GLUCOCORTICOID RECEPTOR GENE MUTATIONS ASSOCIATE WITH GLUCOCORTICOID-RESPONSIVE LEUKOCYTE AND PRODUCTION TRAITS IN CATTLE

By

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ABSTRACT

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Glucocorticoids hormones were named for their effects on glucose metabolism, but are also known to effect many other physiological processes, including embryonic development, blood pressure, lactation, behavior, and immunity. These hormones effect physiology through regulation of glucocorticoid-responsive gene expression. Glucocorticoids bind their cellular receptors, glucocorticoid receptor (GR), which are ligand activated transcription factors. Following hormone binding, activated GR bind to regulatory sequences in glucocorticoid-responsive genes and regulate transcription via their first transactivation domains (called $\tau 1$).

Studies of human GR have identified naturally occurring mutations in DNA encoding the ligand-binding (Karl et al., 1993; Mendonca et al., 2002), DNA-binding domain (Koper et al., 1997; Ruiz et al., 2001), and first transactivation domains (Karl et al., 1996a; de Lange et al., 1997), which change GR numbers and function. In addition to natural mutations identified in human GR $\tau 1$-encoding DNA, recent work has shown that bulls challenged with glucocorticoids display significant additive genetic variation in glucocorticoid-responsive leukocyte traits (Tempelman et al., 2002; Burton et al, in review). This implies that variation in glucocorticoid-responsive leukocyte traits, mediated through bovine GR, are inherited.

Therefore, the hypothesis of this study was that bovine glucocorticoid receptor genes harbor naturally occurring mutations in DNA encoding the

bovine τ1 region, and that these mutations are biologically relevant. Following PCR amplification of GR τ1-encoding exon 2 (GR2) from bovine genomic DNA and subsequent sequencing, multiple single nucleotide polymorphisms (SNPs) were identified in and across six dairy and beef cattle breeds. Several of these mutations change predicted amino acid sequences, possibly resulting in a change to GR2 structure and (or) function. In order to identify whether these amino acid-changing SNPs were biologically relevant, SNPs were associated with glucocorticoid-sensitive leukocyte and milk production traits. Seventeen SNPs were found to be significantly associated ($P \leq 0.05$) with leukocyte and milk production traits, and several were associated with more than one trait. These findings imply that SNPs found within bovine GR2 loci are partially responsible for the way in which a bull's leukocytes and metabolism respond to glucocorticoid challenge. Furthermore, three-dimensional homology modeling of two divergent Holstein alleles revealed changes to β-pleated sheet and loop structures resulting from identified amino acid-changing SNPs in bovine GR2 τ1-encoding DNA.

This study has therefore shown that glucocorticoid receptor DNA which encodes the first transactivation domain is polymorphic in cattle, and that these mutations may enable some animals to display a stress-resistant phenotype.

To my Father, who gave me the love of nature
and my Mother, who gave me the love of learning

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Laboratory, an ever-changing world of graduate students, undergraduates, and post-docs, and never a dull moment. Sally, thanks for the midnight calvings....I didn't know until then that I could tail-bleed a cow upside-down and stuffed into a corner. Dr. Anantachi Chaiyotwitayakun, pope goon mai na ka, and Khob kun ka. My buddies; Valencia, Rebecca, Chris. The world (and South Lansing) will never quite be the same. Have you been 'Kissed' today? We solved many a problem and laughed ourselves silly over coffee, ah bliss! Dr. Peter Saama, thank you for your patience and your critique, I like the way you think. Marquita, thank you, for everything. Always take The Long Way Home...I won't forget what has brought me here.

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LIST OF ABBREVIATIONS

ACTH - adrenocorticotrophic hormone

AI - artificial insemination

AIPL-USDA - Animal Improvement Program Laboratory-United States Department of Agriculture

AJCC - American Jersey Cattlemen's Club

ANOVA - analysis of variance

AP-1 - activator protein-1

AR - androgen receptor

ATP - adenosine triphosphate

BLUP - best linear unbiased prediction

bp - base pair

C/EBP - CAAT/enhancer binding protein

cAMP - adenosine 3',5'-cyclic monophosphate (cyclic AMP)

CBP - p300/CREB binding protein

CD11a - cluster of differentiation 11a

CD18 - cluster of differentiation 18

CD40 - cluster of differentiation 40

CD62L - cluster of differentiation 62L

cDNA - copy deoxyribonucleic acid

CIITA - major histocompatibility complex class II transactivator

CRE - cAMP response element binding protein (CREB) response element

CREB - cAMP response element binding protein

CRH - corticotrophin releasing hormone

dATP - deoxynucleotide adenosine triphosphate

dCTP - deoxynucleotide cytosine triphosphate

DBD - DNA binding domain of the glucocorticoid receptor

dGTP - deoxynucleotide guanine triphosphate

DNA - deoxyribonucleic acid

DRB3 - bovine major histocompatibility complex class II locus

DTT - di-thiothreotal

dTTP - deoxynucleotide tyrosine triphosphate

E-box - response element for myc/max transcription factors

EDTA - ethylenediaminetetraacetic acid

ER - estrogen receptor

Fcγ - Fc receptor gamma

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GCG - Genetics Computer Group

GM-CSF - granulocyte-macrophage colony-stimulating factor

GnRH - gonadotrophin releasing hormone

GR - glucocorticoid receptor

GRα - glucocorticoid receptor alpha isoform

GRβ - glucocorticoid receptor beta isoform

GRP - glucocorticoid receptor isoform rho

GR2 - glucocorticoid receptor exon 2

GRE - glucocorticoid response element

GRIP1 - glucocorticoid receptor interacting protein 1

hGR - human glucocorticoid receptor

HPA - hypothalamic pituitary adrenal axis

hsp - heat shock protein

ICAM-1 - intercellular adhesion molecule 1

IFN-γ - interferon gamma

IL-1 - interleukin 1

IL-1α - interleukin 1 alpha

IL-1β - interleukin 1 beta

IL-2 - interleukin 2

IL-4 - interleukin 4

IL-5 - interleukin 5

IL-6 - interleukin 6

IL-8 - interleukin 8

IPTG - isopropylthiogalactoside

Kb - kilobase

kDa - kilodalton

LBD - ligand binding domain of glucocorticoid receptor

mGR - membrane glucocorticoid receptor

MHC II - major histocompatability complex class II

MR - mineralcorticoid receptor

mRNA - messenger ribonucleic acid

NAAB - National Association of Animal Breeders

NF-1 - nuclear factor-1

NF-AT - nuclear factor-AT

NFκB - nuclear factor kappa B

nGREs negative GRE

NLS - nuclear localization signal

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEPCK - phophoenolpyruvate carboxykinase

PIC - preinitiation complex

PKC - protein kinase C

POMC - proopiomelanocortin

PR - progesterone receptor

PTA - predicted transmitting ability

PVN - paraventricular nuclei

RANTES - regulated upon activation, normal T cell expressed and secreted

RAR - retinoic acid receptor

RFLP - restriction fragment length polymorphism

rGR - rat glucocorticoid receptor

RNA - ribonucleic acid

RT - room temperature

RXR - vitamin D receptor

SAS - Statistical Analysis Software

SCM - synergy control motif

SCS - somatic cell score

SDS - dodecyl sodium sulfate

SNP - single nucleotide polymorphism

τ1 - first transactivation domain

τ2 - second transactivation domain

TBE - tris boric acid EDTA

TBP - TATA binding protein

TEMED - N,N,N',N'-tetramethyl-1,2-ethanediamine

TGF-β - transforming growth factor beta

TFIIA - transcription factor polymerase II subunit A

TFIIB - transcription factor polymerase II subunit B

TFIID - transcription factor polymerase II subunit D

TFIIE - transcription factor polymerase II subunit E

TFIIF - transcription factor polymerase II subunit F

TFIIH - transcription factor polymerase II subunit H

TFIIJ - transcription factor polymerase II subunit J

T_H1 - type 1 T helper cell (CD4+)

T_H2 - type 2 T helper cell (CD8+)

TNF-α - tumor necrosis factor alpha

TR - thyroid hormone receptor

UTR - untranslated region

UV - ultraviolet

WT - wild type

CHAPTER 1. INTRODUCTION

"There is no need to be a doctor or a scientist to wonder why the human body is capable of resisting so many harmful agents in the course of everyday life...disease does not strike everyone indifferently. For some individuals who go down at the attack, there are others who have immunity to a greater or lesser extent". (Metchnikov, 1908).

The innate ability of an animal's immune system to mount inflammatory responses and fight infection is vital to the maintenance of health and survival. For this reason, the study of genetic diversity between livestock animals that vary in their ability to fight infection is critical. In addition, the ability to predict an animal's inherent sensitivity to harsh or stressful environmental and physiological stimuli, which could be provided by knowledge of an animals' genetic makeup, will be key to improved management of animal health and production on farms (Freeman and Lindberg, 1993). Most cells and organ systems, including the immune system, respond to harsh or stressful stimuli by regulating the expression of various genes. Examples of stimuli that are predicted or shown to affect gene expression include extremes in heat and cold, pain and fear, infection by bacteria, viruses, yeast, and fungi, and a variety of common husbandry practices (Filion et al., 1984; Mitchell et al., 1988; Nanda et al., 1990; Minton, 1994; Wohlt et al., 1994; Burton et al., 1995; Grandin, 1997; Kehrli et al., 1999; Preisler et al., 2000a, Weber et al., 2001; Madsen et al., 2002). In response to these stimuli, the paraventricular nuclei (PVN) of the hypothalamus release corticotrophin releasing hormone (CRH), which activates the hypothalamic-pituitary-adrenal (HPA) axis and the release of adrenocorticotrophic hormone (ACTH) into the blood (See **Appendix A**). ACTH, in turn, targets the adrenals to synthesize and release glucocorticoids. In response to glucocorticoids, responsive cells may either up-regulate or down-

regulate expression of genes that encode such proteins as transcription factors, hormone receptors, heat shock proteins, cytokines, proteins involved in energy metabolism, antigen presentation, and cell-to-cell communication. Such changes in gene expression allow specific adaptation of cell phenotypes such that the whole animal can cope with the harsh or stressful situation.

Regulation of gene expression can occur at several levels, one of which is transcription. Transcriptional regulation of gene expression is mediated through multiple and often complex interactions between a variety of transcription factor complexes and DNA sequences (motifs) contained in the regulatory regions of target genes (Schule et al., 1988; Tjian and Maniatis, 1994; Scheinman et al., 1995; Wolberger, 1998; Brogan et al., 1999). One transcription factor important in mediating phenotypic adaptation during stress belongs to a large superfamily of ligand-activated transcription regulators and is called the glucocorticoid receptor (GR). GR mediates the actions of glucocorticoid stress hormones in animals. Therefore, GR is one transcriptional regulator at the forefront of research aimed at studying gene expression and phenotypic changes in cells of animals exposed to stress-induced increases in blood glucocorticoids.

The superfamily of molecules to which GR belongs represents the largest known family of ligand-activated transcription factors in eukaryotes (Evans, 1988; Tsai and O'Malley, 1994). In addition to GR, other members of this superfamily include the steroid hormone receptors for estrogens (ER), androgens (AR), progesterone (PR), mineralcorticoids (MR), and thyroid hormones (TR). Additional members considered part of this superfamily are the retinoic acid receptor (RAR), the vitamin D receptor (RXR), and a set of proteins whose genes have DNA sequence similarity to steroid hormone receptors, called orphan receptors because their ligands are not well characterized.

