution charactetristic of many of these compounds. RU38486 and ZK98299, receptor antagonists which differ only in the substituents on C17 (Guido *et al.*, 1996) can stimulate GR promoter activity *in vivo*, but with quite different versatilities and with a dependency on promoter context. RU38486, normally viewed to be exclusively antagonistic, can induce GR transcriptional activity in HepG2 cells, an activity which is unattainable with ZK98299 and is absent in CV-1 cells. The binding of ZK98299 to GR in Hek293 cells is able to prevent the efficient interaction between GR and the DNA and results in transcriptional repression (Heck *et al.*, 1994). *In vitro*, there is no evidence of ZK98299-GR binding to GREs of either simple or complex promoters. It is likely that the conformation of RU38486 and ZK98299-GR complexes differ significantly from that of the unliganded receptor since without ligand, GR has no known transcriptional activity. Apart from the effects of different ligands on receptor activity, there is also evidence of unique tissue specific factors interacting with specific liganded forms of GR, which play a role in GR modulated transcription (Cavin and Buettik, 1995).

These data suggest interactions between the carboxyl and amino-terminal ends of liganded GR, such that ligand-specific conformations of GR impose unique influences on amino terminal function. By analogy, the flow of signalling information may also occur in the opposite direction, whereby alteration in the primary polypeptide sequence of the GR amino terminus

has an effect on receptor steroid binding. Preliminary investigations of steroid binding affinity by GR proteins expressed in COS-7 cells (pp 157-159 has indicated that a truncated GR isoform in which the first 100 or so amino acids were predicted to be missing, has a significantly lower affinity for dexamethasone (see Fig. 6.13.), supporting the probability of interaction between the receptor amino terminus and ligand binding domain.

7.4.) Association of Milan rat GR alleles with glucocorticoid related phenotypes and expression of GR alleles in CV-1 cells

The polymorphism in the GR triplet $(CAG)_n$ repeat between the Milan hypertensive (MHS) and normotensive (MNS) strains of rat was used successfully as a genetic marker in breeding experiments. In F2 animals resulting from a cross between MHS and MNS, linkage analysis showed a significant association between the MHS GR allele and hypercalcuria and to a lesser extent with reduced systolic blood pressure in females and reduced body weight in male rats, indicating that either the GR gene or a closely linked locus influences these phenotypes. These data have already been reported as preliminary findings (Heeley *et al.*, 1996b) and a more detailed account has also been submitted for publication.

In terms of GR function, this thesis also shows that in CV-1 cells, the Milan alleles Grl^{CAG20} and Grl^{CAG21} are expressed as polypeptides to an equal extent (Fig. 6.20.) and have indistinguishable steroid binding affinities and capacities (Fig. 6.24. and Table 6.5., pp 179-180). If this applies to these alleles *in vivo*, then it is unlikely that alleleic differences in GR perse between the Milan strains of rat, and hence variation in the steroid binding activity would account for the differences in gross phenotype between MHS and MNS. However, the possibility of other affectors of GR steroid binding affinity should not be ruled out. It is not known whether the differences in polyglutamine tract length in rat GR would cause a difference in its transactivation properties *in vivo*.

7.5.) Modulators of glucocorticoid activity

The contrast in binding characteristics between Milan rat alleles in cytosol from rat liver and CV-1 cells may signify the existence of a cytosolic factor(s) which is present in liver, but not CV-1 cells. Such a component could be a metabolite, for example, of steroid biosynthesis or an actively expressed regulator of steroid receptor function. Several possible extranuclear affectors of glucocorticoid activity have been identified, which includes the following; 1.) heat shock proteins (HSPs), 2.) cholesterol and fatty acids, 3.) aminophosphoglycerides, 4.) 'unknown' cytoplasmic factor(s) and 5.) calreticulin. For rat GR, there is the added possibility of modulation of steroid binding by truncated GR isoforms which result from translation initiations downstream of met1 in the GR message. The relevance and possible association of each of these factors with altered GR binding affinity, described in this thesis for rat strains MHS and Zucker obese, is considered below.

7.5.1.) Heat shock proteins (HSPs)

The critical effects of heat shock proteins, particularly HSP90, in determining sensitivity to glucocorticoids is well documented (see Bamberger *et al.*, 1996 and references therein). However, a role for HSP90 as a modulator of steroid binding in MHS and Zucker obese rats has not been determined. There is no evidence of amino acid substitutions within the HSP90 binding domain of GR from either strain of rat which might affect interactions between these two proteins. Mutations in HSP90, however, are still possible although recent observations by Panarelli *et al.*, (1995) suggest no difference between MHS and MNS in temperature sensitivity of GR to steroid binding.

7.5.2.) Truncated GR isoforms

Rat GR expressed both *in vivo* and in tissue culture shows evidence of additional isoforms which are predicted to result from translation events downstream of met1 in the rGR message (Miesfeld *et al.*, 1986; see also chapter 6, part 1, pp 157-163). In COS-7 cells, expression of GR allele Grl^{CAG21} from vector pcDNA1Neo produces two distinctive bands

immunoreactive with GR monoclonal antibody Mab250, one of around 94 kd and the other around 79 kd (p 157). The smaller product is indicative of translation from GR met3. This is likely to be a tissue specific effect, since the isoforms expressed in CV-1 cells and in liver were either different or expressed to a different degree. Steroid binding assays performed on whole cell preparations of COS-7 cells expressing the 94 and 79 kd forms of GR (see p 159) indicated two distinctive and independent binding entities, one with the characteristics for dexamethasone binding of full length native GR (high affinity, low capacity) and the other of a much lower binding affinity. *In vivo*, such a molecule could act as a modulator of GR binding activity by competing for steroid hormone. However, this is an unlikely explanation for the Milan strain differences given the similarities in the levels of these isoforms in each strain (Ch 5, Fig. 5.2.).

In other species, including mouse and man (having a similar arrangement of Kozak sequences at the beginning of the GR mRNA to that of rat: see Table 7.1., below), alternate GR translation products may also be generated. Such products have so far not been reported. Their expression may have a strict species or tissue-specific dependency.

Species	Kozak sequence **('optimum' = (CC)ACCATGG)**				
	met1	met2	met3	met4	met5
Rat	CCAATGG	GTAATGG	*TCCATGG*	TATATGG	GTGATGG
Mouse	CCAATGG	GTGATGG	*TCCATGG*	TATATGG	GTGATGG
Human	CTGATGG	GTGATGG	*TCAATGG*	TATATGG	GTGATGG
Guinea pig	GGAATGT	GTAATGC	*TCAATGG*	TATATGG	GTGATGG

Table 7.1. Comaparison of Kozak sequences in and around the first 5 methionine codons of GR from different species.
The Kozak sequence closest to 'optimum' (Kozak, 1986) for translational initiation, is shown in italics.

7.5.3.) Cholesterol and fatty acids

The apparent glucocorticoid resistance in MHS and Zucker obese rats is correlated with elevated plasma cholesterol levels, a phenotypic variable also found in normal human subjects with reduced affinity for cortisol

binding (Panarelli *et al.*, 1994; Walker *et al.*, 1995). A possible association between glucocorticoid activity and cholesterol metabolism is well established. However, the level at which the glucocorticoid and cholesterol metabolic pathways interact, is unclear. One body of evidence suggests that glucocorticoids may have a direct effect on cholesterol synthesis. For example, glucocorticoids have been demonstrated to inhibit cholesterol biosynthesis in mouse thymocytes by a mechanism which requires functional GR (Picard *et al.*, 1980). In addition, the blockage of glucocorticoid signal transduction in partial GR knockout mice in which the number of active GR molecules is reduced (Pepin *et al.*, 1992) is manifest as obesity.

Alternatively, the increased levels of cholesterol may have the capacity to interfere with steroid binding by GR. It is concieveable that any molecule with the basic steroid ring structure and appropriate substitutions is able to interact with steroid receptors, affecting steroid binding. Cholesterol, the precursor of steroid biosynthesis might be an excellent candidate. It should be stressed that this possibility is least likely, given that the binding of all steroid ligands should be affected in a similar way. The binding of dexamethasone to GR in the Milan and Zucker rat models is not as greatly affected compared with control strains. On the other hand, dexamethasone is considered a stronger ligand than corticosterone and thus, may escape these modulating effects.

Hypercholesterolaemia and hyperlipidaemia are often associated with reduced GR function. It is therefore possibile that the function of GR in these rat strains is also affected by fatty acids. For example, the short chain fatty acid, sodium butyrate affects GR interaction with its GRE by preventing nucleosome displacement and represses transcription by inducing a modification of chromatin (Bresnick *et al.*, 1990). The decreased binding of dexamethasone to rat liver cytosol glucocorticoid receptors has also been demonstrated by physiological concentrations of non-esterified fatty acids, with a dependency on dose, degree of unsaturation and chain length (Vallette *et al.*, 1991). The effects of fatty acids would be particularly applicable to the Zucker obese rat. Hepatocytes from the obese strain in

culture show evidence of fatty acid-like deposition, absent in lean rat cells (personal observations). The possible added influence of fatty acids might explain the steroid binding difference between MHS and MNS rats (MHS are mildly hyperlipidaemic).

7.5.4.) Aminophosphoglycerides

Novel ether aminophosphoglycerides, also known as modulators have been isolated from rat liver preparations, which inhibit GR activation by interfering with its dissociation from heat shock proteins (Bodine and Litwack, 1990; Robertson *et al.*, 1995). From a functional standpoint, modulator represents the endogenous molybdate factor, stabilising both the activated and unactivated forms of GR. These molecules appear to further interfere with GR function by inhibiting translocation to the nucleus. The molecular effects of modulators may be commonplace among steroid receptors. The activity of rat distal colon MR is also affected by these molecules (Schulman *et al.*, 1992). Interestingly, in five unrelated patients with the syndrome of aldosterone resistance, no mutations of pathophysiological significance, either in the MR coding sequence, or in 0.9 kb of the 5' regulatory region were identified, which would explain the resistance of these patients to aldosterone (Arai and Chrousos, 1995). These authors suggest that the defect responsible is at a postreceptor level.

7.5.5.) 'Unknown' cytoplasmic factor(s)

In humans, glucocorticoid resistance may be inherited, as an autosomal recessive trait, or may be acquired (Bronnegard *et al.*, 1996 and references therein). Natural animal models of glucocorticoid resistance are also seen in certain species of New World primate. These are the species of predominantly canopy-dwelling tree monkeys, including squirrel monkey, owl monkey and cotton-top tamarin, some of which are strikingly resistant to all of the steroid related hormones. The squirrel monkey for example has elevated cortisol levels and glucocorticoid resistance. In this species, levels of plasma free cortiosol, 100-times higher than in man are maintained by higher ACTH and cortisol synthesis rates, a cortisol binding globulin with decreased capacity for cortisol and a decreased rate of clearance of cortisol from the circulation. These monkeys, however, do not show signs of

clinical glucocorticoid excess. They have normal levels of plasma electrolytes. The binding affinity of lymphocyte GR in this species is 5-times lower than in human (Kd, 20.9 ± 1.8 vs 4.3 ± 0.2 nmol/L, n=3). The LBD of the squirrel monkey GR contains 4-amino acid differences when compared to the human GR, which are present in other New World primates. The amino terminal domain contains 22 differences. None of the LBD differences appear to be compatible with mutations known to affect steroid binding in other species. When expressed in a reticulocyte lysate system, the affinities of human and squirrel monkey GR were similar (Kd, 5.9 ± 1.2 vs 4.3 ± 0.5 nmol/L, n=3). These results suggest that the substitutions in the LBD of the squirrel monkey GR are not responsible on their own for the observed resistance to cortisol binding. Rather, the binding affinity is most likely influenced by cytosolic factors which affect GR function. In cotton-top tamarin B95-8 cells, inhibitors which interfere with glucocorticoid and vitamin D receptor binding have been identified (Brandon *et al.*, 1995) and a cytosolic cortisol binding protein has been proposed (Brandon *et al.*, 1994). It is not yet known whether similar factors exist in squirrel monkey cells.

Steroid resistance in squirrel monkey may result from a low expression of HSP90, or from the expression of a mutant form of this protein (Cadeponed *et al.*, 1994). Mutant forms of HSP90 or low levels of HSP70 have been given to explain glucocorticoid resistance in some human luekemic cell lines (Kojika *et al.*, 1996). The mechanisms of glucocorticoid resistance in the squirrel monkey are likely to be different to those reported for the guinea pig (*cavia porcellus*). Guinea pig GR also has low affinity for cortisol with a compensatory increase in the plasma (Keightley and Fuller, 1994). The LBD of the guinea pig GR differs in 24 places from that of human. Only four of these substitutions (Gly_{612}, Thr_{545}, Glu_{672} and Leu_{755}) are shared with squirrel monkey, rat and mouse, leaving 20 which might contribute to the differences in steroid binding between guinea pig and human GRs. Preliminary studies suggest the unique substitution at position 539 (Tyr-His) is at least partly responsible for the low binding affinity of the guinea pig GR (Keightley and Fuller, 1994).

The detailed molecular mechanism of steroid resistance in New World primates remains elusive. These species may harbour a global inhibitor of steroid receptor action; steroid-specific cytosol binding proteins only explains some aspects of the paradigm. The model answer requires a global solution which applies to all steroid receptors.

7.5.6.) Calreticulin

The general concept of steroid resistance may be explained in part by the protein calreticulin, a widely distributed multifunctional protein found in the endoplasmic reticulum where it acts as a major calcium-binding protein and in the nucleus where its presence implies a possible role in transcriptional regulation. Calreticulin binds a polypeptide motif KLGFFKR, which constitutes part of the DNA binding domain shared by all known members of the steroid hormone receptor superfamily (Fuller, 1991). It is therefore reasonable to suppose that abnormal expression of calreticulin might affect glucocorticoid sensitivity (Burns *et al.*, 1994; Dedhar *et al.*, 1994). The regulation of calreticulin expression is complex involving both transcriptional and post-translational steps. Integrin molecules are also bound by calreticulin at the cell surface. The cytoplasmic domain of all integrin α-subunits contain an almost identical polypeptide sequence motif, KXGFFKR (where X can be either G, A or V), to that found in steroid hormone receptors. Calreticulin has also, been shown to inhibit AR binding to its DNA response element *in vitro* and AR and retinoic acid receptor gene regulatory activities in tissue culture, thus implying a general role in modulation of gene transcription by steroid receptors (Dedhar *et al.*, 1994). Despite the implied general role of calreticulin in modulating the activity of steroid hormone receptors, effects of this molecule on steroid binding are unlikely.

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Publicatioins arising from this thesis

Abstracts:

R. P. Heeley, G. Casari, L. Zagato L. Torielli, R. G. Sutcliffe G. Bianchi and C. J. Kenyon. Glucocorticoid receptor mutations in genetically hypertensive rats: markers of glucocorticoid insensitivity. *Immunology*. (1996). **89** (suppl), p53.

R. P. Heeley, E. Gill, B. van Zutphen, C. J. Kenyon and R. G. Sutcliffe. A bimodal distribution of CAG repeats in the glucocorticoid receptor of wild and inbred rats. *J Endocrinol.* (1996). **148** (Suppl).

C. J. Kenyon, G. Casari, L. Zagato, G. C. Inglis, R. Heeley, L. Torelli, J. M. C. Connell, R. G. Sutcliffe, and G. Bianchi. Glucocorticoid receptor and 11β -hydroxylase genes interact epistatically with adducin in the control of blood pressure of Milan rats. *J Endocrinol.* (1994). **143**, (Suppl).