However, the main members of this family of ligand-activated transcription regulators are GR, ER, MR, AR, PR, and TR, the human and rodent genes of which have all been cloned, sequenced, and their exon-intron boundaries established (Danielsen et al., 1986; Encio and Detera-Wadleigh, 1991). Known promoter regions for the TR, ER, AR, PR, and GR genes resemble promoters of genes that are constitutively expressed in most cells, called housekeeping genes (Arriza et al., 1987; Huckaby et al., 1987; Baarends et al., 1990; Sakurai et al., 1992). Examples of such genes are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cathepsin D. Like the steroid receptor superfamily genes, promoters for these housekeeping genes are characterized by the presence of a GC rich "island", absence of a TATA box, and the presence of multiple transcription initiation sites (Tsai and O'Malley, 1994; Smale, 1997; Segal et al., 1999). This similarity of promoters suggests that, like housekeeping genes, the steroid hormone receptor genes may be continuously expressed in appropriate cells because the receptors they encode are so crucial to animal health, metabolism, reproduction, and survival. Another characteristic of the steroid receptor superfamily of genes is their size and structure. Most of the genes are greater than 60 kilobases (Kb) in length, with a range of 38 Kb for PR to 140 Kb for ER. These genes also possess multiple exons, with intervening introns, where individual exons typically encode specific functional domains in the complex globular receptor molecule (Tsai and O'Malley, 1994). The human GR gene is 80 Kb with 9 exons and 9 introns (Encio and Detera-Wadleigh, 1991), and preliminary copy DNA (cDNA) sequence from our laboratory suggests the same GR gene structure exists in cattle (Weber and Burton; unpublished data). Therefore, GR structure and function is predicted to be similar in cattle and humans.

Use of the synthetic glucocorticoid, dexamethasone, in human patients

suffering from inflammatory diseases and Cushing's-like symptoms has revealed individuals who exhibit variable sensitivity to glucocorticoid therapy (Huizenga et al., 1998). Importantly, dexamethasone has extremely high specificity and affinity for GR (Roth and Kaeberle, 1982; Miller et al., 1994), suggesting that variable sensitivity to this hormone might be due to variation in GR structure and (or) function. This possibility has led to an extensive search for GR gene polymorphism in human patients, in an effort to identify specific DNA mutations that might explain phenotypic variation in glucocorticoid sensitivity (Lamberts et al., 1986; de Lange et al., 1997). Such studies have identified numerous GR gene polymorphisms, some of which appear to impact cellular gene expression and disease status in patients harboring one or more of the mutations (Lamberts et al., 1992; Ashrof and Thompson, 1993; Karl et al., 1993; Malchoff et al., 1993; Karl et al., 1996b; Koper et al., 1997; Huizenga et al., 1998; Mendonca et al., 2002). At the molecular level, most of the phenotype-altering GR mutations discovered to date affect the number of cellular GR expressed, glucocorticoid binding affinity of GR, or degree of interaction between GR and DNA motifs in regulatory regions of glucocorticoid sensitive genes (Koper et al., 1997; Huizenga et al., 1998). All of these human GR gene polymorphisms have been shown to have dominant negative effects in cells of affected patients. However, more subtle glucocorticoid sensitivities most probably exist and may be caused by polymorphism in other important domain-encoding exons of GR genes. This has been shown recently in humans, where clusters of polymorphisms in exon 2 that encodes the main transactivation domain of GR (called tau-1, or τ1) may produce modest but detectable effects on target gene expression (de Lange et al., 1997; Koper et al., 1997). As this domain is critical to transcriptional regulation of glucocorticoid-sensitive genes by GR, molecular genetic variation in its coding DNA

might help explain phenotypic variation in important traits such as the stress response, immunity, and health in humans and other animals. This is particularly relevant to livestock production because modern husbandry practices that lead to unavoidable stress, such as parturition or handling, can also be associated with heightened disease-susceptibility in some animals (Smith et al., 1973; Guidry et al., 1976; Wohlt et al., 1994).

Evidence for the existence of genetic variation in response to stress and experimental glucocorticoid challenge is available in the diary cattle literature, and suggests that a search for bovine GR gene polymorphism is warranted. For example, Detilleux et. al. (1994) found moderate to high heritabilities (h^2 ranged from 0.11 to 0.61) in a wide variety of immune response traits measured *in vitro* on leukocytes from parturient dairy cows. Importantly, these animals undergo an extreme stress response around calving, which results in high concentrations of blood glucocorticoids, progesterone, and estradiol (Smith et al., 1973), and is associated with changes in immune cell gene expression and phenotype (Lee and Kehrli, 1998; Preisler et al., 2000a,b; Burton et al., 2001; Weber et al., 2001; Madsen et al., 2002; Burton and Erskine, 2003). Similarly, experimental dexamethasone challenges in pedigreed Holstein bulls revealed modest to significant genetic variation in altered protein expression in neutrophils (Tempelman et al., 2002; Kelm et al., in review) and mononuclear leukocytes (Abdel-Azim et al., in review; Burton et al., in review; Kelm et al., in review), indicating genetic influences in an animal's immune system sensitivity to glucocorticoids. This suggested to us that, like human GR, bovine GR genes may be polymorphic in regions that encode important functional domains of the receptor protein. This led to our hypothesis that bovine GR genes harbor biologically significant mutations that affect gene expression. The overall goals of this Ph.D. study were to characterize DNA

mutations in the τ1-encoding exon 2 (GR2 locus) of bovine GR genes and to estimate the degree of association between these mutations and key leukocyte traits that are sensitive to glucocorticoids and may influence health and productivity in dairy cattle.

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

Mutations in the first transactivation domain (τ1) of glucocorticoid receptors (GR) may exist and affect GR function and thus the ability of animals to cope with stressful environments. Several biologically relevant mutations have been identified in exon 2 of human GR genes, which encodes τ1 and its flanking protein regions. **The hypothesis of the current study is that the τ1-encoding GR2 locus of bovine GR genes is polymorphic and that this polymorphism is biologically relevant.** The overall goals of this study were to identify mutations in the bovine GR2 locus and determine whether these mutations associate statistically with heritable traits of mammary health and leukocyte sensitivity to glucocorticoids in dairy cattle.

Four specific objectives were developed to test the hypothesis. These were:

Specific Objective i) To determine if bovine glucocorticoid receptor genes are polymorphic in the GR2 locus

Specific Objective ii) To characterize identified GR2 polymorphism at the DNA and amino acid sequence levels, and at the predicted tertiary protein structure level.

Specific Objective iii) To determine the frequency of identified GR2 polymorphisms within defined Holstein and Jersey bull populations.

Specific Objective iv) To determine if GR2 polymorphisms associate with leukocyte sensitivity to glucocorticoids and (or) milk production traits in defined Holstein and Jersey populations.

CHAPTER 3. LITERATURE REVIEW

A. The Glucocorticoid Receptor: Tissue Distribution, Structure, and Function.

Glucocorticoid receptors are present in most cells predominantly as cytoplasmic receptors (GR), but they can also exist as plasma membrane (mGR) receptors in some cell types. The expression of mGR has, to date, been correlated with the induction of apoptosis via initiation of the Jnk signal transduction pathway, particularly in lymphoma cells (Gametchu, 1987; Sackey et al., 1997; Chen et al., 1999; Gametchu and Watson, 2002). Additionally, human monocytes and B lymphocytes express mGR in their membranes, albeit at low levels relative to lymphoma cells (Buttgereit and Scheffold, 2002). Membrane GR has a size of approximately 97 kDa and has been found to be inactive at a lower size (Gametchu and Watson, 2002). Membrane GR binds hormone and triggers activation of the Jnk pathway and cell apoptosis (Gametchu et al., 1991).

Cytoplasmic GR are 94 kDa in size, are the main cellular receptors for glucocorticoid hormones, and the primary mediators of normal steroid actions in target cells. The end result of all glucocorticoid action in normal cells is regulation of glucocorticoid-responsive gene expression for the purpose of cellular adaptation to stress. In order to regulate target gene expression, glucocorticoid hormones bind to and activate GR. Binding of glucocorticoids turns GR into a ligand-activated transcription factor capable of altering gene expression in target cells through a variety of transcriptional regulatory routes, or the stabilization or destabilization of specific messenger RNA (mRNA) transcripts. Because GR is so critical to survival, the domain structure and function of this complex protein have been extensively studied. In fact, human GR (hGR) was the first mammalian transcription factor to be studied any great detail.

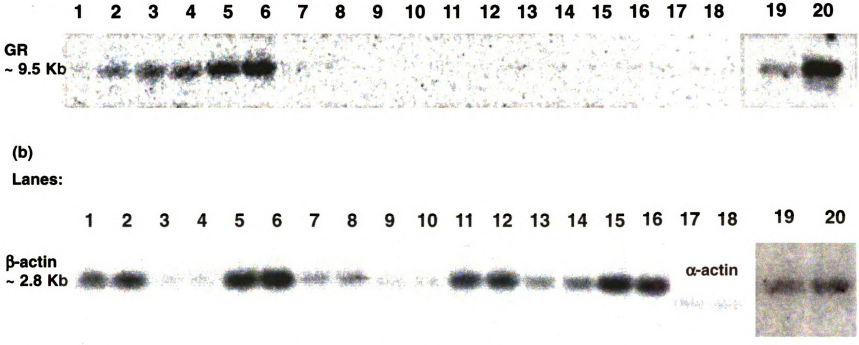
i. Tissue Distribution of the Glucocorticoid Receptor.

As the molecules that mediate the activities of glucocorticoid hormones in target cells, GR are vital to the adaptation of glucocorticoid-responsive tissues to stress. In fact, GR are absolutely required for tissue development and ultimate survival (Cole et al., 1995). Generation of GR knock-out mice showed that although embryos were able to develop into full term fetuses, they died shortly following birth from severe abnormalities including atelectasis of the lung, hyperplasia of the adrenal cortex, and impaired expression of gluconeogenic enzymes in the liver (Reichardt et al., 1998). As implied by their requirement for survival, GR have been found to be present in nearly all cells of the body (Ballard, et. al., 1974; Reul, et. al., 1989). Initially, message for GR was not identified in jejunum, adipose, bladder, seminal vesicle, uterus, prostate tissue (Ballard et al., 1974), or the bovine lens (Jobling and Augusteyn, 2001). However, in 1994 Pedersen et al. identified the presence of hGR in both omental and subcutaneous adipose tissue, and more recently GR has been found in rat embryonic gonadal, gastrointestinal, and urogenital tissue (Kitraki et al., 1997). Although these observations appear to contradict previous findings, it is possible that GR is expressed in certain tissues exclusively during embryonic development and is subsequently undetectable in adult tissue. In fact, the concentration of GR within target tissues varies depending on stage of development, age, cell cycle stage, and endocrine status of the animal (Burnstein et al., 1994 ; Yudt and Cidlowski, 2002). Our laboratory has shown variable but near ubiquitous tissue expression of GR mRNA in adult cattle, with abundance variations dependent on the tissue (**Figure 1**). Interestingly, isolated populations of bovine neutrophils and mononuclear leukocytes cells express relatively abundant levels of GR mRNA and proteins (Preisler et al., 2000a,b; Weber et al., 2001; **Figure 1**) and thus are predicted

Figure 1. Tissue Expression of Bovine Glucocorticoid Receptor mRNA.

In humans, rodents, and other species studied, glucocorticoid receptor (GR) mRNA and protein are nearly ubiquitously expressed but vary in expression levels depending on the tissue. In this figure **(a)**, bovine GR mRNA is shown to be more abundant in spleen (lanes 1 and 2), apidose tissue (lanes 3 and 4), lung (lanes 5 and 6), adrenal gland (lanes 7 and 8), and blood leukocytes (lanes 19 and 20) than in the other tissues represented on the Northern blot, although GR mRNA is still detectable in these other tissues. In panel **(b)** is the corresponding β-actin expression level for each tissue. Cell sensitivity to glucocorticoids is directly related to expression of GR by the cells (Bamberger et al., 1996). Therefore, GR mRNA abundance shown in this figure substantiates previous work from our laboratory, showing that bovine tissues, particularly leukocytes tissues with abundant leukocytes, are sensitive to glucocorticoids (Burton et al., 1995; Burton and Kehrli, 1996; Preisler et al., 2000a,b; Weber et al., 2001; Tempelman et al., 2002; Burton et al., in review).