Papers:

R. P. Heeley, G. Casari, L. Zagato L. Torielli, R. G. Sutcliffe G. Bianchi and C. J. Kenyon. Glucocorticoid receptor mutations in genetically hypertensive rats: markers of glucocorticoid insensitivity. *Biochem Soc Trans.* (1997). **25**, 236S.

R. P. Heeley, E. Gill, B. van Zutphen, C. J. Kenyon and R. G. Sutcliffe. Polymorphisms of the glucocorticoid receptor gene in laboratory and wild rats: steroid binding properties of trinucleotide CAG repeat length variants. *Mammalian Genome.* (1998). **9**, 198-203.

Appendix 1

PRIMERS: For PCR, Reverse Transcription and Sequencing.

Primer name	Sequence (5'-3')	length (mers)	location in GR relative to met1	Application
pG	AATGCTTTCTTCCAGAAGCCG	21	n 421-441	Reverse PCR
pA	CCTGGCAGTTTGCTTGGCCAA	21	n 46-66	Forward PCR
p9	AAAGGCTCCACAAGCAATGTG	21	n 202-221	Forward PCR
p19	GACAGTGAAACGGCTTTGG	19	n 299-317	Reverse PCR
rGR1	GTTGAACCACATGGACTTGG	20	n 2756-2775	Reverse Transc^{n}
rGR2	CTATAAACCACATGTACTGCG	21	n 2582-2602	Reverse PCR
rGR6	CACTTGACGCCCACCTAACAT	21	n 1747-1767	Reverse PCR
rGR10	GGAGCAAAGCAGAGCAGGTT	20	n 1909-1928	Reverse PCR
rGR18	TGATACGCCTATTTTGGATCC AATGCTTTCTTCCAGAAGCCG	42	n 421-441	Reverse PCR/ Cloning
rGR19	ATGTTTGACAGCTTAGGATCC TTGCCAATGGACTCCAAAGAA	42	n $^{-}$6-15	Forward PCR/ Cloning
rGR20	TGGGCAGTTTTTCCTTCGAAT	21	n 1459-1479	Reverse PCR
rGR21	GTTCAGAGCCCCCAAGGAAGT	21	n 1186-1206	Reverse PCR
rGR22	GGGGGAGCAAAGTTCAATGAA	21	n 907-927	Reverse PCR
rGR23	GGACCCAGCGGAAAACTCCAA	21	n 652-672	Reverse PCR
rGR25	TACAAGACAAATTGATAAGTT	21	n 2390-2411	Reverse PCR
rGR28	TTGCCAGTTGTGACTGGAGTT	21	n 2173-2194	Sequencing
rGR29	ATTGCTTGTGGAGCCTTTCGA	21	n 199-219	Sequencing
oligo-CAG	AGCAGCAGCAGCAGCAGCCAGC	22	n 362-384	Hybridisation probe
rGR32	GATCCGCCGTTTCACTGTC	19	Complementary to n 304-317 (sense)	Cloning fragment half-site
rGR33	CATGGACAGTGAAACGGCG	19	Complementary to n 304-321 (antisense)	Cloning fragment half-site
rGR34	ATGGGACTTTCCTACTTGGCA	21	n 266-286	pSTC-specific forward PCR primer
	Biotinylated Primers			
pG-BIO	B-AATGCTTTCCAGAAGCCG	21	n 421-441	Reverse PCR
rGR5-BIO	B-ATTCAGCAAGCCACTGCAGGA	21	n 1555-1575	Forward PCR
rGR19-BIO	B-ATGTTTGACAGCTTAGGATCC TTGCCAATGGACTCCAAAGAA	42	n $^{-}$6- $^{-}$15	Forward PCR
rGR31-BIO	B-TTCCTTCTCGAAGGGGACACG	21	n 763-783	Forward PCR

Compilation of oligonucleotides.
Primers are listed for use in PCR, reverse transcription, sequencing, Southern hybridisation and oligonucleotide half sites for the construction of DNA cloning fragments. Each oligonucleotide is referred to by a unique reference notation. Positioning in the rat GR cDNA sequence, together with length in bases is given. For PCR primers, forward and reverse orientation is stated.

Appendix 2

The following graphs (1-26) show a point-by-point plot of counts bound to CV-1 expressed GR following competition for ^{3}H-dex binding sites by cold dexamethasone or corticosterone. From a total of 55 independent Scatchard determinations, the data for the 20 repeat natural and construct alleles is shown.

Counts (x-axis) are normalised as % counts bound for each curve. The example below shows the calculation of % bound counts, from original counts.

20 CAG Natural : Dex (C):

cpm	Adjusted cpm	Bound cpm	% Bound cpm
177.1	283.4	186.1	50.6
290.7	465.1	367.8	100
241.1	385.8	288.5	78.4
197.7	316.3	219.0	59.5
144.4	231.0	133.8	36.4
93.6	149.8	52.5	14.3
74.8	119.7	22.4	6
69.6	111.4	14.1	4
60.8	97.3	0	0

cpm	Adjusted cpm	Bound cpm	% Bound cpm
164.4	263.0	145.3	44.5
277.7	444.3	326.6	100
263.1	421.0	303.2	92.8
224.1	358.6	240.8	73.7
146.4	234.24	116.5	35.7
107.8	172.5	54.7	16.7
81.8	131.0	13.12	4
73.6	117.8	0	0
76.4	122.2	4.48	1.3

Tables of raw data of duplicate cpm for ^{3}H-dex binding to GR allele 20 CAG Natural (C).
Values of cpm are volume adjusted to take account of the fraction of the total reaction volume taken for counting.

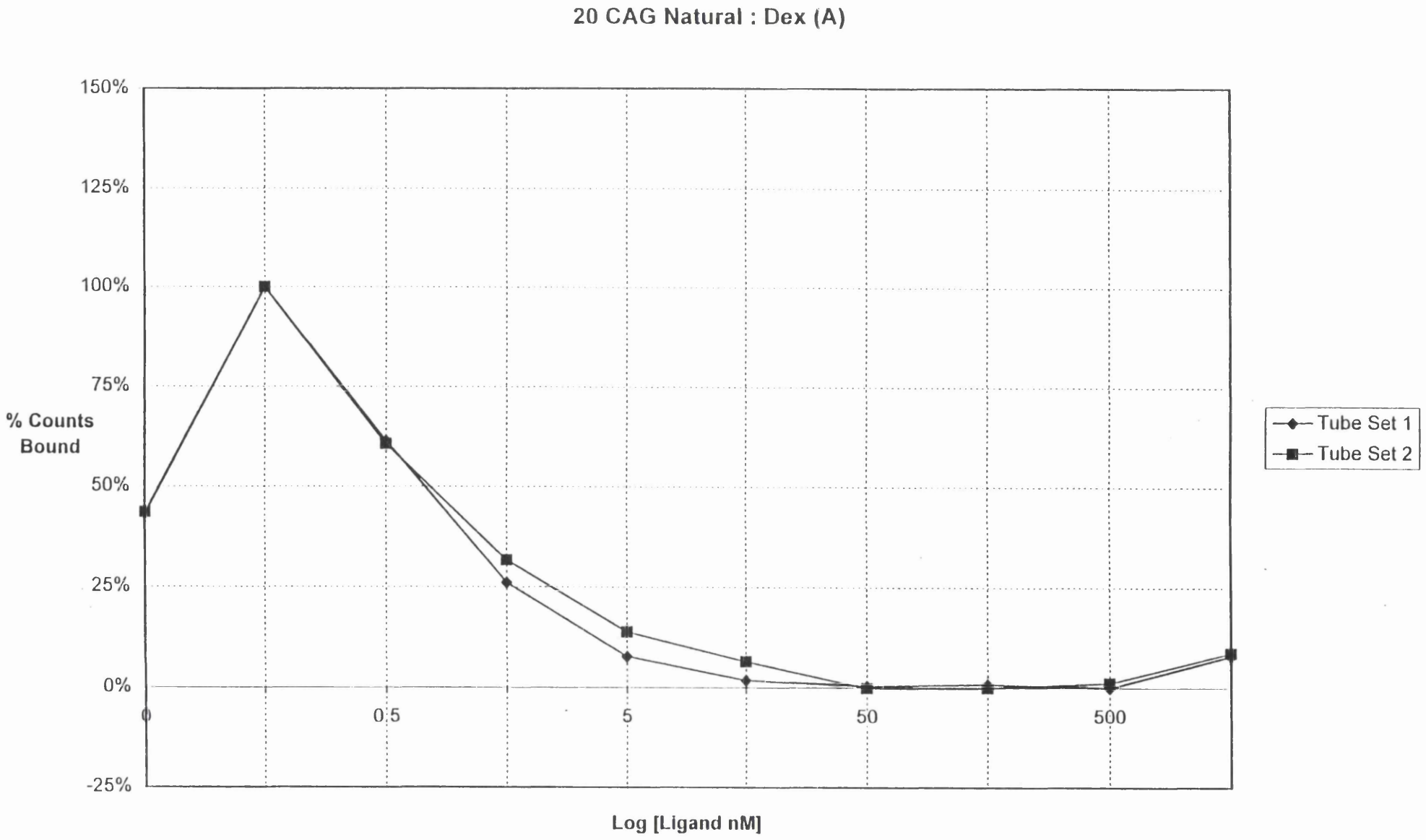
Key:

Circled points, (pages 13, 22 and 26) indicate a set of values in which one of the pair was a clear outlier. In these cases, the outlying point has been adjusted to match that of the value lying closest to the trend of the remaining points.

Points marked by an asterisk (y-axis), pages 9, 14, 15, 16, represent a pair of values which were clearly spurious in the original data. In these cases, the outlying points were omitted from calculations of Kd and Bmax. For the purpose of data plotting, an appropriate value was calculated by averaging the preceding and following data point in respective curves.

Appendix 2

Graph	Original total counts bound	Page
20 CAG Natural : Dex (A)	199.5/185.6	1
20 CAG Natural : Dex (B)	212.5/214.6	2
20 CAG Natural : Dex (C)	283.4/263.0	3
20 CAG Natural : Dex (D)	506.1/501.8	4
20 CAG Natural : Dex (E)	422.9/405.1	5
20 CAG Natural : Dex (F)	405.6/382.1	6
20 CAG Natural : Dex (G)	351.2/383.7	7
20 CAG Natural : Dex (H)	362.4/348.3	8
20 CAG Construct : Dex (A)	464.8/381.8	9
20 CAG Construct : Dex (B)	451.5/452.9	10
20 CAG Construct : Dex (E)	173.3/153.8	11
20 CAG Construct : Dex (F)	204.8/186.6	12
20 CAG Construct : Dex (G)	478.6/403.5	13
20 CAG Construct : Dex (H)	408.1/374.7	14
20 CAG Construct : Dex (I)	436.3/372.3	15
20 CAG Natural : Corticosterone (A)	152.6/158.2	16
20 CAG Natural : Corticosterone (B)	213.4/200.3	17
20 CAG Natural : Corticosterone (C)	327.2/387.7	18
20 CAG Natural : Corticosterone (D)	513.8/493.9	19
20 CAG Construct : Corticosterone (A)	342.7/401.6	20
20 CAG Construct : Corticosterone (B)	477.6/411.8	21
20 CAG Construct : Corticosterone (E)	162.9/161.6	22
20 CAG Construct : Corticosterone (F)	207.4202.9	23
20 CAG Construct : Corticosterone (G)	361.9/359.4	24
20 CAG Construct : Corticosterone (H)	327/346.7	25
20 CAG Construct : Corticosterone (I)	345.6/370.7	26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% Counts Bound