Figure 1. Tissue Expression of Bovine Glucocorticoid Receptor (a) and β-actin (b) mRNA.



Lanes 1 & 2 = spleen
Lanes 3 & 4 = adipose tissue
Lanes 5 & 6 = lung
Lanes 7 & 8 = adrenal gland
Lanes 9 & 10 = bone marrow
Lanes 11 & 12 = gut
Lanes 13 & 14 = thymus
Lanes 15 & 16 = lymph node
Lanes 17 & 18 Muscle
Lane 19 = blood monocytes
Lane 20 = blood neutrophils

to be extremely sensitive to glucocorticoid hormones (Burton et al., 1995; Burton and Kehrli, 1995, 1996). Developmental changes in cellular GR expression, combined with its nearly ubiquitous distribution in cattle and other species, demonstrates the importance and far-reaching effects of this regulator of gene expression during stress (Bamberger et al., 1996).

ii. Domain Structure of the Glucocorticoid Receptor.

In 1985, full length copy DNA (cDNA) for hGR was cloned from human lymphoid and fibroblast cells and the cDNA sequenced (Hollenberg et al., 1985). From this work it was determined that hGR had two splice variants, a 90-94 kDa GRα isoform (dependent on the state of glycosylation) of 777 amino acid residues in length (**Figure 2a)** and a GRβ isoform of 742 amino acids (Hollenberg et al., 1985; **Figure 2b**). A decade later, an additional isoform, GRP, was isolated from lymphocytes of patients displaying glucocorticoid resistance and was found to be 697 amino acid residues in length (Moalli and Rosen, 1994; **Figure 2c**). Therefore, the main structural difference across the 3 GR isoforms appears to be the length of each molecule, with important implications for function (**Figure 2**). Hereafter, the GR acronym will refer to the GRα isoform.

Initially, hGR was thought to be composed of three main functional domains, a region that bound steroid, one that interacted with DNA, and another that interacted with nuclear proteins (Dellweg et al., 1982). In order to study the function(s) of these domains, hGR was randomly fragmented into a number of small pieces by proteolytic cleavage and each piece studied for various activities (Vedeckis et al., 1983). In that work, certain fragments retained some but not all the characteristics (DNA-binding, ligand binding, and transactivation) of the full receptor. Importantly, this work verified for the first time that hGR contains several autonomous domain regions with ligand-binding, DNA bind-

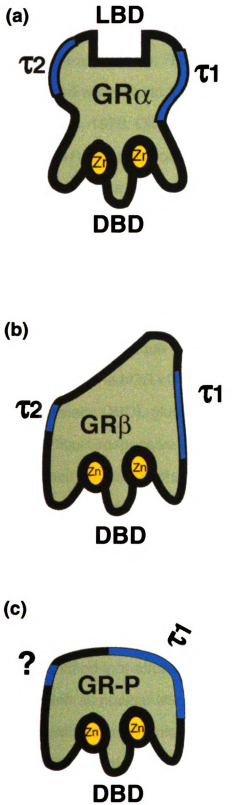


Figure 2. Functional Domains of Three Documented Isoforms of the Human Glucocorticoid Receptor (hGR).

hGR has been identified as having three different splice isoforms; α, β, and P. The hGRα **(a)** has a full ligand binding domain (LBD) and DNA binding domain (DBD) as well as two transactivation domains (τ1 and τ2) and is thus considered the "normal" GR as it binds glucocorticoid and profoundly affects the expression of glucocorticoid-responsive genes in target cells.The hGRβ **(b)** has a truncated LBD and is thus unable to bind glucocorticoids. One paradigm put forward in the literature is that hGRβ provides a fast negative feedback loop for hGRα, turning off glucocorticoid signaling by competing with hGRα for protein binding and (or) glucocorticoid response element (GRE) binding in promoters of hormone-responsive genes. Little is known about hGRP **(c)** although it is thought to lack both a LBD and τ2. It retains the ability to bind DNA through its DBD and increased expression of this isoform by cells appears to be associated with resistance to glucocorticoids (Moalli and Rosen, 1994).

ing, and transactivation activities (Vedeckis et al., 1983). In 1985, Hollenberg et al. refined the original three-domain model of hGR to include an additional immunogenic domain. This domain was located in the amino-terminus of the protein and was able to bind and cross-react to both monoclonal (Govindan and Sekeris, 1978; Okret et al., 1981; Gametchu and Harrison, 1984; Harmon et al., 1984) and polyclonal antibodies (Govindan, 1979; Eisen, 1980; Okret et al., 1981) within a species. In addition, locations of the DNA-binding region (in the middle of hGR) and the glucocorticoid binding site (near the carboxy-terminus) were identified during those studies. However, it was not until 1986 that Giguere et al. (1986) identified the precise boundaries of what are now established as the four main functional domains of hGR (**Figure 2a**). This work employed the technique of random mutational analysis to determine the precise exons in the hGR cDNA that encoded the domains involved in DNA-binding (1 domain; DBD), glucocorticoid-binding (1 domain; LBD), and regulation of glucocorticoid-responsive gene transcription (2 domains; τ1 and τ2). By reason of their degree of effects on transcription of target gene expression *in vitro*, the two transcription regulation domains of hGR were called the first transactivation domain (tau-1, or τ1, having the greatest effect on gene transcription), and the second transactivation domain (tau-2, or τ2, more subtle effects on gene regulation), respectively.

In addition to the four major functional domains, other regions of hGR were soon identified that afforded the molecule other important functional characteristics, such as nuclear translocation, phosphorylation, and protein-protein interactions (Muller and Renkawitz, 1991). Shortly following the elucidation of these hGR domains and subdomains, the murine GR gene was cloned, sequenced, and similar domains for ligand-binding, DNA-binding, and transactivation defined (Danielsen et al., 1986). Elucidation of boundaries between

the functional domains allowed further biochemical and functional analyses of each domain and their contributions to the actions of glucocorticoids on hormone-responsive genes.

One of the first GR domains to be studied in such depth was the ligand-binding domain (LBD). Because this domain binds glucocorticoid hormone, it is primarily responsible for initiating translation of ligand message into observed biological effects in target cells. The LBD of hGR is 16 kDa in size and spans amino acid residues 537 through 777 (Simons et al., 1987; Carlstedt-Duke et al., 1988; Simons et al., 1989). In effect, LBD makes up the entire carboxy-terminal end of the hGR molecule. In particular, residues M622, C656, and C754 bind steroid and are located within hydrophobic segments of the LBD (Carlstedt-Duke et al., 1988). This would indicate that there is a hydrophobic pocket within the three-dimensional hGR, which binds hormone and activates the receptor to regulate target gene expression. Recent X-ray crystallographic structures of this domain depicts an α-helical "sandwich" structure of LBD, where the hormone is buried in the middle (**Figure 3a**; Huang and Simons, 1999 and references therein). In addition to the hormone-binding function of LBD, this domain appears to repress *in vitro* transactivation when not ligated with hormone (Muller and Renkawitz, 1991). Using mutagenesis, GR LBD truncation mutants were created as well as LBD mutants with inserted amino acid residues. GR LBD mutants containing amino acid residues 1 through 532 were not active, while GR containing residues 1 through 582 retained 40% of "normal" or wild-type (WT) activity. By fusing amino acid residues 533 through 777 back into the mutants displaying reduced activity, two subdomains of the LBD were identified that possess inhibitory effects on GR activity (Hollenberg et al., 1989). These subdomains lie between amino acid residues 530 and 582 and residues 697 to 777 (Hollenberg et al., 1989).

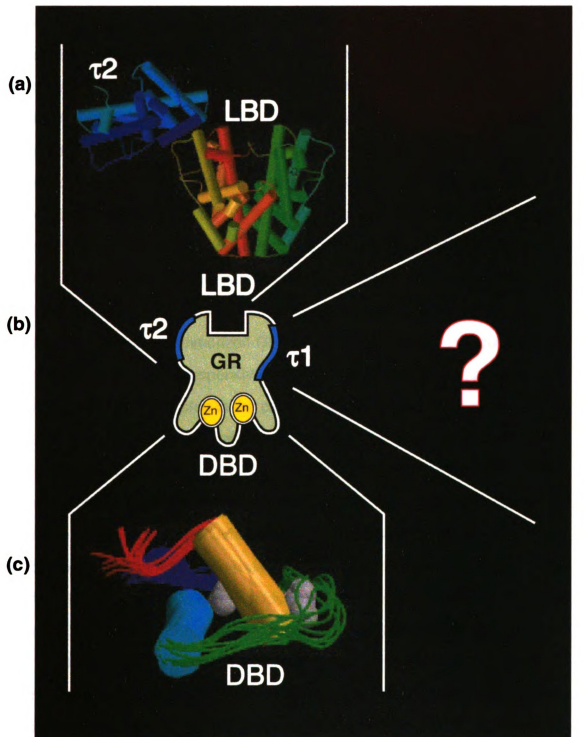
Therefore, the unligated LBD acts as a ligand-dependent "switch" that keeps the remainder of the GR molecule in an inactive state. While the LBD of the carboxy-terminus of GR is critical for initiation of hormone activity in cells, studies have also shown that loss of the amino-terminus of GR leads to only weak transactivation of target genes in the presence of glucocorticoids (Encio and Detera-Wadleigh, 1991). It was thus concluded from these studies that initial receptor activation via ligand binding in the LBD is not enough to effect full regulation of target gene expression, and subsequently led to further studies into the DNA-binding and transactivation domains of GR.

The DNA-binding domain (DBD) is responsible for linking GR with specific DNA targets in regulatory regions of glucocorticoid-responsive genes. The general properties of such DBD were first characterized for the *Xenopus* transcription factor IIIA, which was observed to possess peptide loops that form "fingers" stabilized by centralized zinc ions (Brown et al., 1985; Miller et al., 1985). Since then, this general zinc finger motif has been identified in numerous DNA-binding proteins, including the large family of steroid hormone receptors. The DBD of GR is comprised of 93 amino acids, spanning residues 440 to 510 in full-length GR (Luisi et al., 1991; van Tilborg et al., 1995). GR's DBD contains two subdomains, each with a peptide loop finger containing four cysteines bound to a central zinc ion (Luisi et al., 1991; **Figure 3c**). The amino-terminal or "first" finger contains aromatic and hydrophobic residues that form a β-sheet structure. This acts as a core to stabilize the relative orientation of both zinc fingers within the DBD and allows interaction of the first finger with a second GR molecule following homodimerization (Luisi et al., 1991). Due to its stable orientation, the first zinc finger is considered 'specific' in its binding to regulatory DNA in glucocorticoid-responsive genes. In contrast, the carboxy-terminal or "second" zinc finger forms an α-helix structure followed by a β-

Figure 3. Tertiary Protein Structures of hGRα Functional Domains Predicted from X-ray Crystallography.

Two functional domains of GR have been x-ray crystallized; **(a)** the ligand binding domain (LBD; PDB Accession #1QKU; Eiler et al., 2001) and **(c)** the DNA binding domain (DBD; PDB Accession #1RGD; van Tilborg et al., 1995). The τ2 domain forms part of the LBD and is shown in **(a)** to lie just above the groove of LBD that forms the hormone binding pocket or "sandwich". The LBD and τ2 appear to be mostly α-helical (cylinders) in structure. The DBD **(c)** is comprised of two zinc finger regions, which are positioned in the GR molecule for effective interaction with the major groove of the DNA double helix as well as with glucocorticoid response elements in the regulatory DNA of target genes. Though τ1 **(b)** is critical for transactivation or transrepression of glucocorticoid-responsive genes by GR, this domain has proven elusive in x-ray crystallography experiments due to its lack of tertiary structure at neutral pH. Therefore, the crystal structure of GR's τ1 domain is still unknown.