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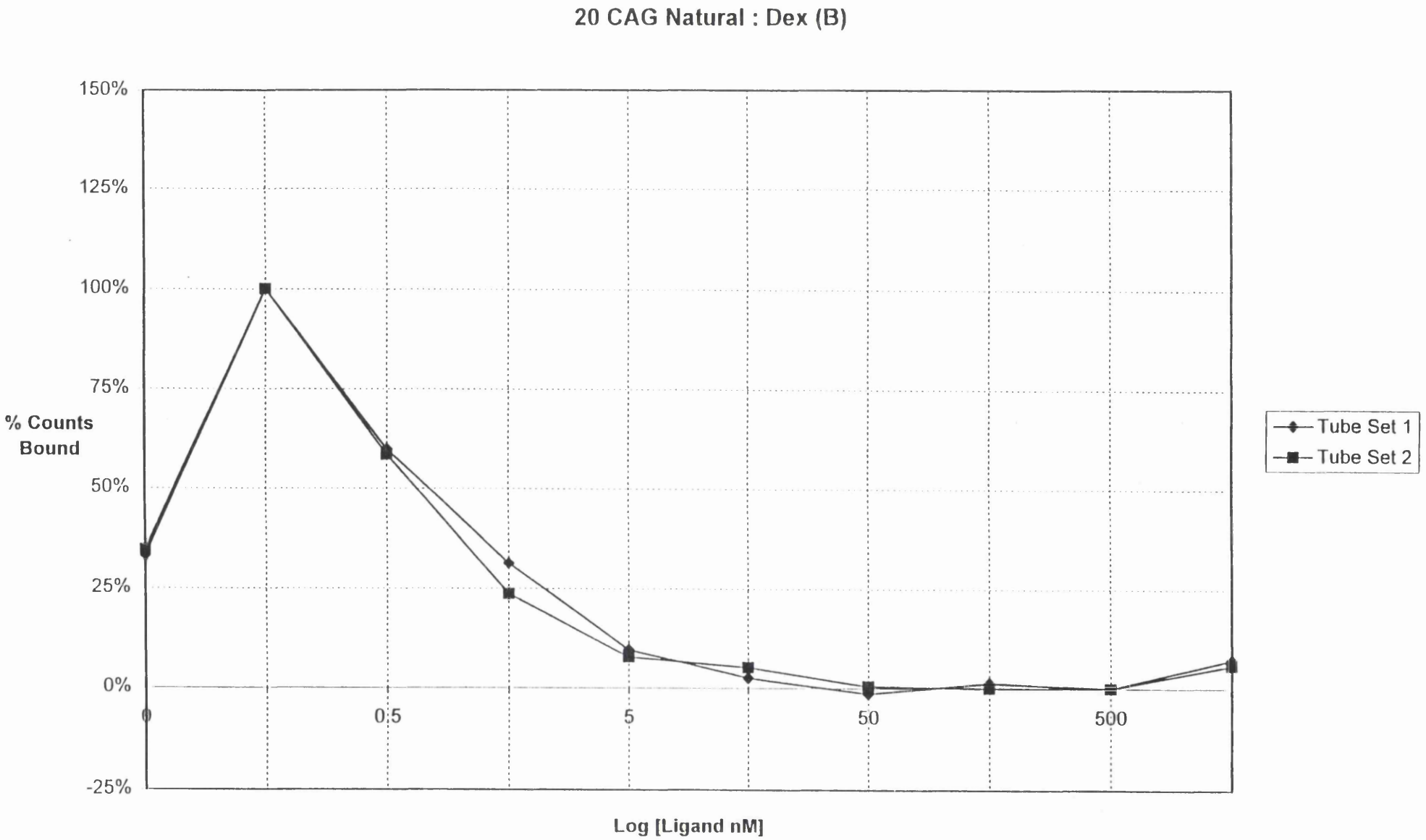
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2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-25%
% Counts Bound
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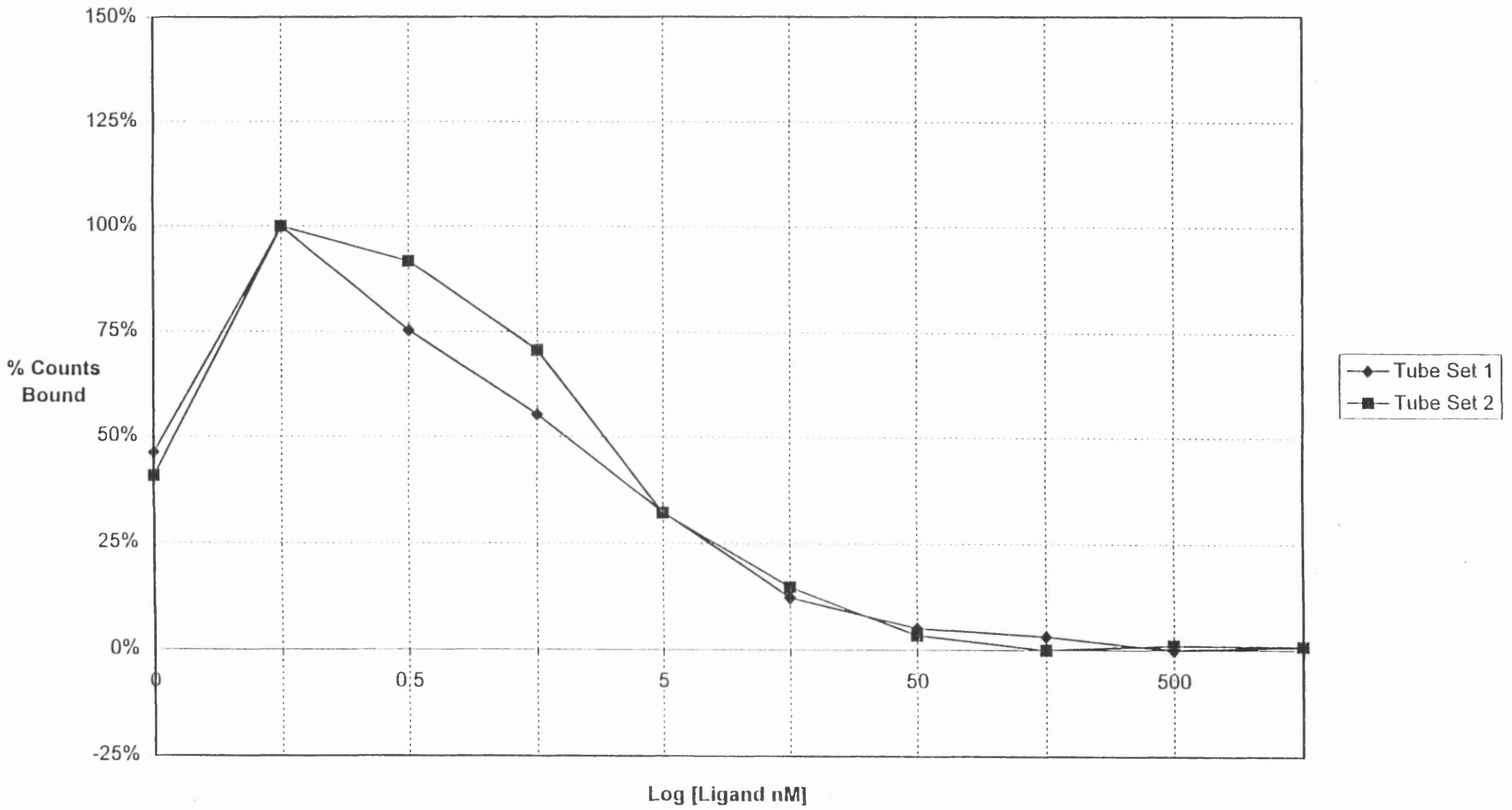
500
Log [Ligand nM]
Tube Set 1
Tube Set 2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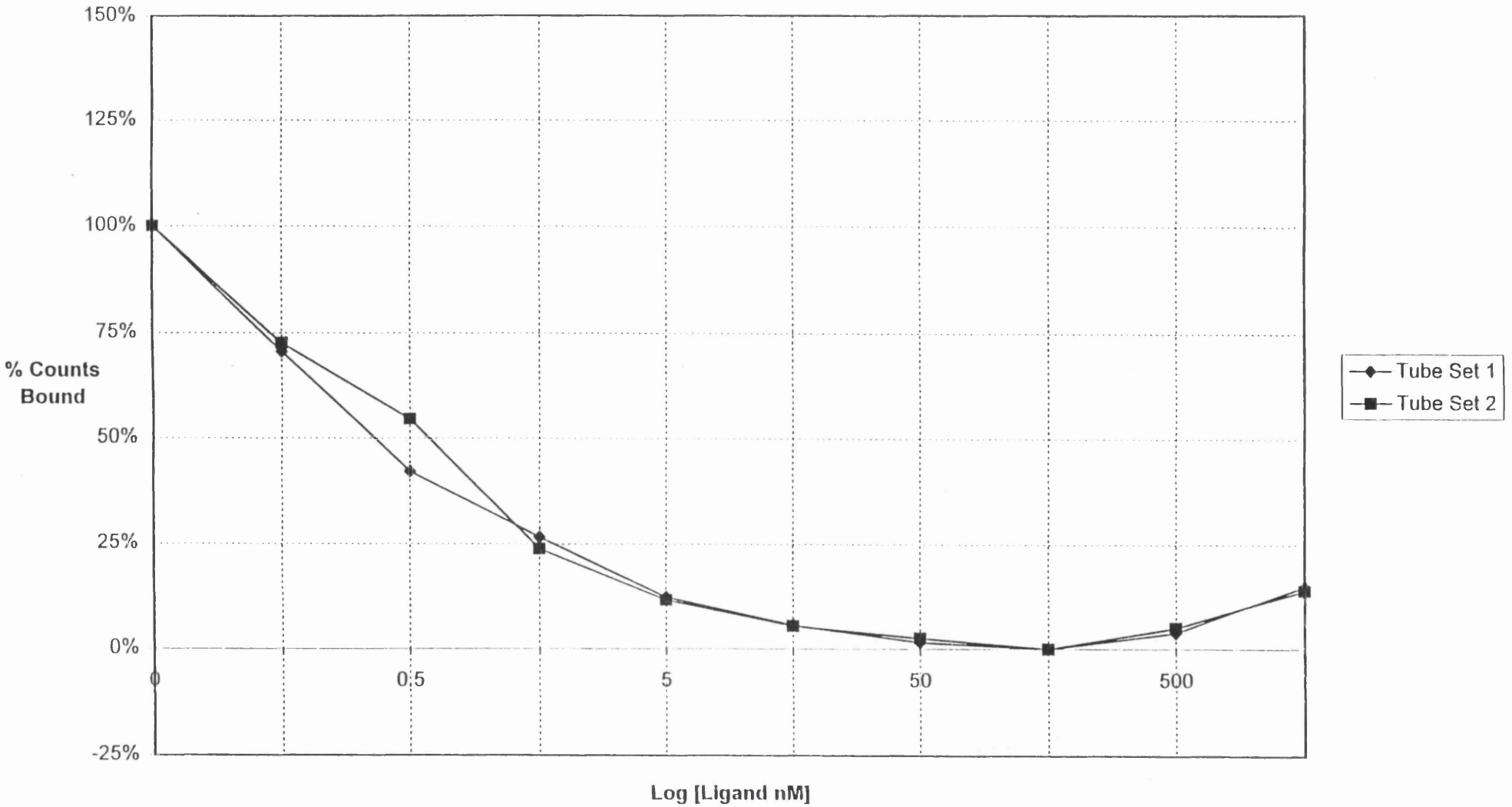
Tube Set 2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% Counts Bound
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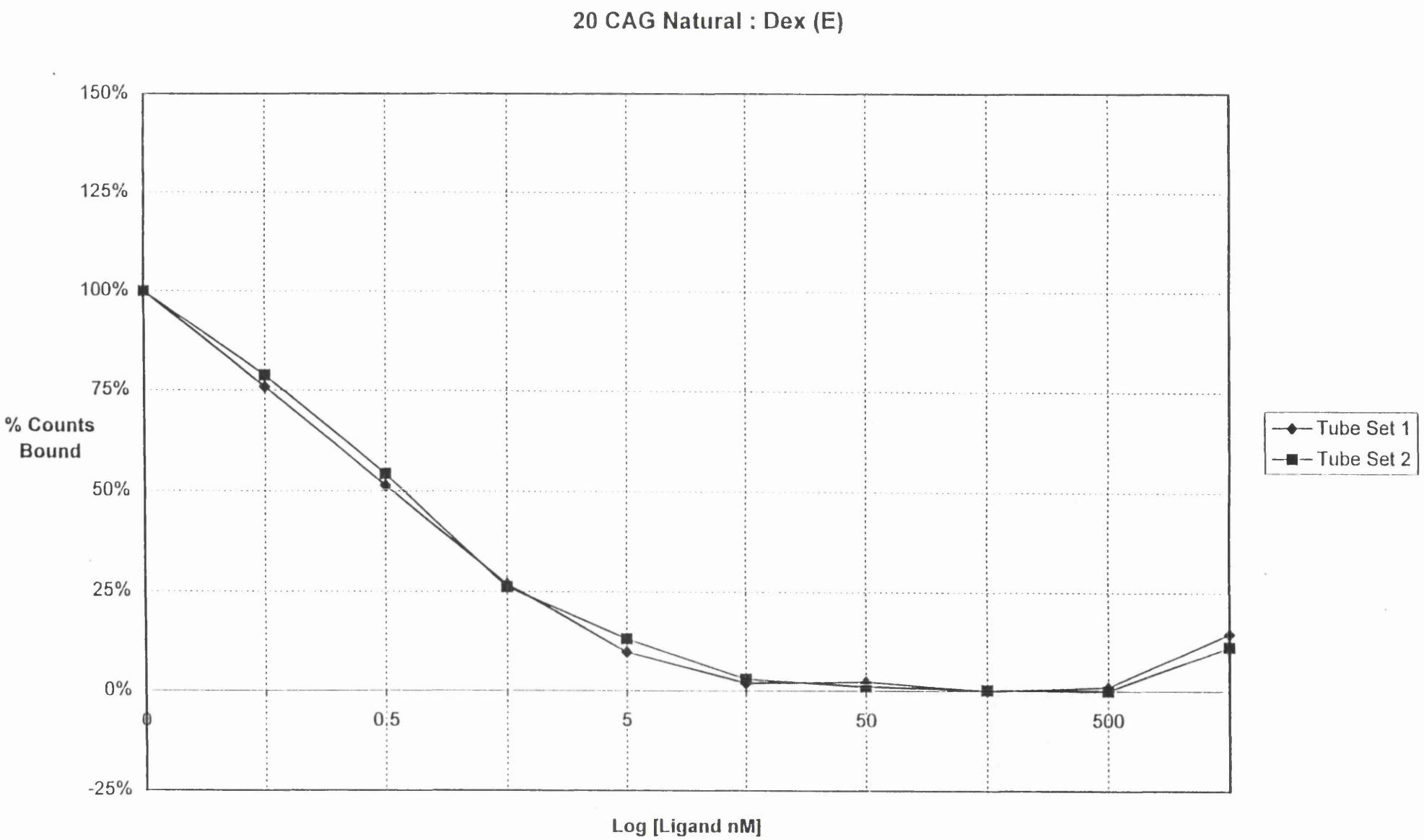
Tube Set 1
Tube Set 2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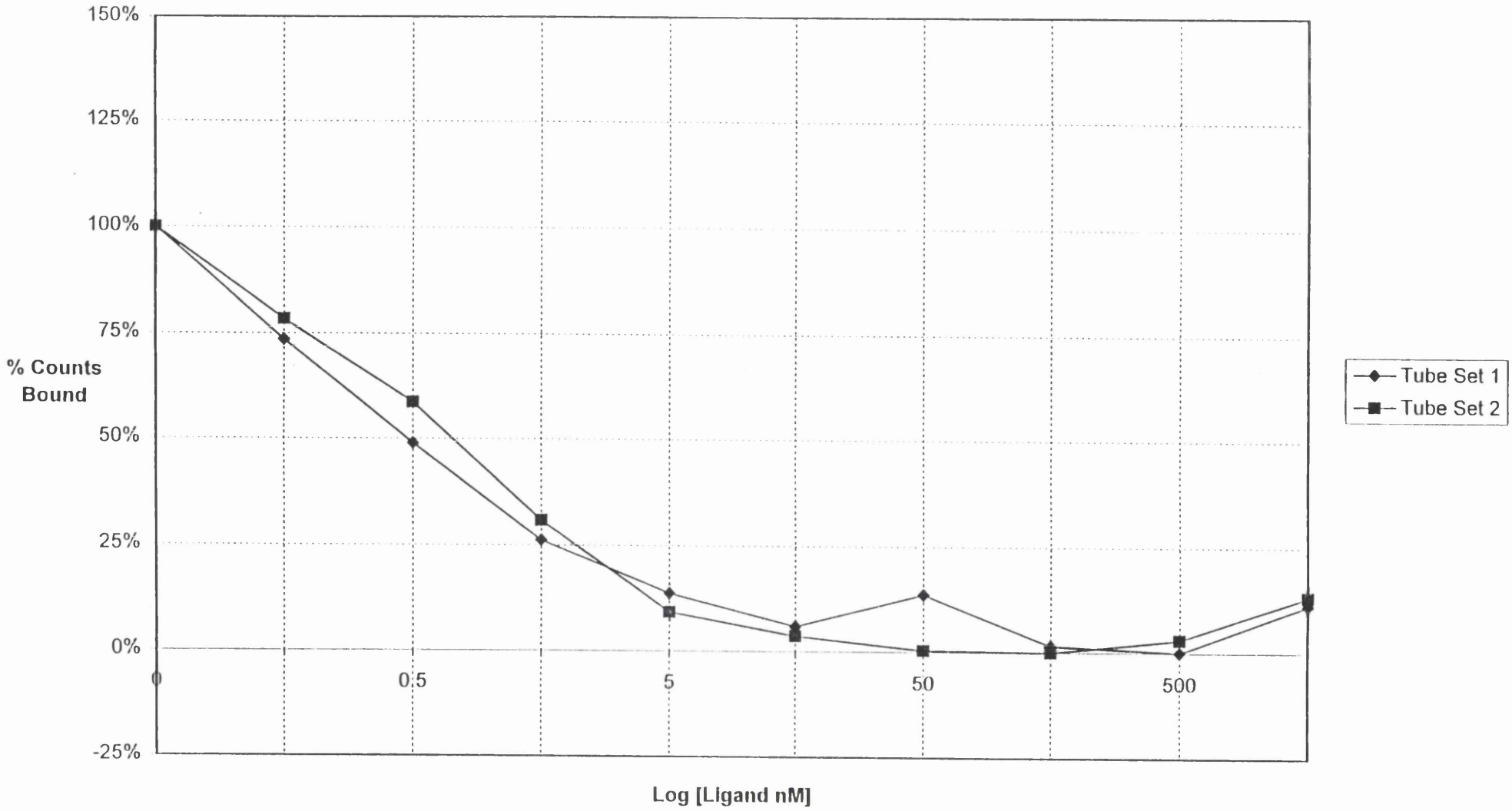
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2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% Counts Bound
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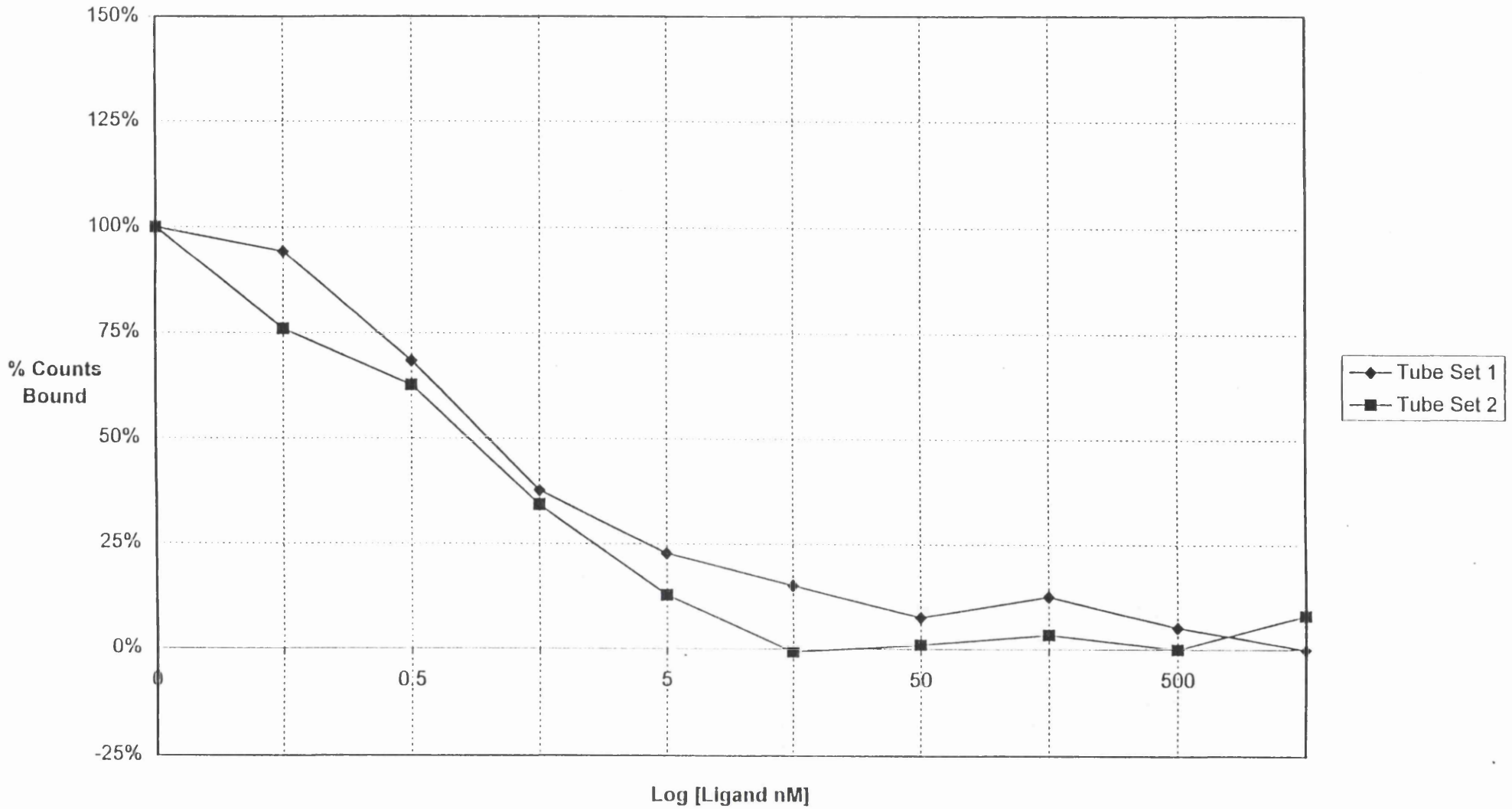
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2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% Counts Bound
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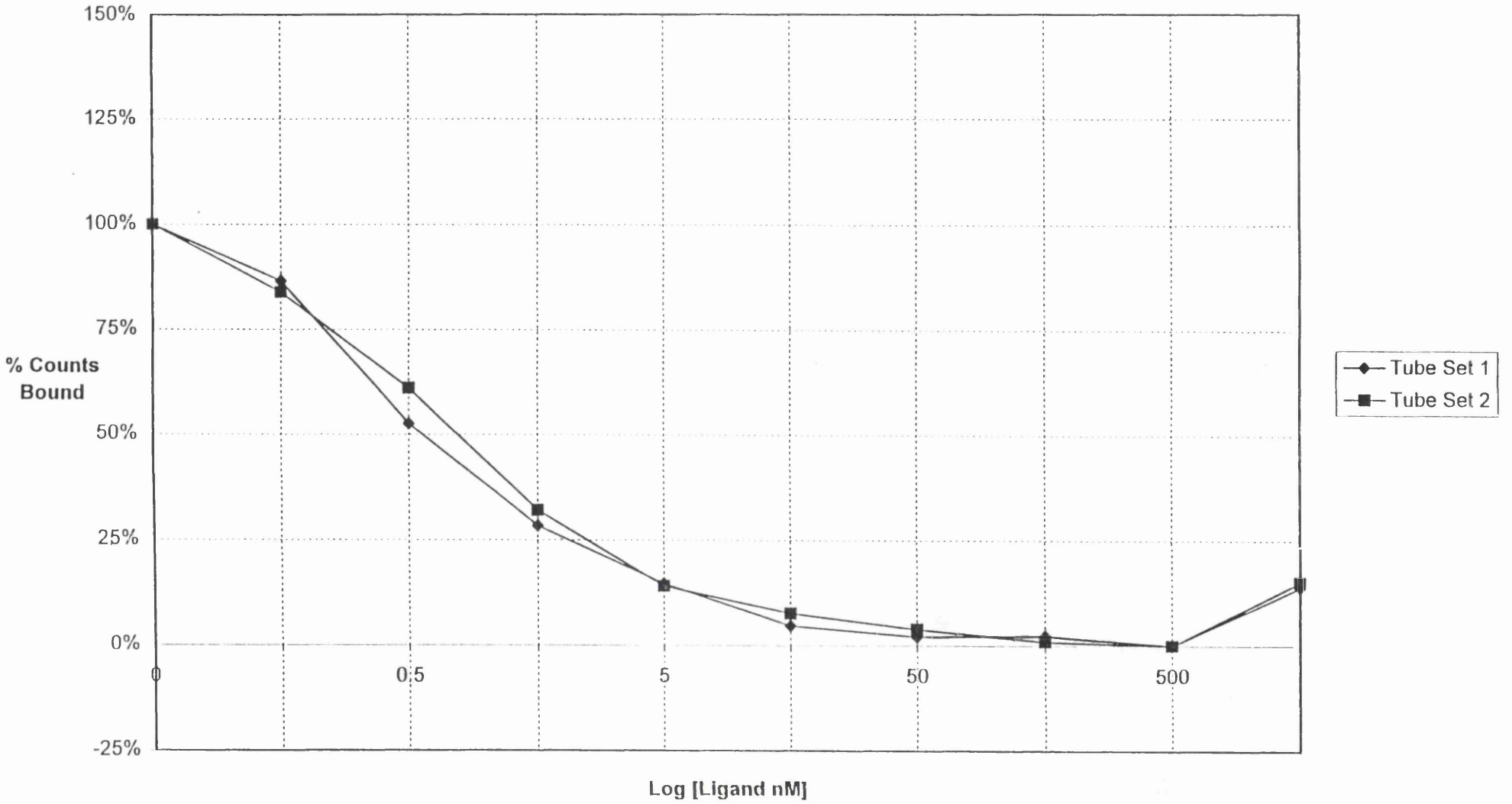
Log [Ligand nM]
Tube Set 1
Tube Set 2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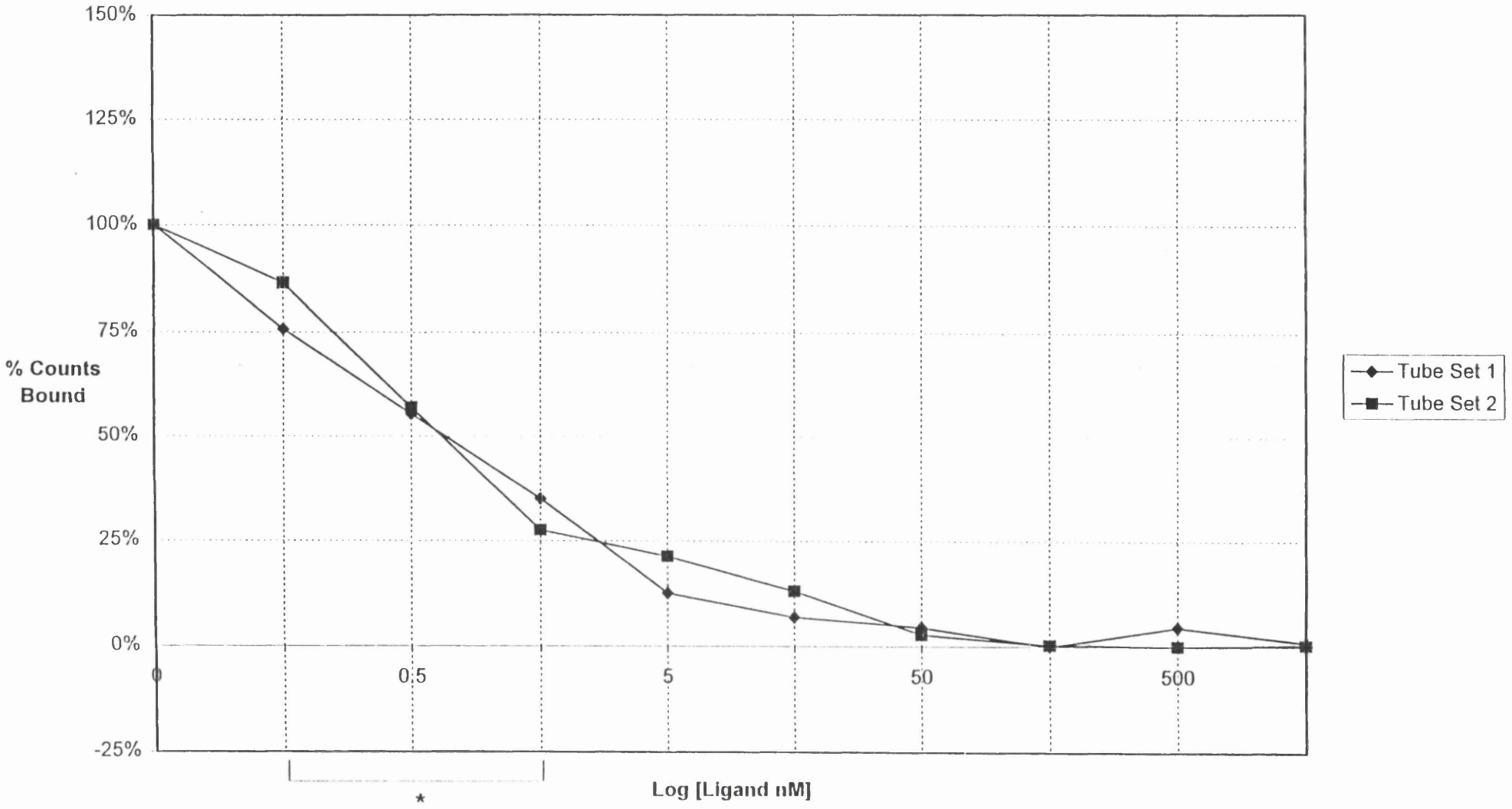
Tube Set 1
Tube Set 2

Appendix 2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% Counts Bound
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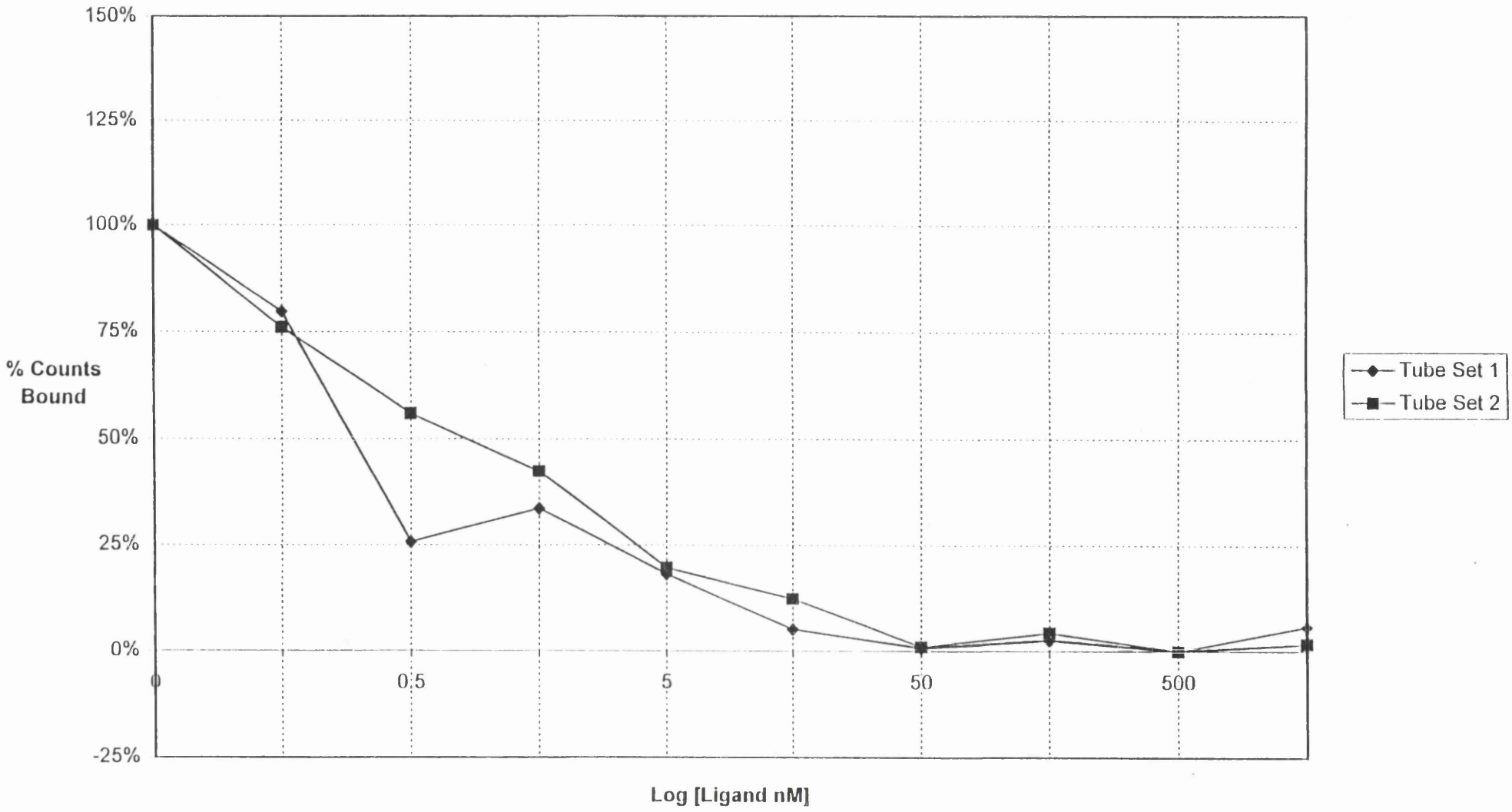
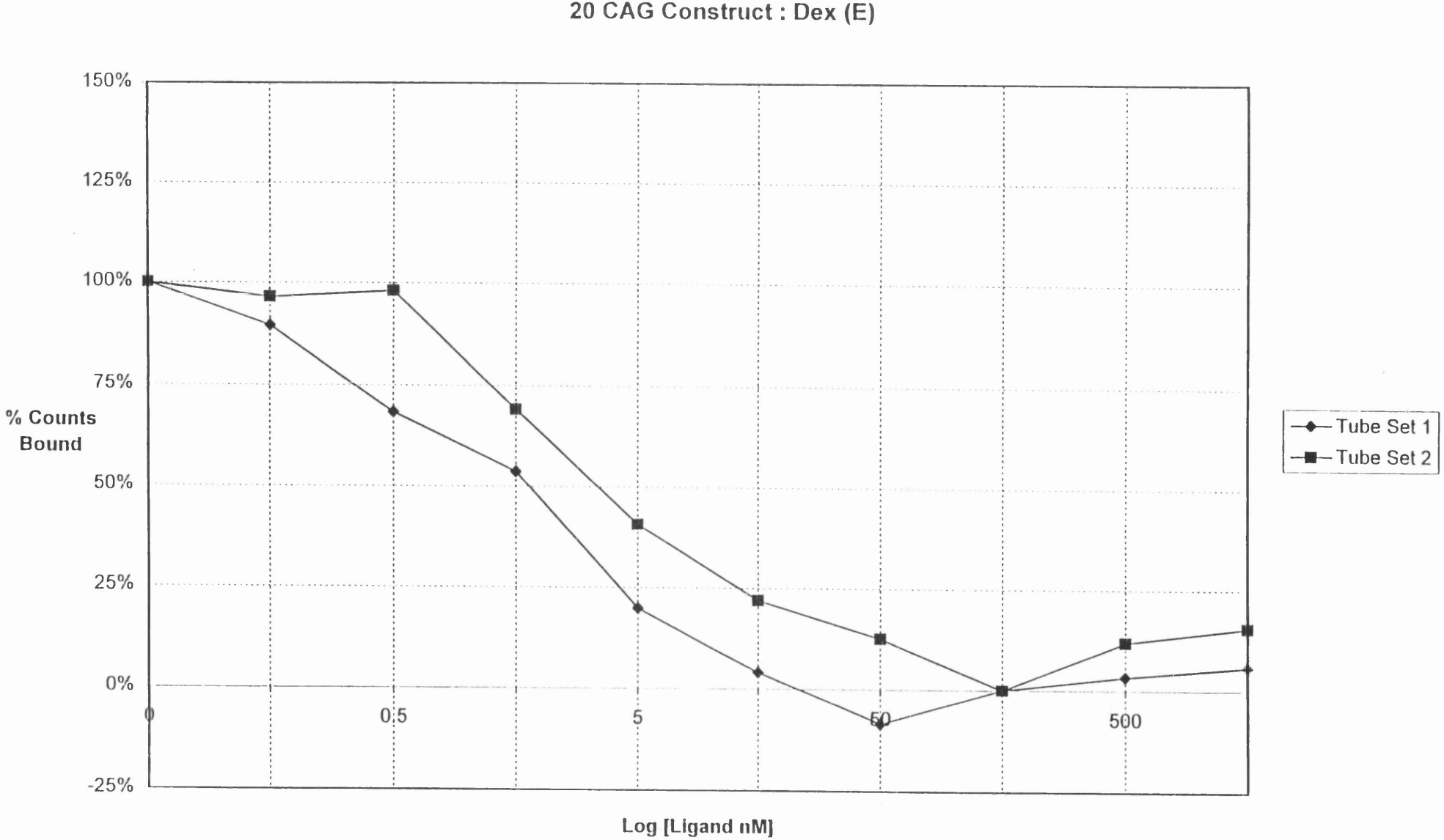
Tube Set 1
Tube Set 2