Figure 3. Tertiary Protein Structures of hGRα Functional Domains Predicted from X-ray Crystallography



sheet, leading to a finger with a more flexible orientation. The role of this second zinc finger appears to be to make contact with DNA-associating proteins (Luisi et al., 1991). In addition, this is the finger that binds to DNA with particular affinity for sequence contained in the major groove of the double helix. Interestingly, the second fingers of GR homodimers bind to successive major grooves while orienting around, but not binding to, the minor groove of the double helix (Luisi et al., 1991; Tsai and O'Malley, 1994). This may allow GR to orient the double helix in such a way that the first fingers of GR's DBD can then access promoters of target genes, and (or) stabilize dimerized GRs on the double helix. The second zinc finger is considered to have a more 'non-specific' DNA binding role than the first zinc finger due to its ability to bind to multiple DNA sequences and their associated proteins. The two zinc fingers of the DBD thus act in concert to modify the DNA double helix for subsequent binding of activated and dimerized GR to specific DNA motifs in regulatory regions of glucocorticoid-responsive genes. Therefore, DBD domain activity provides another critical step in the cascade of events leading to glucocorticoid-induced regulation of target genes by ligand-activated GR.

Lying within and flanking the LBD and DBD are additional regions, or subdomains, of GR that have important functions in the activity of the receptor molecule. One of these subdomains lies directly downstream of the DBD and is composed of 15 amino acids (residues 510 through 525; Dahlman-Wright, et al., 1991), with a core of 5 amino acids called the dimerization or D-loop. The D-loop is responsible for homodimerization of ligand-bound GR (Luisi et al, 1991). Specific amino acids that flank the D-loop and lie within the second zinc finger of DBD also contribute to efficient dimerization of ligand-bound GR. In fact, mutation of just one of these amino acids (eg. A458T) completely abolishes GR dimerization (Newton, 2001). Dimerization of ligand-activated GR is

important because it contributes to the specificity and affinity of GR interactions with DNA (Dahlman-Wright et al., 1995).

Another important subdomain of GR is a stretch of basic amino acids located directly downstream of the DBD and its D-loop region. This region carries one of two nuclear localization signals present in the GR molecule (Muller and Renkawitz, 1991; Sackey et al., 1996). The first nuclear localization signal is located in the amino-terminus of GR (Muller and Renkawitz, 1991). In rat GR, this DBD-associated nuclear localization subdomain is 28 amino acids long and helps mediate nuclear localization of GR molecules that become dimerized upon ligand binding (Sackey et al., 1996). The importance of this subdomain was elucidated when a single point mutation (R484H) was shown to reduce the number of nuclear GR by 60%, although whole cell expression of GR was the same as for cells with wild-type GR (Sackey et al., 1996). This indicated that fewer activated mutant GR were able to translocate into the nucleus and regulate expression of glucocorticoid-responsive genes. GR dimerization is thus critical not only for proper interactions with the DNA double helix but also for translocation of the activated receptor complex from the cytosol through the nuclear membrane. This is accomplished when the GR dimers interact with important nuclear import proteins, such as importin α, which enables GR to be transported across the nuclear envelope via specialized nuclear pores (Prufer and Barsony, 2002). Without dimerization and nuclear localization, GR is unable to mediate the effects of glucocorticoids on expression of target genes in otherwise sensitive cells (Maden and DeFranco, 1993; Sackey et al., 1996).

The GR molecule has a third subdomain that is exposed for protein-protein interaction when the receptor is not bound by hormone. This subdomain binds a variety of heat shock proteins (hsp), including hsp90, hsp70, and hsp56

(Schreiber, 1991; Walsh et al., 1992; Czar et al., 1994). While little is known about this subdomain, it appears to form part of the LBD and may mask the nuclear localization site when GR is associated with these hsp complexes and not bound by hormone (Czar et al., 1994). The hsp bound to this subdomain are thought to act as GR chaperones (Caamano et al., 2001), trafficking newly synthesized GR to the cytoplasm of the cell and keeping cytoplasmic GR in a state that promotes high affinity for ligand-binding by LBD (Schreiber, 1991; Walsh et al., 1992). Upon binding of hormone to LBD, the hsp subdomain presumably undergoes a conformational change as the entire hsp complex is released (Czar et al., 1994). Release of the hsp complex would then expose the nuclear localization subdomain, since it is after such release that hormone-activated GR dimerize and translocate into the nucleus (Sackey et al., 1996). Therefore, the main LBD and DBD domains as well as the dimerization, nuclear localization , and hsp subdomains of GR are critical for glucocorticoid binding and subsequent activity of the hormone bound receptor in the nucleus. However, none of these domains and subdomains actually cause changed expression of hormone-responsive genes. This is the job of additional transactivation domains.

Initiation or repression of messenger RNA synthesis is a primary point of control in the regulation of gene expression. Transactivation domains present in transcription factors, including GR, control mRNA synthesis via interactions with other transcription co-factors and proteins of the basal transcriptional complex (Ma and Ptashne, 1987; Sadowski et al., 1988; Mitchell and Tjian, 1989; Jiang et al., 1994). The first transactivation domains to be defined in eukaryotic transcription factors were for the Gal4 (Ma and Ptashne, 1987; Kakidani and Ptashne, 1988; Sadowski et al., 1988) and GCN4 (Hope and Struhl, 1986) transcription factors in yeast. These transactivation domains were found to be

comprised of a high percentage of glutamine, proline, and acidic amino acid residues (Mitchell and Tjian, 1989). Other glutamine-rich transactivation domains have been identified in transcriptional coactivators including oct-1, AP-1, and Drosophila homeotic proteins (Bohmann et al., 1987; Williams et al., 1987; Sturm et al., 1988; Ko et al., 1988), although their specific functions have not been well characterized. Proline-rich transactivation domains are thought to function by disrupting α-helices in proteins that interact with them (Williams et al., 1987; Ko et al., 1988; Struhl et al., 1988; Norman et al., 1988). Transactivation domains that are rich in acidic amino acids have been identified in Gal 4 (Ma and Ptashne, 1987) as well as hGR (Hollenberg and Evans, 1988) and may facilitate transcription initiation by interacting in a relatively non-specific manner with other transcriptional regulators. One characteristic shared by acidic transactivation domains is their ability to remain highly unstructured at the tertiary protein level when placed at physiological (neutral) pH (Sigler, 1988; Dahlman-Wright et al., 1995). It has been theorized that these acidic transactivation domains remain unstructured until they contact other regulatory proteins, at which time they develop the tertiary protein structure necessary for regulation of transcription initiation and repression (Sigler, 1988).

The τ1 of hGR is contained within a major immunogenic region in the amino-terminus of the molecule (Hollenberg et al. 1985; Weinberger et al., 1985; Hollenberg et al., 1986). This region was considered immunogenic due to its ability to cross-react with species-specific monoclonal (Govindan and Sekeris, 1978; Okret et al., 1981; Gametchu and Harrison, 1984; Harmon et al., 1984) and polyclonal antibodies (Govindan, 1979; Eisen, 1980; Okret et al., 1981), implying that it is variable. Immunogenic domains are present in all members of the superfamily of steroid hormone receptors (Evans 1988).

While there is variation in the size of this domain across family members, each contains amino acid sequences that confer strong *in vitro* transactivation activity (Giguere et al., 1986; Hollenberg et al., 1987; Godowski et al., 1988). In fact, the transactivation activity from τ1 contained within these immunogenic domains occurs independent of hormone or DNA binding of a full length receptor molecule (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987; Reichardt et al., 1998; Wright et al., 1991; Dahlman-Wright et al., 1995; Scheinman et al.,1995). Also, when the τ1 domain is relocated in GR or other members of this steroid receptor family, it still exhibits strong transactivation activity (Dahlman-Wright, et al., 1994). Therefore, GR's τ1 domain is considered to have constitutive transactivational capacity and, as such, is predominantly responsible for the regulation of glucocorticoid-responsive genes by hormone-activated nuclear GRs (Hollenberg and Evans, 1988).

The τ1 of hGR spans amino acid residues 77 through 262 (Wright et al., 1991). The τ1 domain region has a "core" region consisting of 41 amino acids, spanning residues 187 through 227. Although this core region is absolutely necessary for transactivation from τ1, alone it only displays 60-70% of the full τ1 activation of glucocorticoid-responsive genes (Dahlman-Wright et al., 1994). Thus, the protein sequences flanking the τ1 core region are also important determinants of transactivation. Indeed, a 58 amino acid stretch (residues 187 through 244) of τ1 in hGR has been identified that retains almost all of the transactivation activity of the core (Wright et al., 1991). However, the remaining transactivation activity from the immunogenic region of GR must come from flanking amino acid sequences, as mutations in these flanking regions have been shown to both positively and negatively affect transactivation from GR's τ1 (Wright et al., 1991).

GR τ1 works by binding to either the transcription preinitiation complex (PIC) or a multitude of other transcriptional co-factors, and by that means, either activates or represses the expression of glucocorticoid-responsive genes (**Figure 4**). Human GR τ1 contains clusters of acidic residues, which classifies it as an acidic transactivator (Mitchell and Tjian, 1989). Multiple phosphorylation sites on conserved serine residues contribute further to the overall acidity of τ1. This is important in light of the fact that a strong positive correlation exists between the acidity of such domains and their transactivation activity (Danielsen et al., 1987). Despite this observation, it is also likely that certain hydrophobic amino acids in τ1 domains are critical for full transactivation activ-

Figure 4. General Transcriptional Regulatory Mechanism of GR τ1.

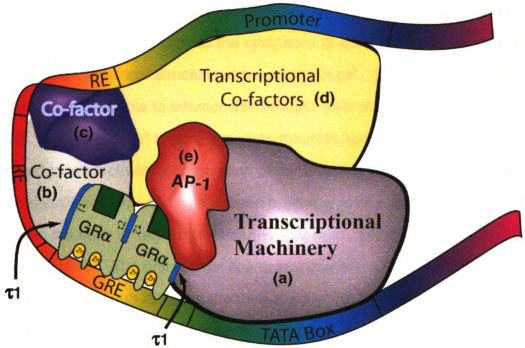


Figure 4. General Transcriptional Regulatory Mechanism of GR τ1.

Glucocorticoid-responsive gene expression is regulated via GR τ1 (arrows), through protein-protein interactions with the basal transcriptional machinery **(a)** as well as interaction with other transcriptional co-factors (**b**, **c**, and **d**), such as AP-1 **(e)**. Thus τ1 of GR are intimately involved in the regulation of target gene expression.

ity as these are highly conserved across such proteins (Cress and Treizenberg, 1991; Reiger et al., 1993). Thus, it appears that a combination of domain charge and tertiary protein structure following protein-protein interactions dictate the transactivation potential of τ1.

A prediction of hGR τ1 tertiary structure was recently reported using circular dichroism and nuclear magnetic resonance spectroscopy (Dahlman-Wright et al., 1995). Unlike other domains of GR, τ1 is largely unstructured at neutral pH (6.9-7.7; Sigler et al., 1988). However, τ1 forms α-helices in the presence of trifluoroethanol, an α-helical stabilizer (Dahlman-Wright et al., 1995 and references within). No evidence of β-sheet formation was observed even in the presence of the β-sheet stabilizer, SDS (Dahlman-Wright et al., 1995). This work indicates that hGR τ1 is a conformationally flexible domain in neutral physiological solutions (such as the cytoplasm of a cell) but acquires a more rigid, possibly α-helical structure upon change in pH. Such flexibility would suggest that τ1 is able to interact with multiple different proteins, readily adapting its "binding groove" to accommodate the wide variety of proteins it is required to interact with for regulation of gene expression. In support of this proposition, segments of τ1 have been predicted to contain α-helices, two that reside in the core τ1 region (Dahlman-Wright et al., 1995; Dahlman-Wright and McEwan, 1996). The first α-helix spans amino acids 189 to 201, the second residues 216 through 227, and the third residues 234 through 240, with the first and second helices making up the 41 amino acid τ1 core (Dahlman-Wright et al., 1995; Dahlman-Wright and McEwan, 1996). Mutation of proline residues at the edges of these three helices disrupt helical structure and cause dramatic reductions in transactivation activity of τ1 (Dahlman-Wright and McEwan, 1996). This suggests that proline residues contributing to τ1 α-helices are absolutely required for transactivation. It is thus not surprising that

these same proline residues are highly conserved in the τ1 domains of GR across mammalian species (Dahlman-Wright and McEwan, 1996). To date, the precise tertiary protein structure of τ1 remains unknown. Despite this, the research reported above is conclusive: τ1 is the primary domain of GR that is responsible for transcriptional regulation of glucocorticoid-responsive genes.