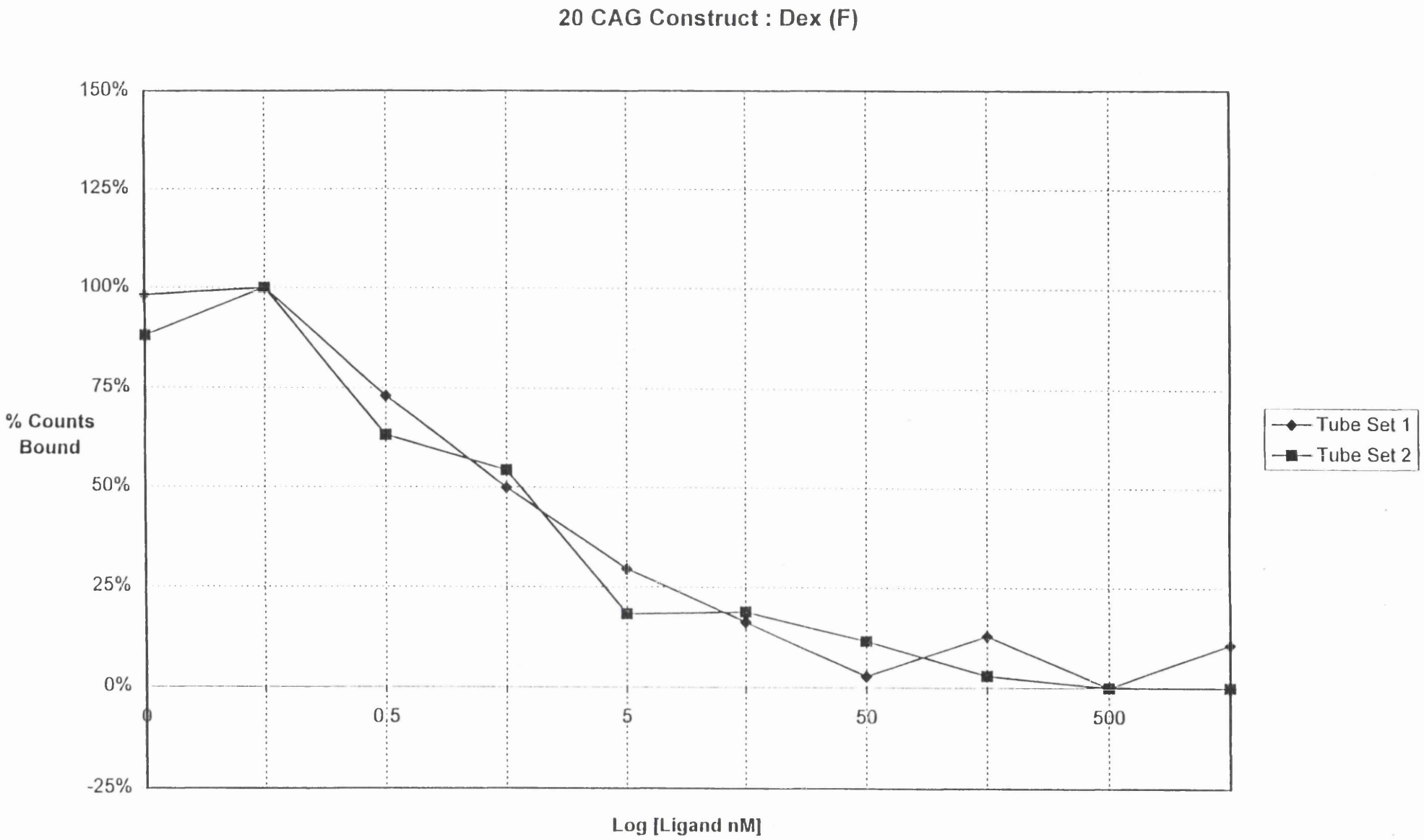
Appendix 2


20 CAG Construct : Dex (B)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

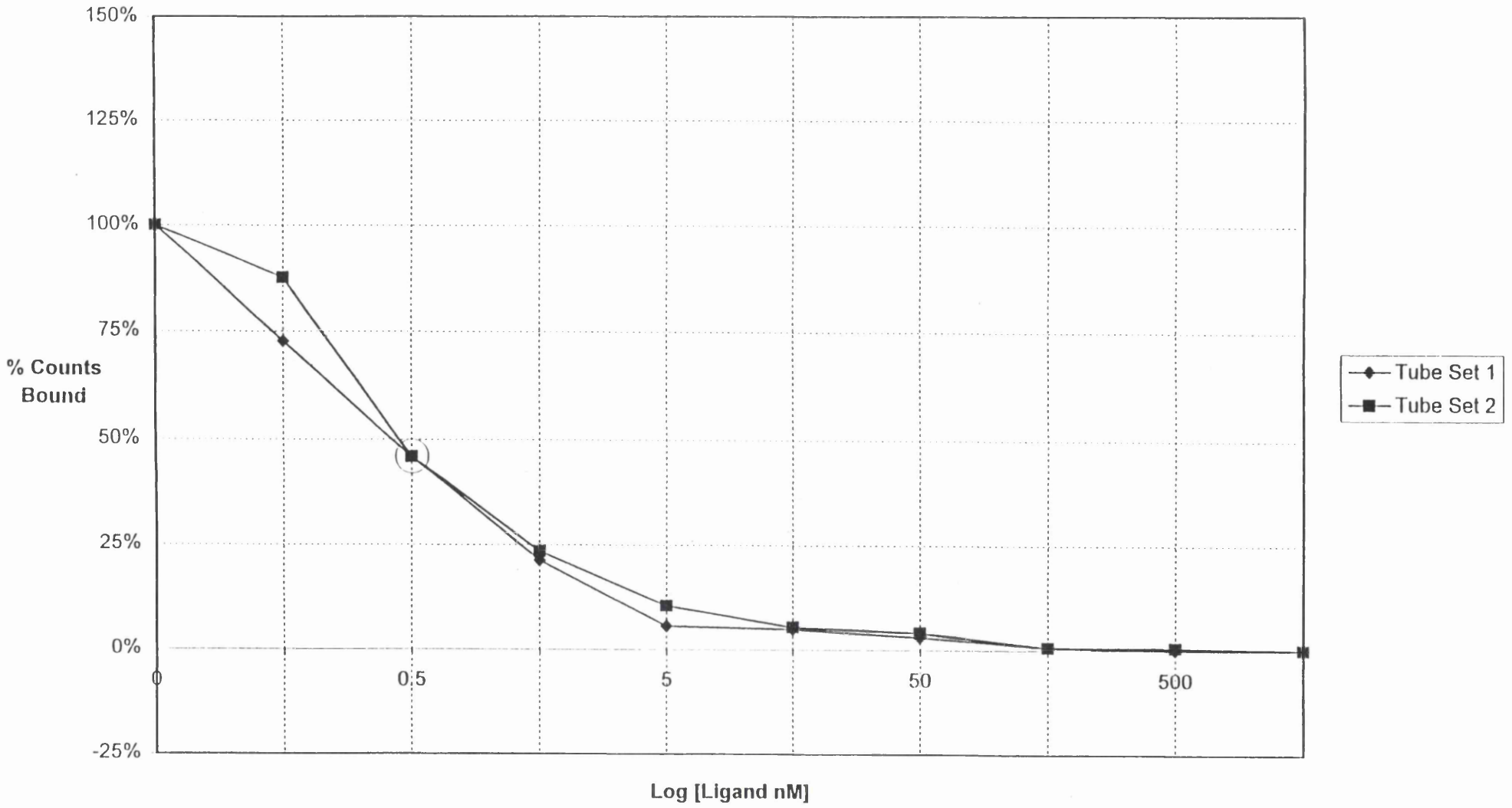
Appendix 2


20 CAG Construct : Dex (E)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

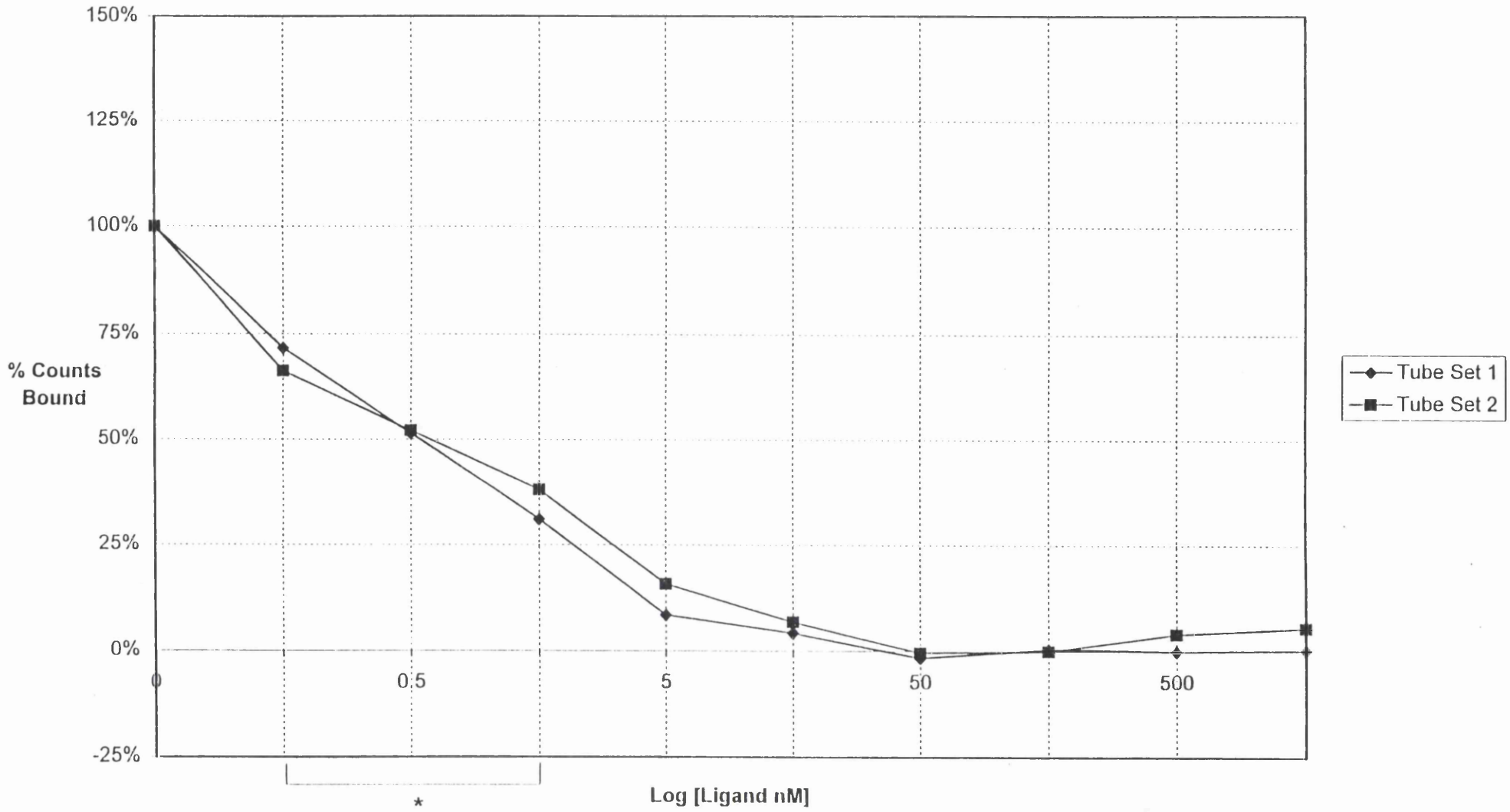
Appendix 2


20 CAG Construct : Dex (F)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

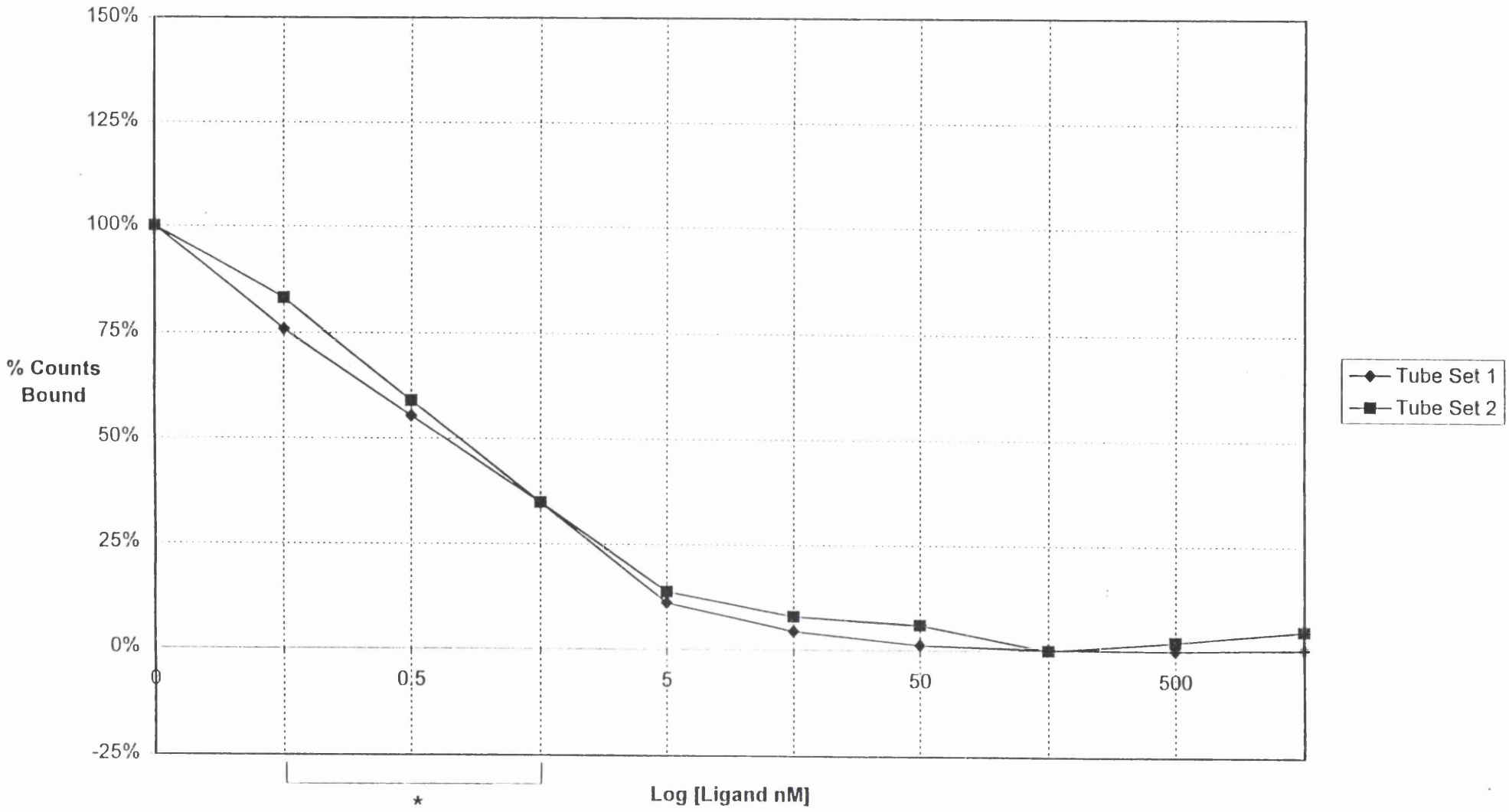
Appendix 2


20 CAG Construct : Dex (G)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

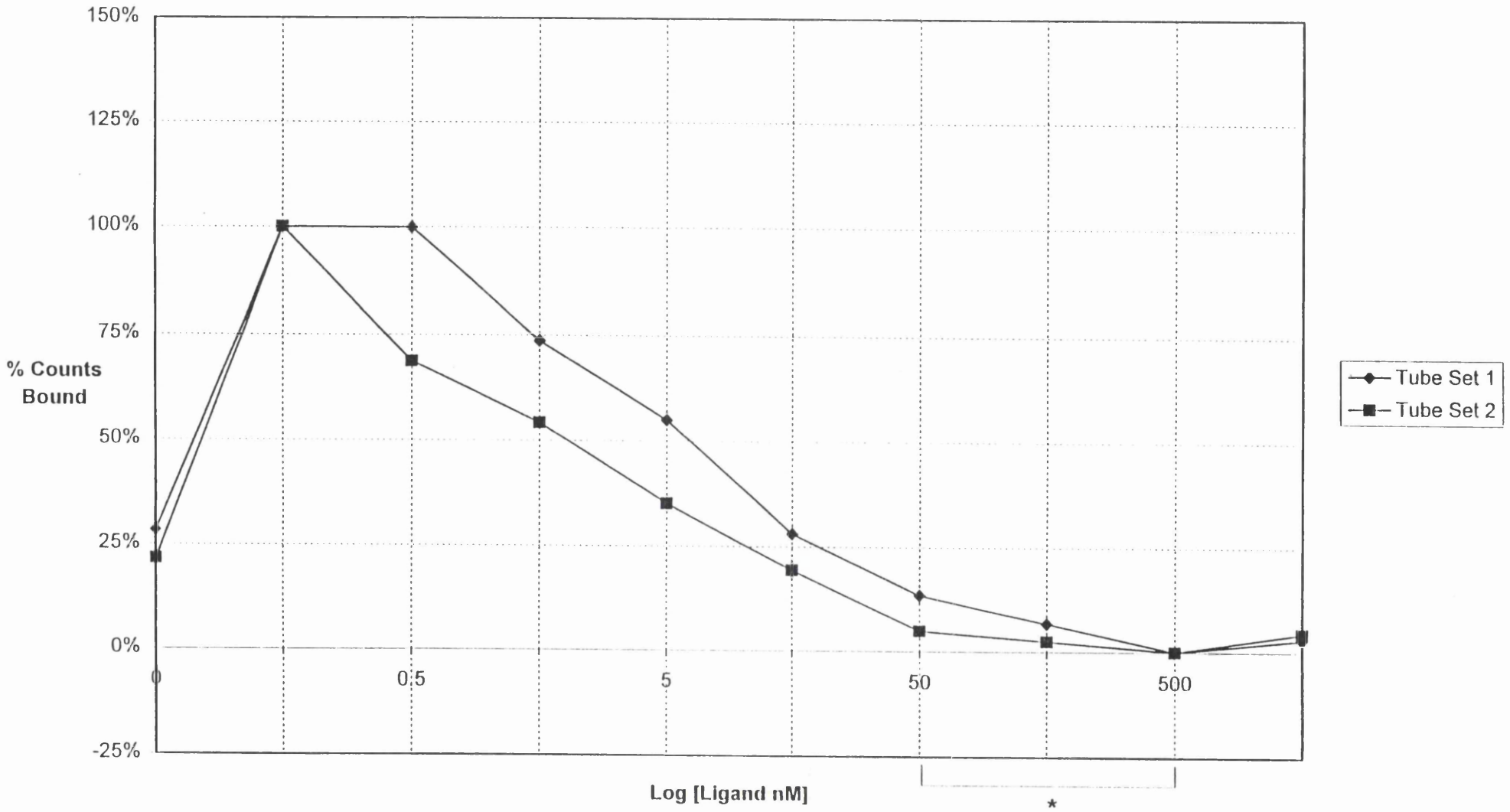
Appendix 2


20 CAG Construct : Dex (H)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
*
Log [Ligand nM]
Tube Set 1
Tube Set 2

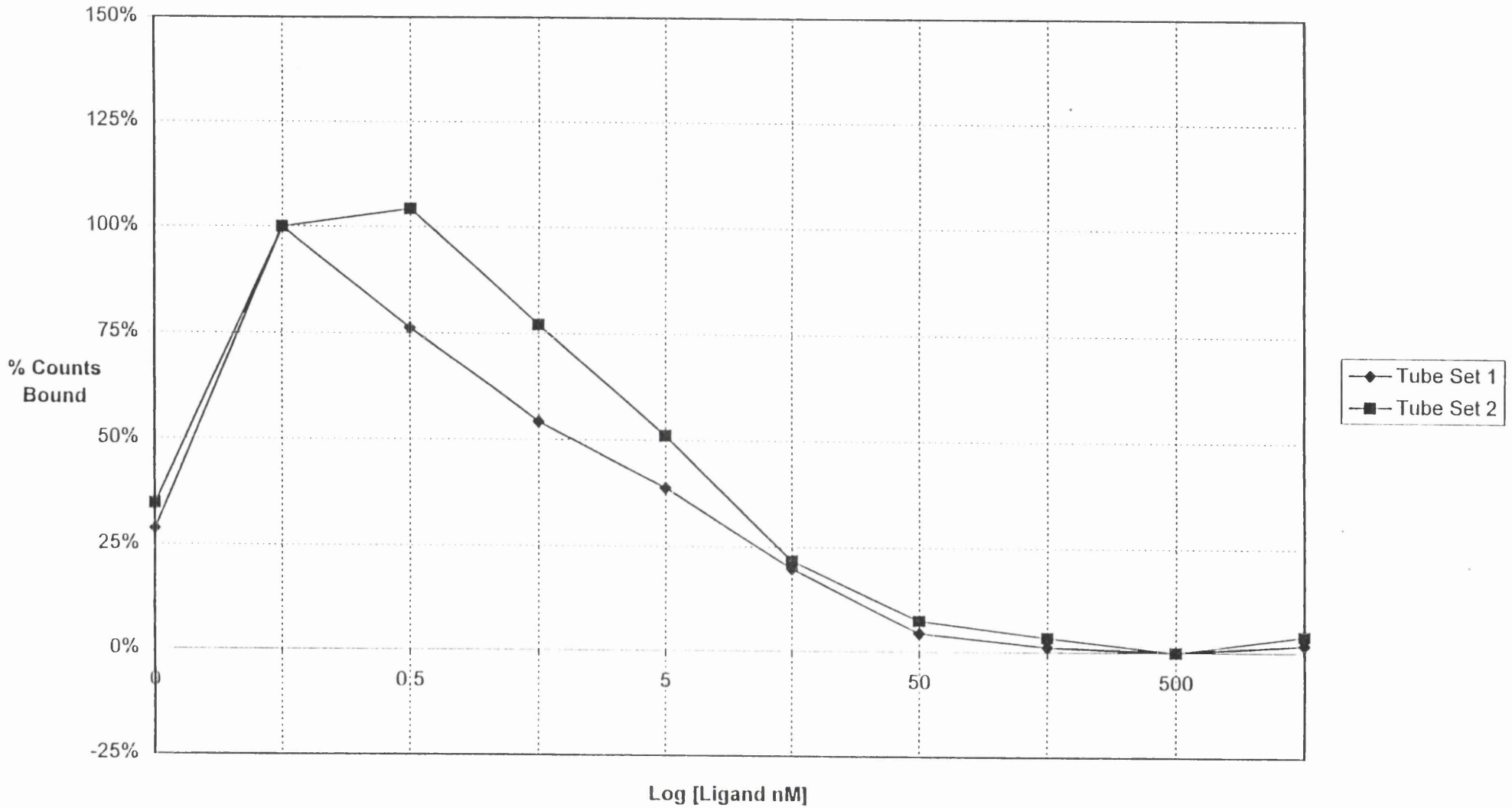