An additional region of GR critical to the regulation of target gene transcription was identified in the mid-1980's by Giguere et al. (1986). Mutagenesis of hGR revealed a region within the amino-terminus of the LBD (between amino acids 526 and 556) that retained wild-type steroid binding affinity but displayed reduced *in vitro* transcriptional activity (Giguere et al., 1986; Milhon et al., 1997). Further study of this region revealed that it retained transactivation activity independent of its position within hGR (Hollenberg and Evans, 1988). These findings confirmed the presence of a second weaker transactivation domain contained within the main LBD of GR; as such the domain was called the second transactivation domain, or τ2. The GR τ2 is composed of two structurally and functionally divergent subdomains. The amino-terminal subdomain spans amino acids 532 to 547 and is predicted to have an α-helical structure (Milhon et al., 1997). Importantly, one face of the τ2 α-helix makes contact with α-helices in the main part of the LBD, forming a sandwich structure into which a glucocorticoid hormone molecule fits (**Figure 3a**; Milhon et al., 1997). In effect, the α-helix of τ2 functions to stabilize the LBD of GR following hormone binding. Another face of the τ2 α-helix is exposed to the surface of GR and is thus accessible for interacting with other proteins (Milhon et al., 1997). Therefore, the α-helices of τ2 may effect hormone-dependent transactivation by their stabilizing effects on the LBD and through contact with other transcription factors and co-factors. The carboxy-terminal subdomain of τ2 spans residues 548 and 561 and is predicted to form

ɔ exposed to the outside of the GR molecule and thus is fully accessible
teractions with other proteins (Milhon et al., 1997). It is thought that this
area is the primary transactivation region of τ2. Indeed, a reduction in
t gene expression of up to 50% was observed following mutagenesis
lues L550A and S561A) of this hGR τ2 region (Milhon et al., 1997).
efore, the full GR τ2 domain acts both as a stabilizer of the hormone-
d LBD as well as a regulator of target gene expression.
n addition to its position within the GR molecule, τ1 differs from τ2 in sev-
egards. Importantly, τ1 can regulate transcription in *in vitro* gene expres-
systems independent of hormone binding while τ2 requires hormone bind-
) display full transactivation activity (Schmitt and Stunnenberg, 1993).
ermore, τ2 has a well-defined tertiary protein structure whereas τ1
ins unstructured until it encounters other regulatory proteins. Therefore,
ay interact with a smaller selection of similar proteins whereas τ1 is pre-
d to have the capacity to interact with a large variety of diverse proteins.
may explain the relatively strong transactivation activity of τ1 compared to
Because of these differences, the two transactivation domains of GR most
co-activate receptor regulation of glucocorticoid-responsive gene expres-
regardless of cell type involved.
The domain structure of GR described thus far has been for the full-length
form of this receptor. As previously mentioned, however, other isoforms
R also exist, namely GRβ (Hollenberg et al., 1985) and GRP (Moalli and
n, 1994; Krett 1995; de Lange 2001; **Figure 2**). Full GR regulation of glu-
rticoid-responsive genes probably involve all three isoforms. GRβ is
ɔlogous with GRα between amino acids 1 and 727 (Hollenberger et al.,
) but differs in its carboxy-terminus (Encio and Detera-Wadleigh., 1991).
difference occurs because of an alternative splice site in exon 9β of the

GR gene that splices out a large region of the LBD-encoding DNA during transcription (Encio and Detera-Wadleigh, 1991; Oakley et al., 1996). Therefore, GRβ has a truncated LBD and is unable to bind glucocorticoids. GRβ is also devoid of an intact τ2 region (Encio and Detera-Wadleigh, 1991). Together with the truncated LBD, GRβ is incapable of ligand-dependent transactivation. Although GRβ possesses all other domains and subdomains found in GRα, it has been found to be transcriptionally inactive (Yudt and Cidlowski, 2002). Despite the ability of GRβ to bind hsp molecules, they are located primarily in the nucleus as monomers in the absence of ligand, and form dimers with hormone-activated GRα (Oakley et al., 1996; Oakley et al., 1997). One paradigm put forth in the biomedical literature is that GRβ acts as an important modifier of GRα activity on target gene expression. As such, GRβ blocks ligand-dependent transactivation of the target gene by competing with hormone-activated GRα for dimerization and DNA binding (Bamberger et al., 1995; Oakley et al., 1996; Oakley et al., 1997; Brogan et al., 1999; Oakley et al., 1999). In this way, GRβ may act in a short-loop fashion to curtail a cell's responsiveness to an excessive or prolonged glucocorticoid stimulus through competition with hormone-activated GRα. Similarly, GRP is associated with glucocorticoid resistance, although specific regulatory mechanisms of this isoform are not known.

In summary, GR is present in cells in at least 3 known isoforms, all bearing the important DBD, hsp, and τ1 domains, but each containing varying amounts of LBD and τ2 domains. Only GRα can bind glucocorticoid hormones to regulate target gene expression in response to these hormones. In this case, the τ1 domain appears to possess most of the transactivation activity of the molecule with τ2 contributing some activity. However, GRβ and GRP may have important modifying functions on the activity of GRα, possibly protecting cells

from "hyper-responsiveness" during extreme or chronic exposure to high glucocorticoid concentrations. While the DBD is important for gene regulation in systems that require DNA-binding by GR, the τ1 domain acts independently of hormone binding and thus initiates transactivation (or transrepression) in all glucocorticoid-responsive gene systems. The importance to gene regulation of τ1 will be highlighted further in the subsequent section on GR function.

iii. Regulation of Transcription by the Glucocorticoid Receptor.

In the absence of ligand, GR is found mainly in the cytoplasm of glucocorticoid-sensitive cells. Inactive GR is bound to multiple accessory proteins including the hsp complex. At least four heat shock protein molecules are known to bind to inactive GR, 2 molecules of hsp90 and one molecule each of hsp70 and hsp56 (**Figure 5b**). These hsps act as chaperones that facilitate the transport of newly synthesized GR from the Golgi apparatus to the cytoplasm following translation of the receptor protein. Hsp90 molecules chaperone protein folding; hsp70 has protein 'unfoldase' activity and binds to other proteins during translation, while hsp56 plays a role in both GR folding and trafficking (reviewed in Czar et al., 1994). Binding of hsp70 is a prerequisite for subsequent binding of two hsp90 molecules, after which hsp56 binds to hsp90 (Czar et al., 1994). The main function of this hsp heterocomplex is to keep GR inactive in the cytoplasm and in a high affinity state for hormone binding in the LBD (Bamberger et al., 1996).

Because glucocorticoid hormones are lipophilic, they readily diffuse across the lipid bi-layer making up the plasma membranes of cells (**Figure 5a**). Once in the cytoplasm, the hormone can bind with high affinity to the GR-hsp complex. This ligand binding induces a conformational change in GR that causes dissociation of all hsp (**Figure 5c**; Bamberger et al., 1996). Though poorly understood, the ligand-induced conformational change in GR is believed to be

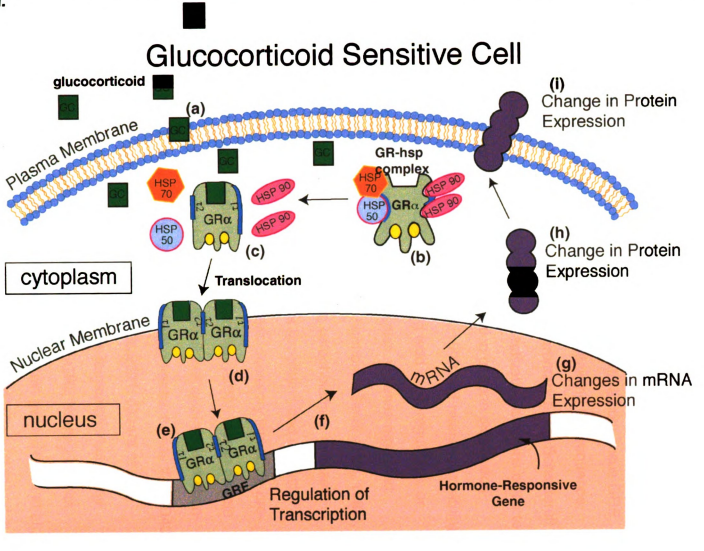
the most important consequence of ligand binding, exposing the dimerization sequence, nuclear localization signal (NLS), and perhaps the τ2 domain of the receptor (Tsai and O'Malley, 1994). Shortly following hsp dissociation, GR becomes hyperphosphorylated on conserved serine residues, which appears to activate the receptor even further (Bamberger et al., 1996). Two ligand-activated GR dimerize, either in the cytoplasm just prior to nuclear localization (Bamberger et al., 1996), or just following translocation. The activated GR are transported across the nuclear membrane through nucleoporins, most probably through binding of nuclear transport protein importin α (or β) to the GR NLS (**Figure 5d**; Prufer and Barsony, 2002). The relevance of the process from ligand binding to nuclear translocation is that it allows GR to become appropriately positioned in the nucleus of the cell for subsequent transcriptional regulation of hormone-responsive genes (**Figure 5e**). However, GR's target DNA is compressed and packaged tightly around histone and non-histone proteins into a nucleosome. In order to gain access to appropriate DNA motifs in regulatory regions of target genes, GR must remodel the nucleosomes. To accomplish this, translocated GR interacts with co-factors including the SWI/SNF and Ada-Gcn5 complexes (Henriksson et al., 1997; Robyr and Wolfe, 1998; McEwan, 2000). These complexes use energy from ATP hydrolysis to alter histone-DNA interactions, such that GR's DBD can gain access to appropriate docking motifs (see below) in the target DNA (McEwan 2000 and references within; **Figure 5e**). The zinc fingers of the DBD then bind to the DNA's double helix, keeping the underlying DNA exposed and allowing regulation of gene expression by GR (Luisi et al., 1991; Tsai and O'Malley, 1994; **Figure 5f**, **g**, **h**, and **i**).

Transcriptional regulation by the ligand-activated GR is complex, occurring via several direct and indirect mechanisms, and involves physical interaction of GR with several other transcriptional regulatory proteins. In general, these mechanisms can be divided into two categories; regulation by direct GR-DNA

Figure 5. Model of Direct Action of Hormone-Bound GRα on Glucocorticoid-Responsive Gene Expression in a Glucocorticoid-Sensitive Target Cell.

Lipophilic glucocorticoids cross the plasma membrane of target cells **(a)** for binding with GR molecules. Heat shock proteins (hsp) bound to GRα act as chaperones to keep unbound receptors in the cytosol and in a state of high affinity for the steroid **(b)**. Upon binding by glucocorticoid, GRα sheds hsp **(c)** and forms homodimers with other hormone-activated GR, which then translocate into the nucleus via nucleoporin transfer **(d)**. To affect gene expression, the DNA binding domains of GR homodimers bind to the major groove of the DNA double helix and to specialized motifs (glucocorticoid response elements, or GREs) in the regulatory DNA of glucocorticoid-responsive genes **(e).** DNA binding allows for interaction of τ1 and τ2 with transcriptional co-factors to either activate or repress gene expression **(f)**. Altered gene expression may lead to changes in mRNA abundance **(g)** and subsequently, protein abundance **(h** and **i)** of the affected gene, ultimately changing the cell's phenotype.