Appendix 2


20 CAG Construct : Dex (I)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
*
Log [Ligand nM]
Tube Set 1
Tube Set 2

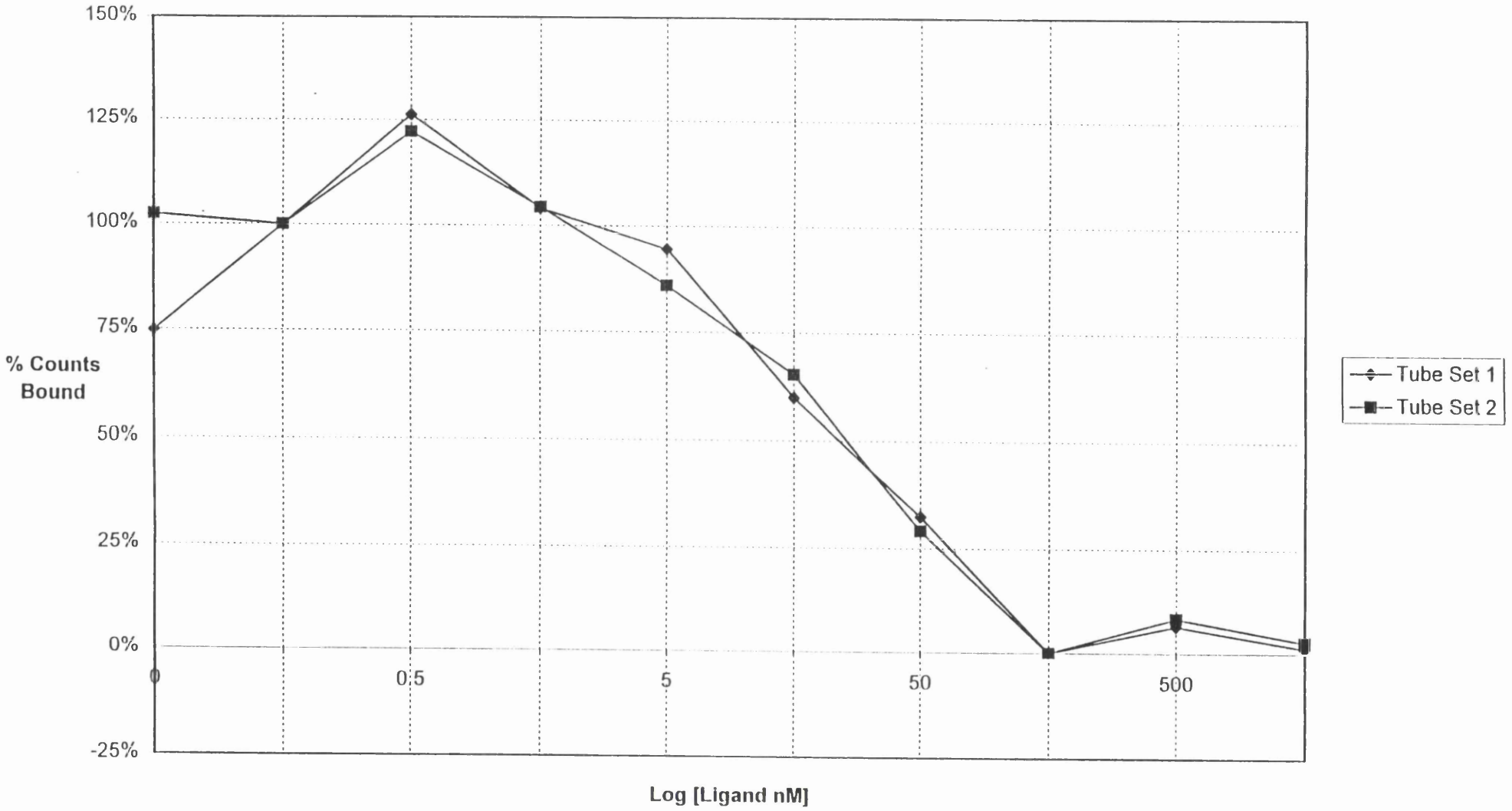
Appendix 2


20 CAG Natural : Corticosterone (A)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
*
Tube Set 1
Tube Set 2

Appendix 2

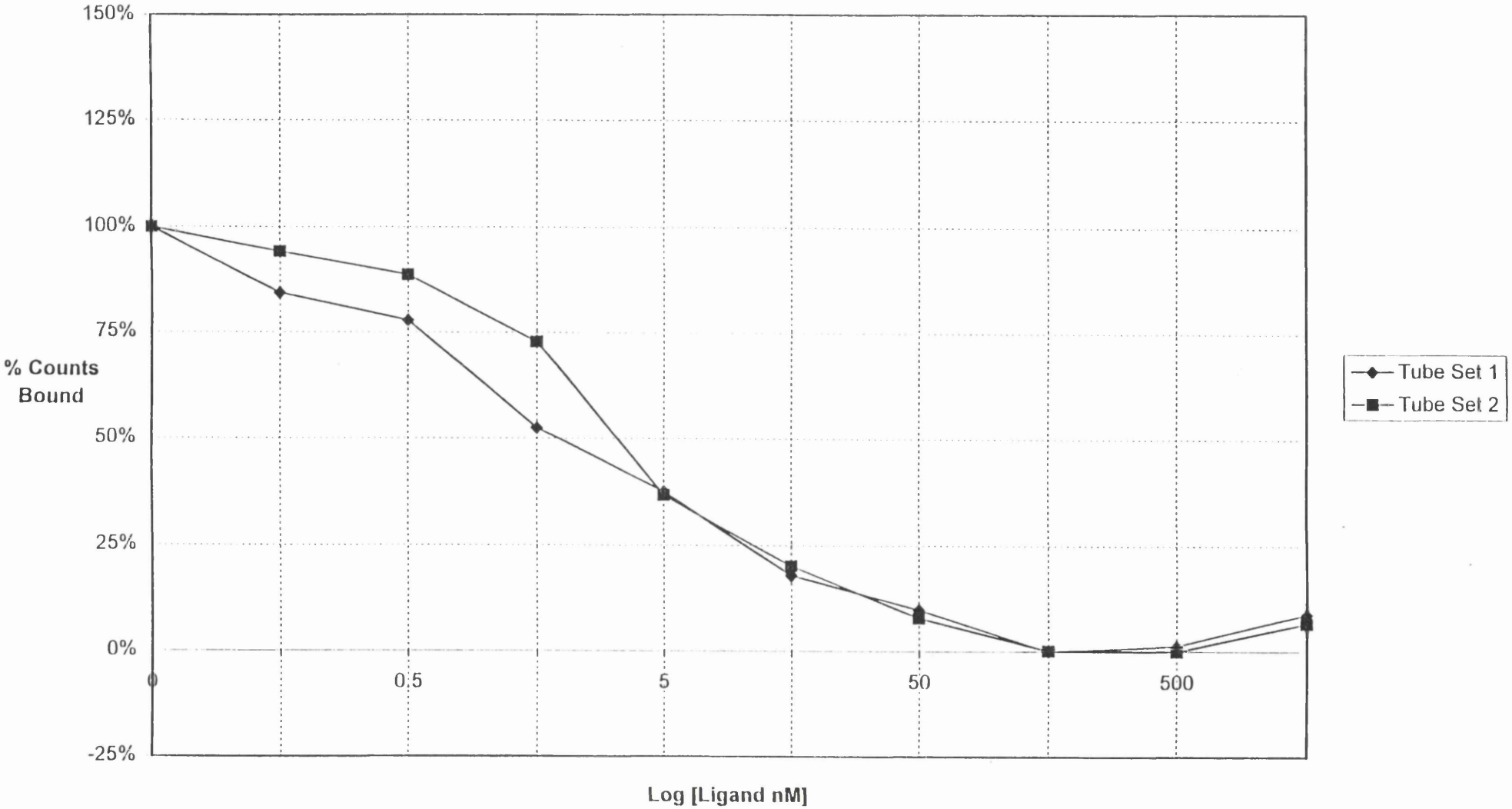

20 CAG Natural : Corticosterone (B)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2


20 CAG Natural : Corticosterone (C)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

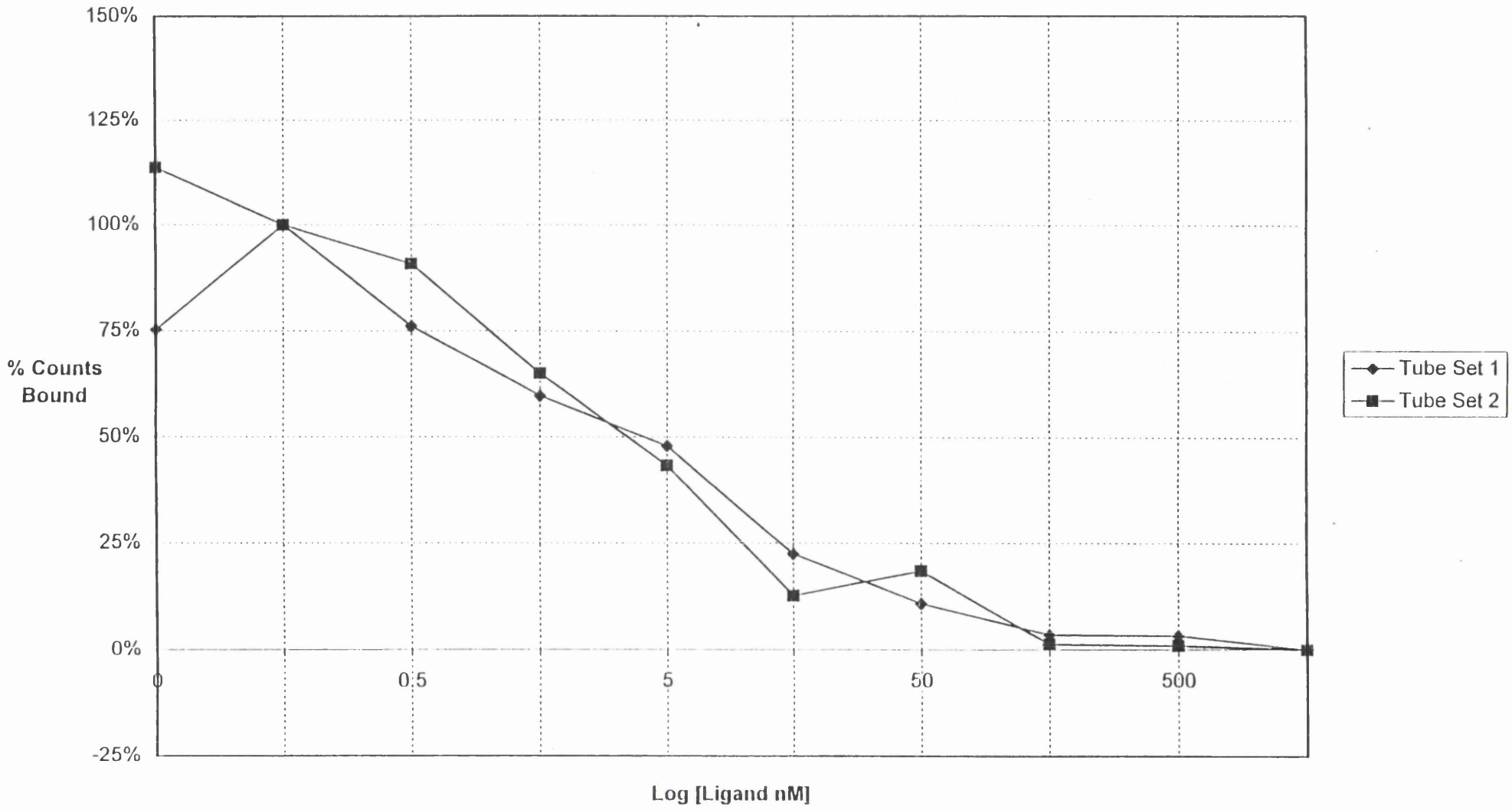
Appendix 2

20 CAG Natural : Corticosterone (D)

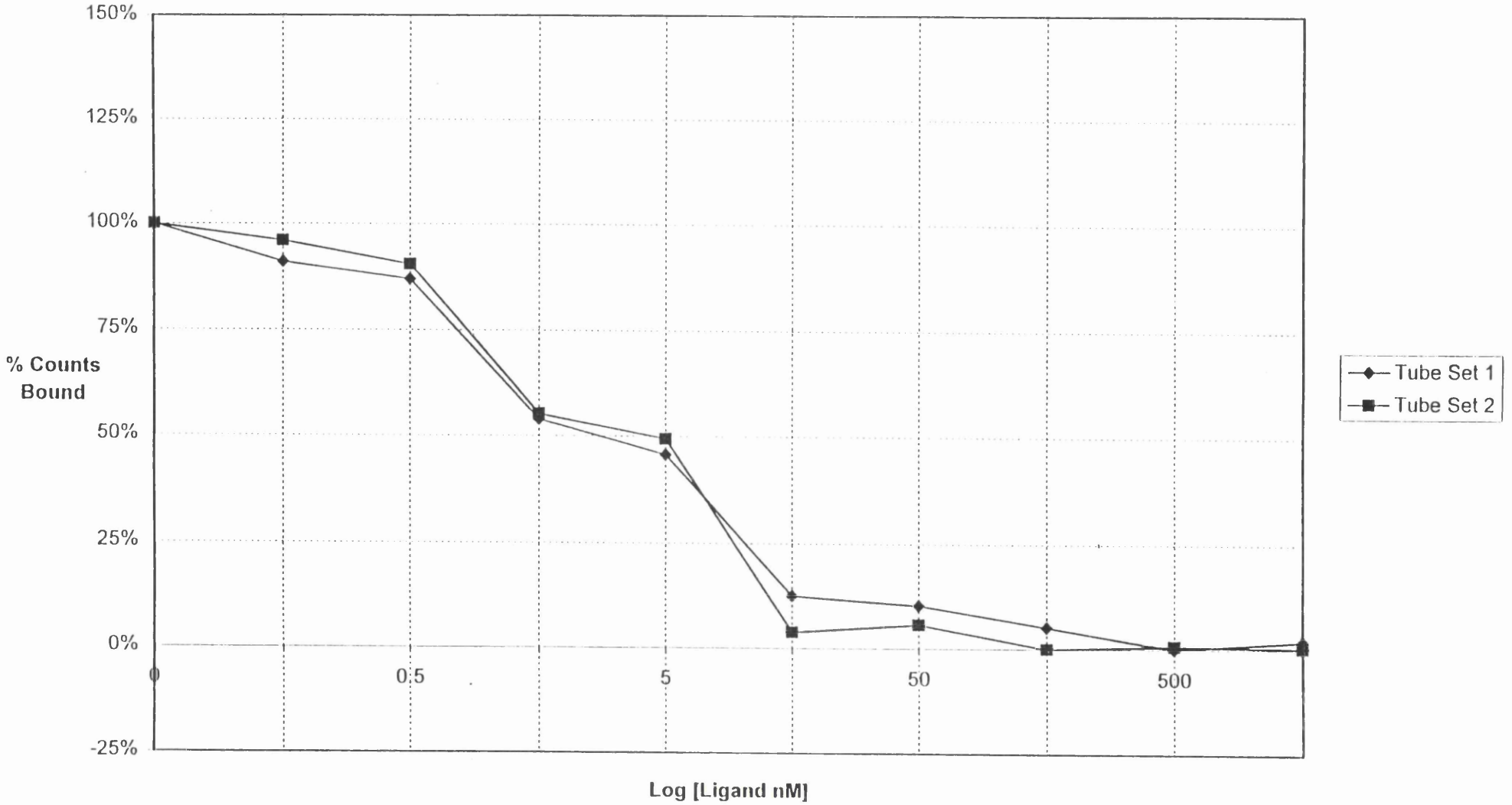


Appendix 2

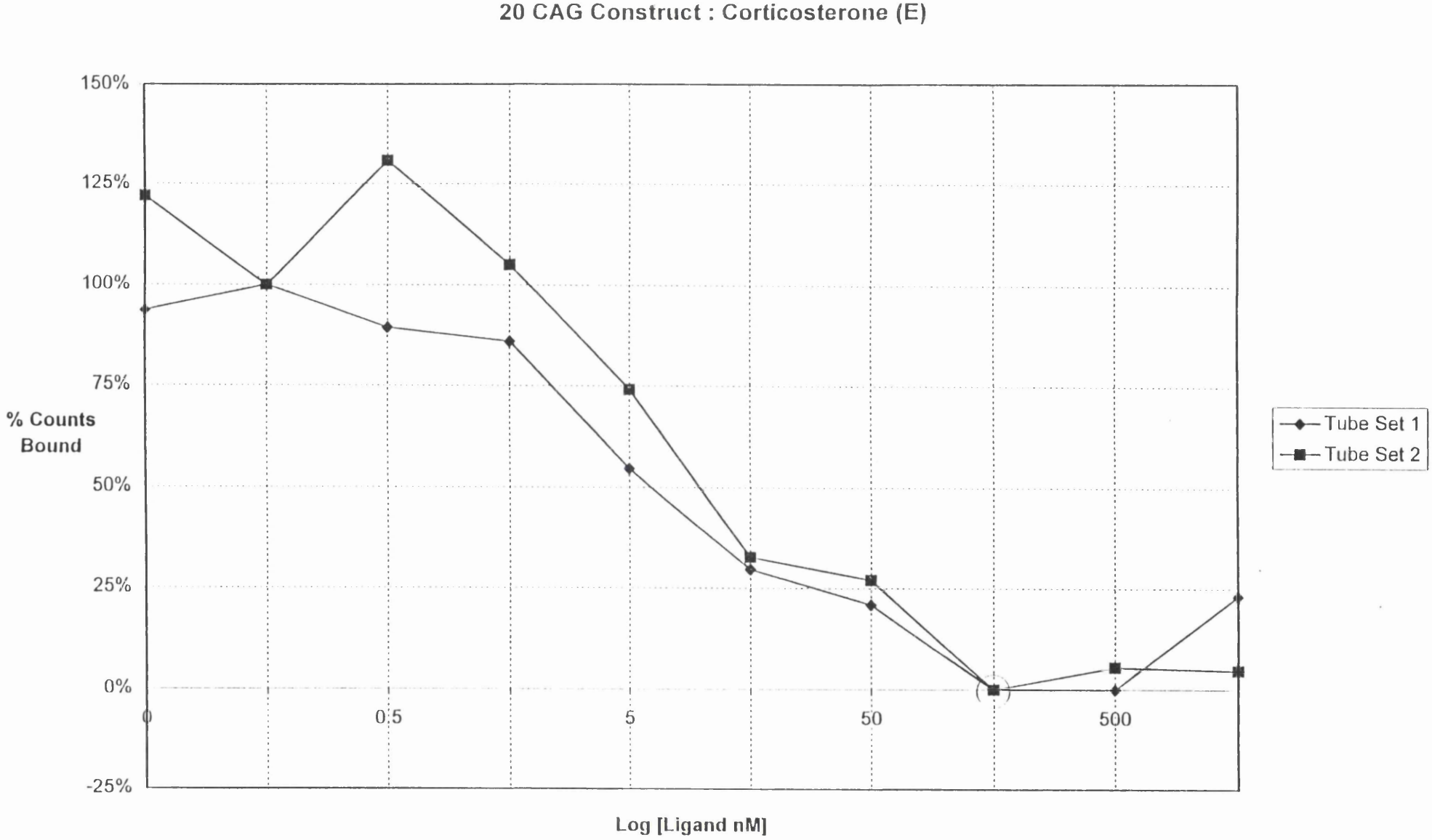
20 CAG Construct : Corticosterone (A)