Figure 5. Model of Direct Action of Hormone-Bound GRα on Glucocorticoid-Responsive Gene Expression in a Target Cell.



binding, and regulation that is independent of GR binding to DNA. Direct regulation of gene expression involves the binding of GR homodimers to specific DNA motifs within regulatory regions of glucocorticoid-responsive genes (**Figure 5e**). These motifs are called glucocorticoid response elements (GRE) and occur in several forms, depending on the target gene.

The simplest mechanism of action is binding of GR to a consensus GRE (Newton, 2000; Reichardt et al., 2000; **Figure 6a**) containing the sequence, GGTACAnnnTGTTCT (where the 'n' represents an arbitrary base; Scheidereit et al., 1983). These GREs act to tether GR's transactivation domains close to promoters of target genes, allowing for GR regulation of gene expression (Beato, 1989; Beato, 1991; Miner and Yamamoto, 1992 and references within). Most known cases of this simple GR-GRE binding lead to up-regulation, or transactivation, of target gene expression. Examples of genes regulated through simple GR-GRE binding include growth hormone (Slater et al., 1985), tyrosine hydroxylase, (the rate limiting enzyme in catecholamine release; Hagerty et al., 2001), the inflammatory regulators lipocortin I and calpactin binding protein (Newton 2000), β_2-adrenoreceptors, secretory leukocyte protease inhibitor, the decoy IL-1 type II receptor (Newton 2000), myelin protein 22 (Desarnaud et al., 2000), and sgk1 (a regulator of epithelial sodium channels; Itani et al., 2002). Additionally, GR-GRE binding transactivates the CD40 ligand gene in both T_H2 and B lymphocytes (Barnes 2001; Jabara et al., 2001). GR binding to simple GREs can also repress transcription of many immune and inflammatory genes. In this context, GR-GRE binding inhibits expression of the acute phase protein α1-acid glycoprotein gene (Nishio et al., 1993), multiple cytokine genes (IL-1α, IL-1β, IL-4, IL-8, and IFN-γ; reviewed in Almawi et al., 1996), adhesion molecule genes (CD18; Agura et al., 1992; and CD11a, Pitzalis et al., 2002), the macrophage Fcγ receptor gene (Sivo et al.,

Figure 6. Models of Six Mechanisms by Which Hormone-Activated GRα Can Regulate Glucocorticoid-Responsive Gene Expression.

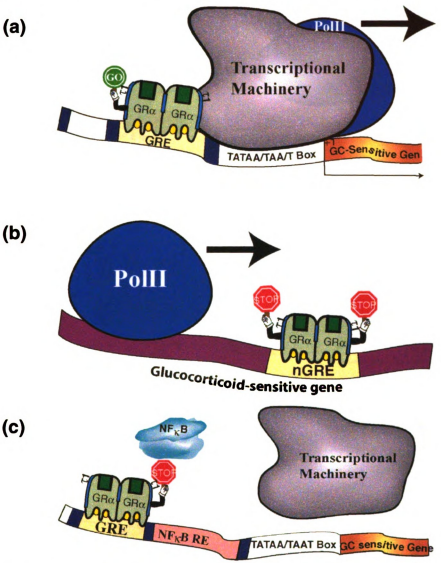
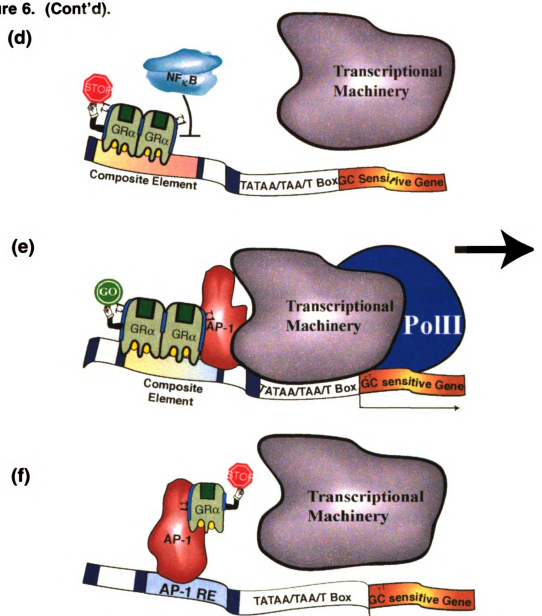


Figure 6. Models of Six Mechanisms by Which Hormone-Activated GRα Can Regulate Glucocorticoid-Responsive Gene Expression.

In panel **(a)**, hormone-activated GR binds to a consensus DNA sequence motif, called the glucocorticoid response element (GRE), in the promoter region of glucocorticoid-responsive genes. In doing so, the τ1 domain of GR is well positioned to recruit proteins of the basal transcription machinery, causing transactivation of the affected gene. In panel **(b)**, a less well defined glucocorticoid response element, called nGRE, binds hormone-activated GR, but in this case the receptor transrepresses the hormone-responsive gene. A variety of nGRE motifs have been found in promoters, introns, and untranslated regions (UTRs) of glucocorticoid-responsive genes and may act by blocking movement of RNA

Figure 6. (Cont'd).



polymerase during transcription of the affected gene. In panel **(c)**, competitive nGREs are GRE sequence motifs closely placed to response elements for other transcription factors (as shown for NF-κB) resulting in the inability of that transcription factor to bind the DNA once GR has bound. In this model of gene regulation, GR blocks normally active gene expression. In panels **(d)** and **(e)**, composite GREs are GREs that overlap the response element for another transcription factor [shown are NF-κB in **(d)** and AP-1 in **(e)**], leading either to inhibition **(d)** or activation **(e)** of gene transcription through protein-protein interactions. In panel **(f)**, a tethering GRE is shown in which a transcription factor (AP-1) already bound to its response element recruits hormone-activated GR and thus blocks binding of other transcriptional co-factors to AP-1. This ultimately represses normal activation of the down-stream gene.

1993), and the TGF-β inhibitor gene TGF-β1 (Parrelli et al., 1998), and MHC II genes (Chen et al., 2000). Therefore, this GR-GRE mechanism of action that is considered simple and straightforward causes complex phenotypic changes in cells by virtue of both activation and repression of glucocorticoid-responsive gene expression.

Some genes contain poorly defined GREs whose expression, when bound by GR, is repressed. These GREs have been termed "negative GREs" or nGREs (Newton, 2000; **Figure 6b**). Because nGREs have no consensus sequence, their existence is rather controversial. In fact, some investigators believe that nGRE are actually GRE half-sites (Sakai, et al., 1988; Drouin, et al., 1989; Subramaniam, et al., 1997; Zhang et al., 1997) that have lower affinity for GR dimers than full GREs (Malkoski and Dorin, 1999), or that bind with monomers of GR, possibly GRβ (Subramaniam et al., 1997). One of the best characterized nGREs is in the promoter of the pro-opiomelanocortin gene (POMC; Drouin et al., 1989a,b,c; Nakai et al., 1991). This nGRE has been identified as CGTCCA in one strand of DNA and TGACC in the complementary DNA strand (Drouin et al., 1989a). Other genes identified as harboring nGREs in either their promoter or other regulatory regions include prolactin (Sakai et al., 1988; Subramaniam et al., 1997, 1998), and osteocalcin (Newton 2000). To date, only one cytokine gene has been identified as containing a nGRE, the IL-1β gene (Zhang et al., 1997). In another mechanism of action, GR can bind to a GRE or nGRE, and compete with another transactivating factor for response element binding (**Figure 6c**), resulting in inhibition of gene transcription. In fact, several cytokine genes are regulated through this mechanism; IL-2, IL-6, and TNF-α gene expression is inhibited by competition for binding between GR and nuclear factor-κB (NF-κB; Almawi and Melemedjian, 2002). Additionally, GR binding to a GRE in the ER gene, inhibits normal binding of

the C/EBP transcription factor in ER's promoter, thereby repressing transcription of ER (Lethimonier et al., 2002). Thus, predominantly negative effects on transcription occur through GR-nGRE binding, with increased transcriptional inhibition from steric interference of other transcription factors by bound GR.

In addition to simple GREs and putative nGREs, composite GRE motifs also exist in promoters of some glucocorticoid-responsive genes. Composite GREs are GRE sites that overlap the binding site(s) of other transcription factors (Newton, 2000), including activator protein-1 (AP-1; Diamond et al.,1990; Miner and Yamamoto, 1992), NF-κB (Scheinman et al., 1995; de Bosscher et al., 1997; Liden et al., 2000), cAMP response element (CRE; Scott et al., 1998), the binding site of the myc/max family (E-box; Tseng et al., 2001), and oct-1 (Chandran et al., 1996). GR binding to composite GREs can either block binding by other transcription factors, effectively inhibiting transactivation of the target gene by these other activators (**Figure 6d**), or enhance transcription by the co-activator sharing the composite GRE (**Figure 6e**). In the case of AP-1, this transcription factor can be made up of c-fos homodimers, c-jun homodimers, or c-fos/c-jun heterodimers (Herrlich 2001), determined by the ratio of c-fos to c-jun within the cell (Diamond et al., 1990). GR binding to a composite GRE in the presence of c-fos homodimers caused no change in proliferin gene expression, whereas the presence of c-jun homodimers resulted in activation of transcription and c-fos/c-jun heterodimers resulted in repression of proliferin gene transcription in the presence of GR (Diamond et al., 1990). Examples of genes harboring composite GREs, which are transrepressed by GR, include phophoenolpyruvate carboxykinase (PEPCK) through a composite GRE/CRE site (Imai et al., 1993; Scott et al., 1998), gonadotrophin-releasing hormone (GnRH) through a GRE/oct-1 binding element (Chandran et al., 1996), the β1-adrenergic receptor through GRE/E-box binding (Tseng et al., 2001), and the

CRH (Malkoski and Dorin, 1999), osteocalcin (Morrison and Eisman, 1993), and proliferin genes (Pearce and Yamamoto, 1993; Miner and Yamamoto, 1992; Diamond et al., 1990) through composite GRE/AP-1 binding sites. Binding of GR to GREs, nGRES, or composite GREs in disrupted chromatin allows other regulators and transcription factors to access GR's τ1 and τ2 domains, thus initiating transcriptional regulation of glucocorticoid-responsive target genes (McEwan, 2000).

Many glucocorticoid-responsive genes do not contain GRE, nGRE, or composite GRE motifs, suggesting that glucocorticoid regulation of such genes is not dependent on GR-DNA binding. Instead, these glucocorticoid-responsive genes are regulated through interactions between activated GR and other transcription factors that ultimately contact DNA. These DNA elements that use GR-protein interactions to regulate gene expression are termed "tethering" GRE or nGREs (Newton 2000). Tethering elements are the response elements for transcription factors, and GR binds with the transcription factor already bound to its response element (**Figure 6f**; Jonat et al., 1990; Schule et al., 1990; Yang Yen et al., 1990; Konig et al., 1992). Transcription factors identified capable of tethering GR include AP-1 (Schule et al., 1990; Pfahl 1993; Heck et al., 1994; Herrlich 2001), NF-κB (Baeuerle and Henkel, 1994; Ray and Prefontaine, 1994; Scheinman et al., 1995; De Bosscher et al., 1997; Wissink et al., 1997; McKay and Cidlowski, 1998), GRIP1 (Hong et al., 1999), nuclear factor AT (NF-AT; Refojo et al., 2001), and p300/CREB-binding protein (CBP; Kino et al., 2002b; Refojo et al., 2001; Kamei et al., 1996). Most of the genes identified to be regulated through GR tethering involve the AP-1 or NF-κB transcription factors. NF-κB is a p50/p65 heterodimer (Kawakami et al.,1988; Baeuerle and Baltimore, 1989) and, when activated, is involved in a number of immunological responses such as toxic shock, acute inflammatory responses,

graft-versus-host reactions, and the acute phase response (Baeuerle and Henkel, 1994). GR interacts with the p65 subunit of NF-κB (Ray and Prefontaine, 1994; Wissink et al., 1997) and prevents NF-κB binding to DNA and the resultant activation of gene transcription (Scheinman et al., 1995; de Bosscher et al., 1997; Liden et al., 2000) and, by this means, regulates transcription of glucocorticoid-responsive genes. Interaction between GR and AP-1 generally results in decreased in transcription of genes normally induced by AP-1. Examples of genes that are regulated through GR binding to AP-1 are protein kinase C (PKC; Maroder et al., 1993), collagenase (Liu et al., 1995), collagenase I (Jonat et al., 1990), TGF-β (Periyasamy and Sanchez, 2002; AyanlarBatuman, et al., 1991), the proapoptotic gene Bax (Amsterdam et al., 2002), and T_H1-mediating cytokines IL-2, IL-2 receptor, and IFN-γ (Refojo et al., 2001). Likewise, GR interaction with NF-κB generally results in inhibition of transcription. Genes affected via GR-NF-κB interaction include the adhesion molecules E-selectin (Ray et al., 1997) and ICAM-1 (Liden et al., 2000; Caldenhoven et al., 1995), the chemokine RANTES (Wingett et al., 1996), and cytokines IL-6 (Vanden Berghe et al., 1999) and GM-CSF (Refojo et al., 2001). A less studied transcription factor believed to interact with GR is NF-AT, which results in suppression of GM-CSF (Smith et al., 2001), IL-2 and IL-4 in T cells, TNF-α in B cells, and IL-4 and IL-5 in mast cells (Refojo et al., 2001). Additionally, interaction between GR and cAMP-response element-binding protein transcription factor, or CREB, stimulates uteroglobin gene expression (involved in differentiation of lung epithelium; Cato et al., 1984; Nord et al., 2000), and possibly the MHC Class II *trans*-activator gene (CIITA; van der Stoep et al., 2002). The CD18 gene harbors not only binding elements for AP-1, but also oct-1 and CREB (Rosmarin et al., 1992), any or all of which could interact with GR to suppress gene expression. Therefore, activated GR regu-

lates glucocorticoid-responsive gene expression through a variety of mechanisms in target immune and other cells, underscoring the potent and broad-reaching effects of glucocorticoids.

Despite the specific mode of transcriptional regulation by GR, the τ1 domain is almost always involved. As early as 1982 it was suggested that GR regulation of transcription via τ1 may be through direct interaction of this domain with transcription initiators like RNA Polymerase II (Dellweg et al., 1982). Initiation of transcription by RNA Polymerase II requires an assembly of general factors (the basal transcriptional machinery) and polymerase at or near the transcription start site. The basal transcription factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIJ and comprise a pre-initiation complex (PIC) (Tjian and Maniatis, 1994). TFIID is in itself a multiprotein complex including the TATA-binding protein (TBP) and tightly associated factors (Ford et al., 1997). Binding of TBP to the TATA box of target gene DNA is required for subsequent formation of PIC and gene activation. In addition to PIC, numerous transcriptional co-factors have been identified and work by modifying the activity of basal transcriptional machinery to form a more stable complex (McEwan et al., 1993; Tjian and Maniatis, 1994). In 1991, Wright et al. observed interaction of GR's τ1 with the DNA of a basal yeast promoter. In this study, it was noted that co-factors were unnecessary for transcriptional regulation by τ1, and that the core τ1 region was identified as being responsible for τ1's interaction with the basal transcriptional machinery (Wright et al., 1991). However, Cordingley et al. (1987) showed that chromatin remodeling by GR exposes a nuclear factor-1 (NF-1) binding site, suggesting that τ1 transactivates via cofactor proteins. Models of direct PIC interaction or cofactor interactions with τ1 are not mutually exclusive in light of GR's ability to regulate transcription with or without DNA binding. These studies, and the fact

that τ1 is unstructured prior to protein binding, makes it probable that τ1 regulates transcription by binding basal transcriptional machinery as well as transcriptional co-factors. Studies performed by McEwan (et al., 1994) and Dahlman-Wright (et al., 1994) found that although τ1 bound basal transcription factors, it did not directly bind TBP. Direct interaction of τ1 with basal transcriptional machinery, however, is not fully explained by τ1 binding kinetics (McEwan et al., 1995). The τ1 displayed maximum transactivation function between 20 and 40 minutes, while the PIC is fully assembled within 25 minutes. This suggests that τ1 interacts with not only the PIC but also with other transcriptional co-factors and that transcriptional regulation by τ1 is more complex than previously thought (McEwan et al., 1995). Indeed, it was shown that τ1 does interact with the SWI/SNF (Dahlman-Wright et al., 1994) and GRIP (Hong et al., 1996; Eggert et al., 1995) cofactor proteins to regulate transcription. In 1997, Ford et al. revisited the question of a τ1-TBP interaction and showed that τ1 does indeed interact with the TFIID complex and at least partly with TBP itself. Interaction and recruitment of TFIID by τ1 is yet another mechanism for regulation of glucocorticoid-responsive genes by hormone-activated GR. Regardless of whether or not GR binds DNA to effect regulation of target gene expression, τ1 is involved through its interactions with either the preinitiation complex and (or) transcriptional co-factor such as AP-1 or NF-κB (McEwan, 2000). Therefore, τ1 affords GR its main power to effect transcriptional regulation of glucocorticoid-responsive genes.

The GR has an additional mechanism whereby it can regulate gene expression, without directly influencing transcription of the gene. In this capacity, GR does not necessarily use τ1. Ligand activated GR has been shown to bind directly to the 3' ends of certain mRNA transcripts, ultimately decreasing the stability of the message and leading to inhibition of protein expression.

This mechanism of GR regulation is best characterized for its own mRNA (Okret et al., 1986; Kalinyak et al., 1987; Rosewicz et al., 1988; Burnstein et al., 1990), where hormone-activated GR effectively limits GR gene expression by destabilizating its mRNA (Dong et al., 1988; Burnstein et al., 1991; Burnstein et al., 1994). Other proteins affected through this mechanism include cytokines IL-1α, IL-1β, and IL-6 (Amano et al., 1993). Alternatively, mRNA for the pro-survival protein Bcl-x is stabilized by GR (Amsterdam et al., 2002). Therefore, cells may be able to modify target gene responsiveness to glucocorticoids through ligand-activated GR destabilization of mRNAs in addition to the other GR regulatory mechanisms.

A final series of gene regulation events in the face of glucocorticoid challenge are provided by the other isoforms of GR, namely GRβ and GRP (Bamberger et al., 1996; Oakley et al., 1996; de Lange et al., 2001). Early studies of the non-hormone binding GRβ isoform suggested that it inhibited activity of the GRα isoform (Bamberger, et. al., 1996), implicating an auto-regulatory function for GRβ in a cell's response to glucocorticoids. However, there are no clear mechanisms for this inhibition. GRβ has been found to be bound to heat shock protein 90, can bind to activated GRα (de Castro et al., 1996; Oakley et al., 1999), and has an inhibitory effect on the activity of GRα *in vitro* (Oakley et al., 1996; Brogan et al., 1999; Oakley et al., 1999). Also, GRβ is expressed at significantly higher levels in macrophages, eosinophils, and polymorphonuclear cells from patients with glucocorticoid-insensitive asthma (Christodoulopoulos et al., 2000; Gagliardo et al., 2000; Webster et al., 2001) and arterial hypotension (Bamberger et al., 1997) than in cells from normal patients, suggesting that the GRβ inhibition of GRα function is in part responsible for these dysfunctions. However, GRβ has not been found to affect transrepression by GRα of glucocorticoid-sensitive genes on GRE, AP-1, or NF-κB

sites, showing that the only known function of GRβ is competitive inhibition of GRα transactivation.

In direct opposition to the observed actions of GRβ, GRP is theorized to up-regulate the activity of GRα. Recently, patients that were diagnosed with hematological malignancies and examined for the presence and prevalence of GR isoforms in bone marrow cells, were shown to express significantly lower GRβ than GRα or GRP (de Lange et al., 2001). Reduced expression of GRβ with normal expression of GRα as well as GRP suggested that the regulatory function of GRα is supported by GRP. Obviously, more research of this isoform is warranted. However, if GRP does activate GRα, it may be indirectly involved in GR regulation of glucocorticoid-responsive genes.

In summary, GR is the primary mediator of the effects of glucocorticoids on target cells and tissues. It is present in nearly every cell in the body in either the cytoplasm (GR) and (or) membrane (mGR), and is alternatively spliced into several isoforms (GRα, GRβ, and GRP). GRα is the main and most abundant GR isoform, primarily responsible for transcriptional activities of GR on glucocorticoid-responsive target genes. Scant literature indicates that the GRβ and GR-P isoforms may act to modify the activities of GRα on target gene expression. GR is composed of four main functional domains and several regulatory subdomains. The LBD with it's τ2 subdomain is responsible for forming a stable hormone "sandwich", while other GR subdomains are responsible for hsp binding, receptor dimerization, nuclear localization, and chaperone receptor folding and movement to and from the cell's cytoplasm. The DBD uses its zinc fingers to interact with the major groove and regulatory DNA of target genes once the hormone-activated receptor translocates into the nucleus. Lastly, the τ1 transactivation domain plays a critical and direct role in gene regulation by recruiting both PIC and other important regulatory proteins to the promoters of

glucocorticoid-responsive genes. GR uses numerous, complex, direct and indirect mechanisms to effect activation and (or) repression of target genes in glucocorticoid-responsive target cells. Target genes include those involved in glucose and energy metabolism, cellular differentiation, inflammation and immunity, and reproduction and lactation. The τ1 domain is significantly involved in the regulation of these genes. Therefore, τ1 is a good candidate domain for study of GR regulation of target gene expression and cellular phenotypic changes in response to elevated circulating glucocorticoids.

B. The Glucocorticoid Receptor Gene and mRNA

Animal to animal variation in sensitivity to glucocorticoids can be explained in part by mutations in GR genes. These mutations, or polymorphisms, can result in altered receptor protein molecules or numbers, resulting in variation in glucocorticoid sensitivity of target cells. Following cloning and sequencing of the full-length hGR cDNA, investigators were able to determine that the cDNA sequence is approximately 9500 base pairs (bp) in length, and the genomic gene 80 kb in length (Hollenberg et al., 1985; Encio and Detera-Wadleigh, 1991). Identification of exon/intron boundaries was the next step in elucidating the structure of the hGR gene. GR was shown to be composed of 9 major exons separated by introns (**Figure 7a**; Encio and Detera-Wadleigh, 1991). The first exon of the GR gene is comprised of the first 182 bp, makes up part of the GR gene promoter, and is transcribed into a primary mRNA transcript but not into the secondary transcript, which will be ultimately translated into protein (Zhong et al, 1990; Encio and Detera-Wadleigh, 1991). Sequencing of the full GR promoter revealed up to eighteen binding sites for the activator protein SP-1, four transcription initiation sites, two nGREs, as well as AP-1 and NF-κB binding sites (Zhong et al., 1990; Govindan et al., 1991; Yudt and

Cidlowski, 2002). However, the GR promoter does not harbor either a TATA- or a CAAT-box (Zhong et al., 1990; Govindan et al., 1991; Nobukuni et al., 1995). Promoters that have multiple SP-1 binding sites, contain multiple initiation sites, and lack a TATA box are typical of housekeeping genes, examples including certain growth factors, oncogenes, cytoskeletal elements, and transcription factors (Azizkhan et al., 1993), all of which are required for basic cell structure and (or) function. Accordingly, housekeeping genes typically display ubiquitous and constitutive expression. Since GR is expressed in almost every cell within the body and mediates the activity of glucocorticoid hormones (see **Section 3A**), it is not surprising that the GR promoter fits a profile consistent with that of housekeeping genes. Indeed, the promoter elements of the GR gene indicate that GR proteins are expressed in a continuous manner in glucocorticoid-sensitive cells, consistent with their critical roles in cellular development and adaptation during stress (Bamberger, et al., 1996). The GR genes of both mouse and man have been identified to harbor multiple distinct promoters. Mouse GR contains three promoters (exons 1A, 1B, and 1C; Strahle et al., 1992), while human GR has been found to contain five untranslated exon 1 sequences (Breslin et al., 2001). These multiple promoters have been theorized to give rise to cell type-specific regulation of GR (Breslin et al., 1998), including regulation of mGR (Chen et al., 1999a,b; Yudt and Cidlowski, 2002).