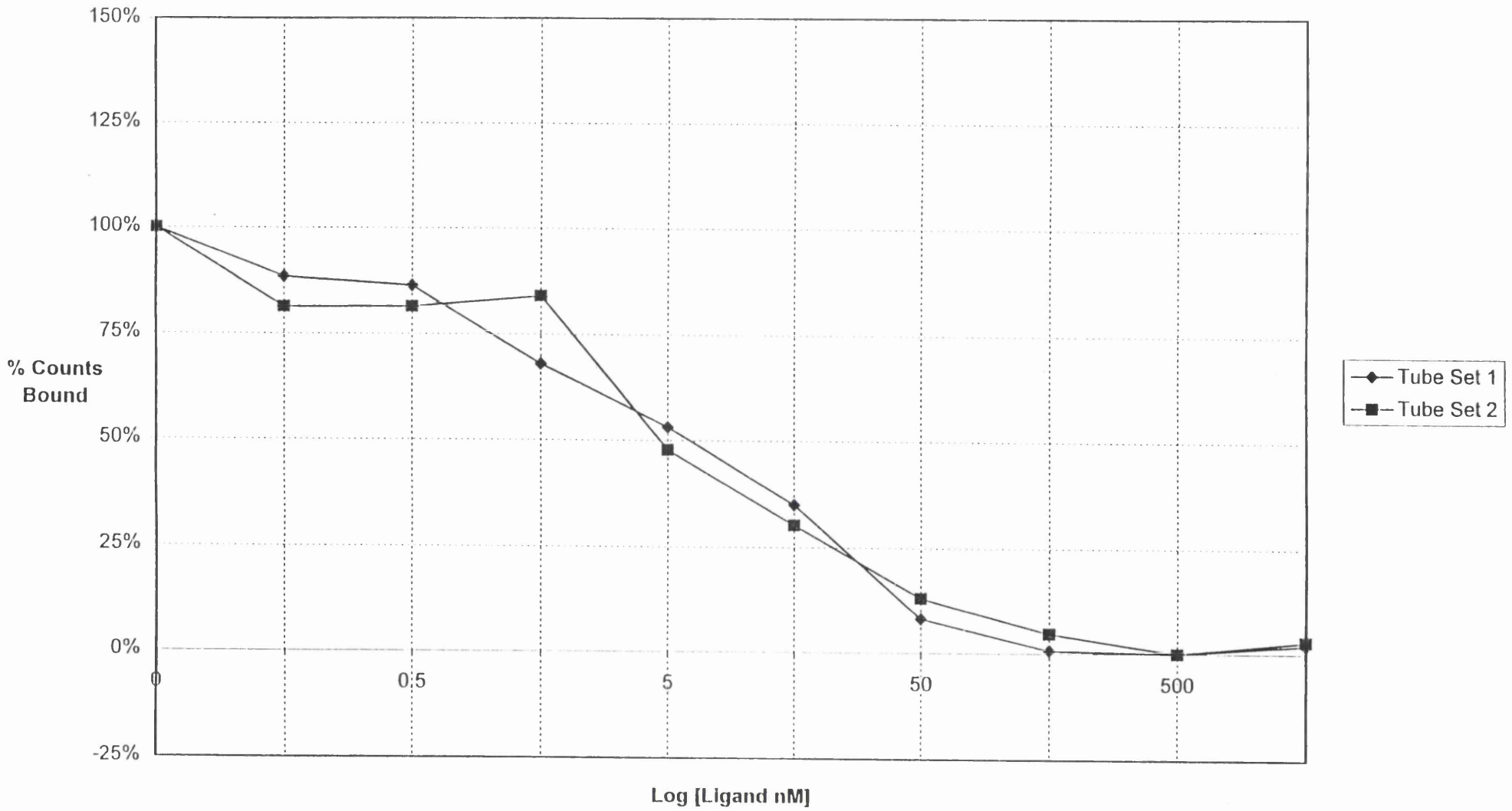
Appendix 2


20 CAG Construct : Corticosterone (B)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

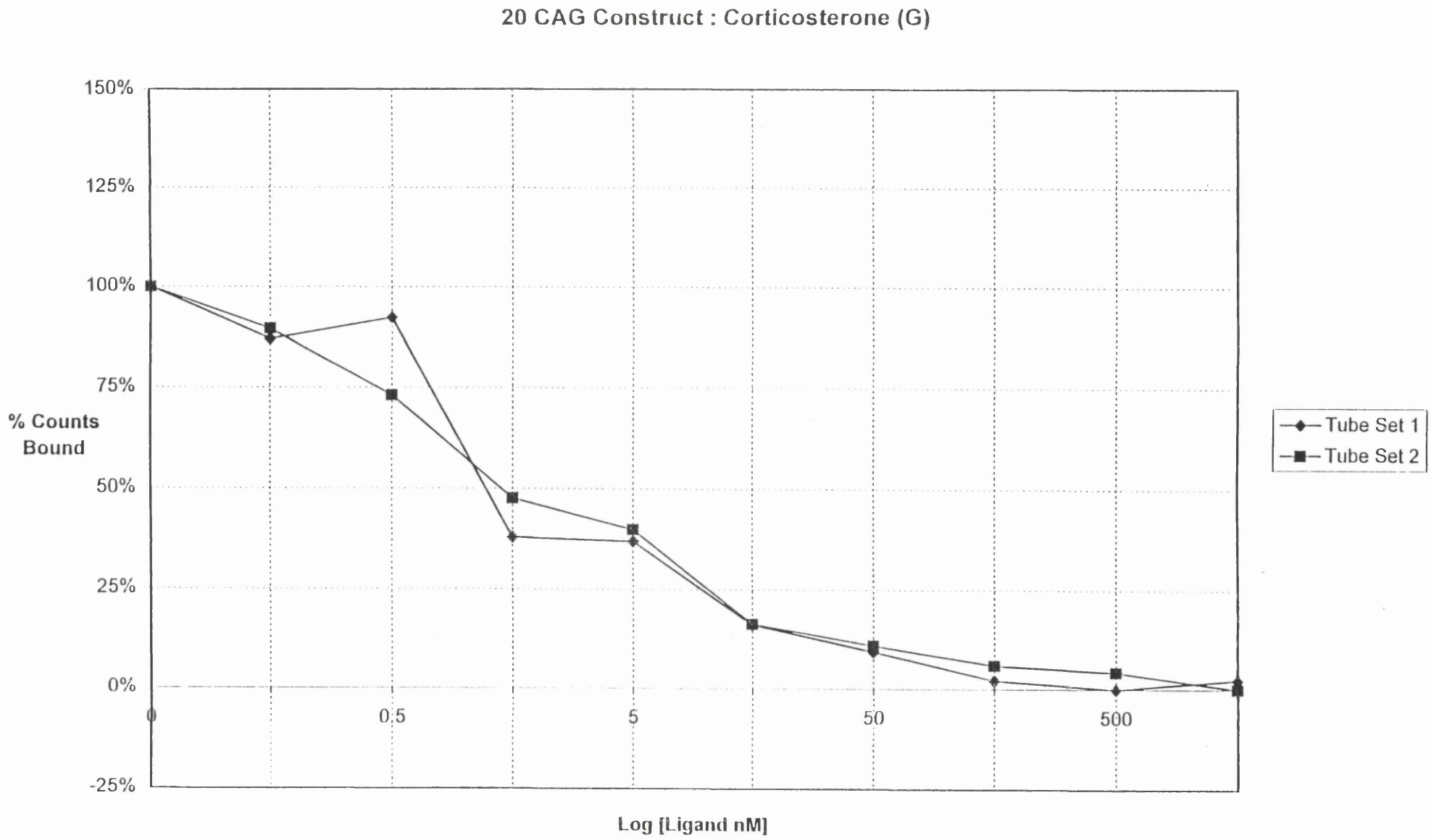
Appendix 2


20 CAG Construct : Corticosterone (E)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

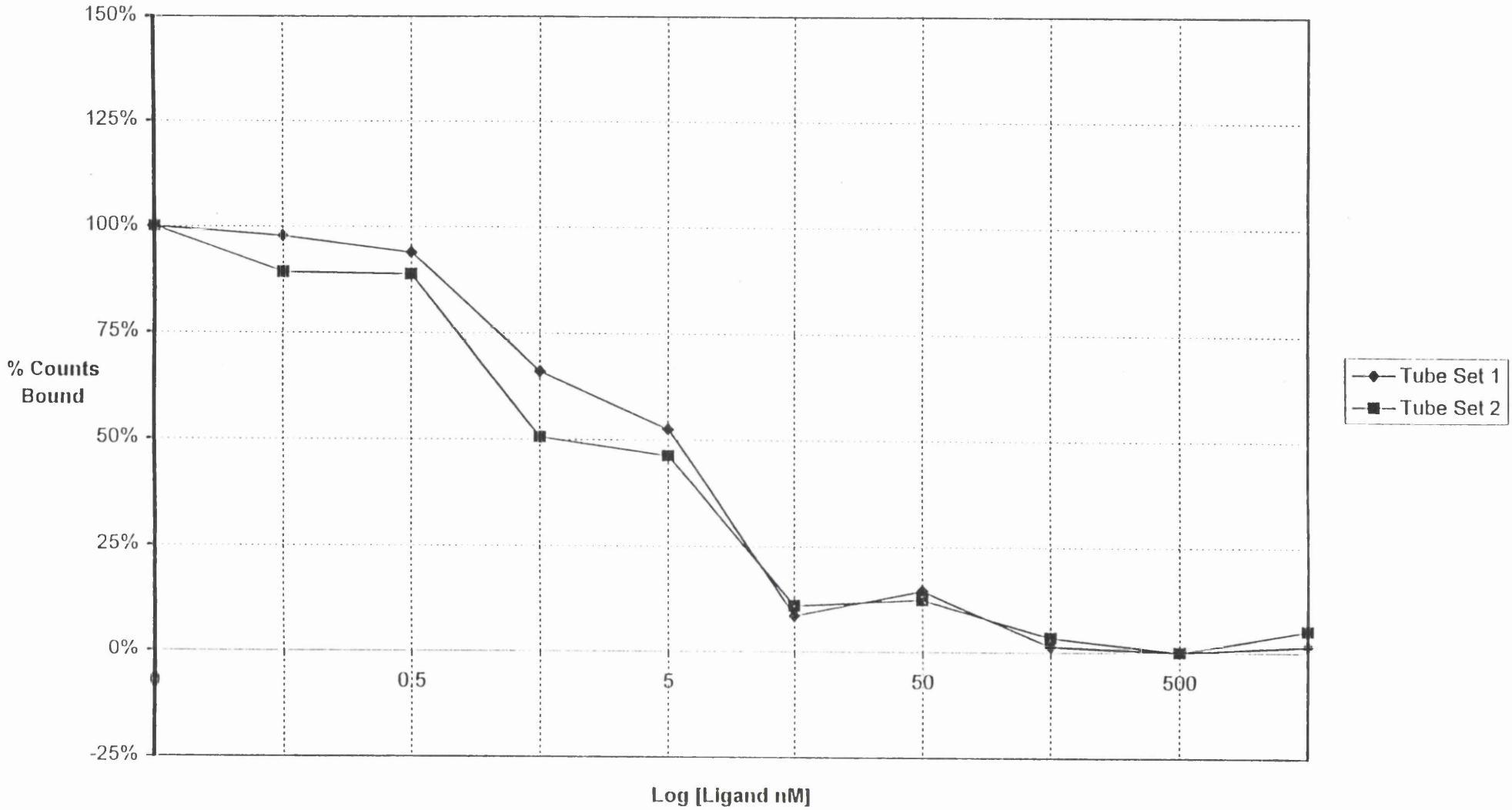
Appendix 2


20 CAG Construct : Corticosterone (F)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2

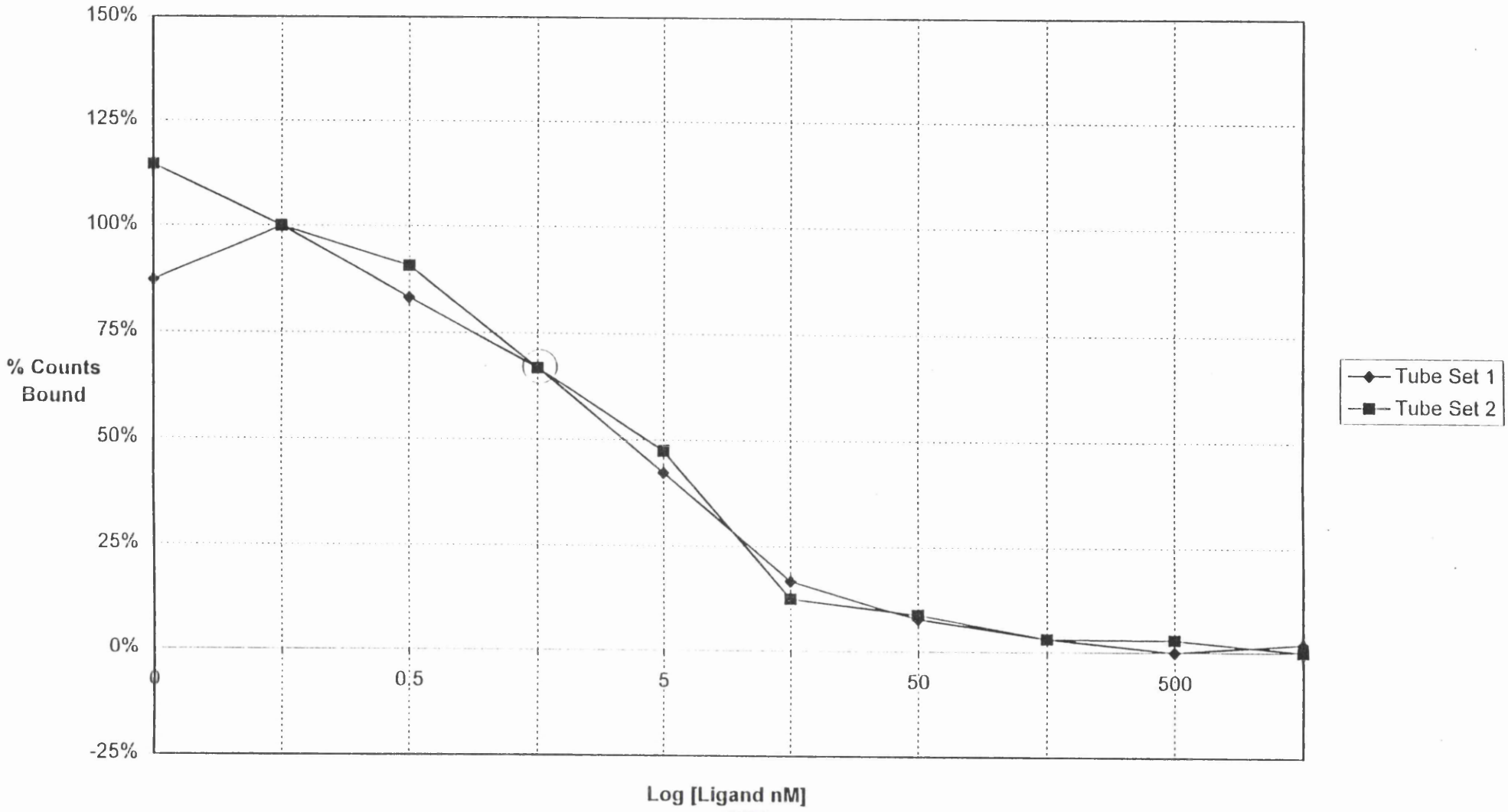

20 CAG Construct : Corticosterone (G)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2


20 CAG Construct : Corticosterone (H)
150%
125%
100%
75%
50%
25%
0%
-25%
% Counts Bound
0
0.5
5
50
500
Log [Ligand nM]
Tube Set 1
Tube Set 2

Appendix 2

20 CAG Construct : Corticosterone (I)



Appendix 2