Exons 2 through 9 of GR are transcribed and translated into the functional domains and subdomains of the receptor protein. The first intron of GR (intron A) is a 4 kb stretch of DNA lying 3' of exon 1 (Encio and Detera-Wadleigh, 1991) and is followed by the 1197 bp exon 2. Exon 2 contains the transcriptional start site and therefore is the first exon of the GR gene that is transcribed. Exon 2 also encodes the entire immunogenic domain, including the τ1 core and its upstream and downstream flanking regions (Giguere, et. al.,

Figure 7. Structure of the Human GR Gene, mRNAs, and Proteins.

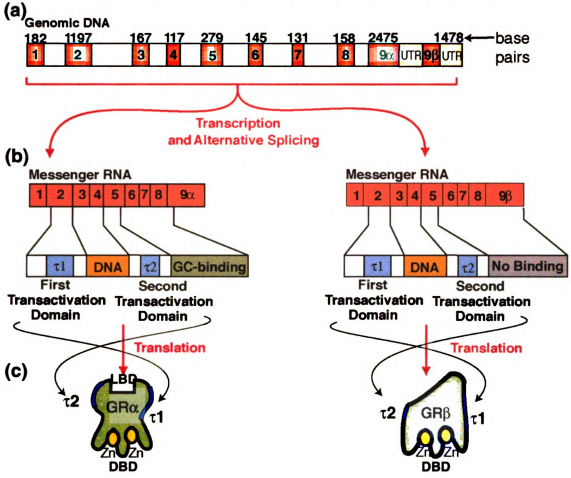


Figure 7. Structure of the Human GR Gene, mRNAs, and Proteins.

The hGR gene **(a)** is composed of 9 exons with interspersing introns. Sizes of the exons (in base pairs) are listed above each one. Exon 1 encompasses the promoter of the GR gene and is transcribed into a primary but not a secondary mRNA transcript. Exons 2 through 9 are transcribed into mRNA **(b)**, which exists primarily as one of two splice variants, GRα mRNA (encoded by exons 2 through 9α) or GRβ (encoded by exons 2 through 9β). These mRNAs are translated into two vastly different GR proteins, the GRα isoform that binds glucocorticoid and the GRβ isoform that does not **(c)**. The main functional domains are the first transactivation domain (τ1) encoded by exon 2, the zinc (Zn) finger containing DNA binding domain (DBD) encoded by exons 3 and 4, the second transactivation domain (τ2) encoded by the 5' end of exon 5, and the ligand binding domain (LBD) encoded by the 3' end of exon 5, and by exons 6, 7, 8, and 9α.

1986; Hollenberg and Evans, 1986; Encio and Detera-Wadleigh, 1991). As previously described, τ1 is the domain primarily responsible for transactivation and transrepression of cellular genes by GR in response to glucocorticoids. Following exon 2 is a 30 kb stretch of DNA called intron B. Next are exons 3 and 4 composed of 167 bp and 117 bp, respectively. A 400 bp intron C lies between exons 3 and 4. Together , exons 3 and 4 encode the DBD and its two zinc fingers, responsible for GR's ability to interact with target DNA. Between exons 4 and 5 is a large intron D, composed of a 16 kb stretch of DNA. Exon 5 is 279 bp long and encodes part of the LBD, dimerization and nuclear translocation domains, and all of τ2. Therefore, the sequence of exon 5 is partly responsible for hormone binding, receptor dimerization, receptor translocation, and transactivation of glucocorticoid-responsive genes (Tsai and O'Malley, 1994). Introns E (2 kb), F (4.6 kb), G (13.5 kb), and H (800 bp) are interspersed between exons 5 and 6, 6 and 7, 7 and 8, and 8 and 9, respectively. Exon 6 is 145 bp long and together with the 3' end of exon 5, all of exons 7 (131 bp long), and 8 (158 bp long), and the 5' end of exon 9 encodes the full LBD of GRα (Encio and Detera-Wadleigh, 1991). In total, exon 9 is comprised of almost 4000 bp and contains an alternative splice site as well as a long (3.8 kb) 3' untranslated region (UTR). The splice site enables exon 9 to give rise to the two predominant GR isoforms, α and β (Hollenberg et al., 1985). GRP remains uncharacterized but may be the result of a second splice site in or somewhere immediately 5' of exon 6.

Upon GR gene transcription, exon 1 and introns A through H are spliced out, exons 2 through 9α, 2 through 9β, or 2 through ~ 6 are ligated together (**Figure 7b**), and the various GR isoforms are translated and expressed as GR proteins in the cell (**Figure 7c**). Exon-specific encoding of GR's individual

functional domains and subdomains has facilitated studies of GR structure, function (described in **Section 3A** above) and dysfunction (see **Section F** below).

C. Human Glucocorticoid Receptor Genes Harbor Multiple Biologically Relevant Mutations

In light of the fact that GR mediates the effects of glucocorticoid hormones in target cells, receptor mutations have long been suspected as being responsible for multiple glucocorticoid-related diseases in human patients. These diseases include extreme sensitivity or resistance to glucocorticoids, and dependence on glucocorticoid therapy for survival. An early study on glucocorticoid-resistance compared GR function in glucocorticoid "resistant " guinea pigs versus glucocorticoid "sensitive" mice (Kraft et al., 1979). Results of this study revealed that guinea pig GR had a 20-fold decrease in glucocorticoid binding affinity as compared to the hormone binding affinity of mouse GR (Kraft et al., 1979). When the guinea pig GR gene was finally cloned, sequenced, and compared with human, monkey, rat, and mouse GR gene sequences, over 24 different changes were observed in the region of DNA that encodes the LBD (Keightley and Fuller, 1994). These differences translated into a dramatically altered LBD in the guinea pig, (Keightley and Fuller, 1995), and are undoubtedly responsible for the characteristic low glucocorticoid affinity of guinea pig GR compared with GR from the more sensitive species (Keightley and Fuller, 1994; 1995). Primates (Marmoset) have also been shown to harbor multiple and significant changes in the functional domains of their GR (Brandon et al., 1991).

Glucocorticoid resistance has since been studied in humans with identifiable glucocorticoid-resistance phenotypes, including hirsutism, pattern baldness, and sexual abnormalities (e.g., irregular menstruation, isosexual precocity, pseudohermaphroditism; Lamberts et al., 1986; Mendonca et al., 2002). As shown in **Table 1**, abnormalities of GR from these patients included reduced affinity for glucocorticoids (Chrousos et al., 1982; Tomita et al., 1986; Nawata et al., 1987; Lamberts et al., 1992; Kamada et al., 1994), dramatic reduction in the number of expressed cytoplasmic GR (Iida et al., 1985; Lamberts et al., 1992; Kamada et al., 1994), and altered thermolability of GR (Bronnegard et al., 1986). These abnormal properties of GR acted to significantly reduce glucocorticoid uptake by target tissues, resulting in dramatically elevated blood glucocorticoid concentrations, glucocorticoid-resistance, and disease phenotypes. By studying families displaying biochemical and/or physical glucocorticoid resistant phenotypes it was found that such resistance was inherited (Tomita et al., 1986; Linder and Thompson, 1989; Hurley et al., 1991; Lamberts et al., 1992). This finding launched numerous studies aimed at the identification of causative mutations in the GR genes of these patients. One of the first suspect domains of GR was the LBD, as many glucocorticoid-resistance abnormalities related to reduced hormone binding affinity by GR. In fact, cloning and sequencing of murine GR genes had revealed that one abnormality in glucocorticoid binding with GR was the result of a single glutamine to glycine substitution at amino acid residue 546 (Danielsen et al.,1986). Later, Linder and Thompson (1989) analyzed the DNA sequence encoding the LBD of hGR from a patient with known resistance to glucocorticoids, and identified the additional of a BglII restriction endonuclease cut site compared with corresponding DNA from healthy patients. Using computer analysis, the LBD-encoding DNA was analyzed and found to contain four bp substitutions that

explained the added BglII site and resulted in altered LBD function in the patient (Linder and Thompson, 1989). Since that study, additional mutations have been found in the LBD-encoding sequence of hGR (**Table 1**). One such DNA mutation was an A2054T point mutation that resulted in an important V641D amino acid substitution (see **Appendix B** for a table of amino acid codes) leading to co-dominant inheritance of glucocorticoid resistance (Hurley et al., 1991). Another mutation of the LBD of hGR is an A2317G nucleotide change that results in an I729V amino acid substitution and a marked decrease in GR's affinity for glucocorticoids (Malchoff et al., 1993). In other studies, a silent A/C change at asparagine residue 766 was not associated with any glucocorticoid resistance phenotypes (Koper et al., 1997), while a mutation causing an I559N amino acid change totally abolished ligand binding (Karl et al., 1996b) and a L753F residue change led to increased ligand-GR dissociation and glucocorticoid-resistance (Ashrof and Thompson, 1993; Palmer et al., 1991). Mendonca et al., (2002) have identified a V571A amino acid change within GR's LBD that results in a 10-50-fold decrease in GR trans-activation and pseudohermaphroditism, while a G679S mutation in exon 8 results in a 50% reduction in GR transactivation (Ruiz et al., 2001) and an I747M mutation results in decreased affinity of GR for hormone (Kino et al., 2002a; Vottero et al., 2002). In addition to these mutations, Karl et al. (1993) identified a 4 bp deletion at the intron-exon boundary of exon 6 in GR genes from patients with overt glucocorticoid resistance. This deletion removes a donor splice site in the affected allele, resulting in an unstable transcript and a 50% reduction in cytoplasmic GR content (Karl et al., 1993). A frameshift insertion of an 'A' at bp 2439 in exon 9, meanwhile, results in decreased num-bers of GR and is associated with Lupus Nephritis (Jiang et al., 2001). Taken together, these studies show that the LBD-encoding sequences of human GR

Table 1. Naturally-Occurring Mutations in the Ligand Binding and DNA Binding Domains of Human GR.

Human GR genes have been shown to harbor numerous mutations in exons encoding the ligand binding (LBD) and DNA (DBD) binding domains of the receptor molecules. Many of these mutations are associated with dysfunctional GR phenotypes and glucocorticoid-resistance in affected patients. A variety of other species have also been identified that harbor polymorphism in their GR genes. The left-hand columns of this table indicate the nucleotide and amino acid positions (if known) of the listed GR gene mutations. Also shown are the GR functional domains and exons effected, the resulting GR phenotypes, and the references that document these changes.

Table 1. Naturally-Occurring Mutations in the Ligand Binding and DNA Binding Domains of Human GR.

Nucleotide	Amino Acid	Domain/Exon	GR Phenotype	Reference
Multiple	Multiple	LBD	Glucocorticoid resistance in guinea pigs	Keightley and Fuller, 1994; 1995
Multiple	Multiple	LBD	Decreased hormone affinity of GR in Marmoset	Brandon et al., 1991
BglII Site	4 bp insertion	LBD	Altered glucocorticoid-binding by GR	Linder et al., 1989
Tth111I Site		Promoter	Increased blood cortisol concentrations	Rosmond et al., 2001
G1430A	R477H	DBD/Exon 4	No Phenotype	Ruiz et al., 2001
G1549C		DBD	No Association with cortisol-resistance	Koper et al., 1997
T1808A	I559N	LBD/Exon 5	Unable to bind glucocorticoids	Karl et al., 1996b
T1844C	V571A	LBD/Exon 5	Pseudohermaphroditism	Mendonca et al., 2002
G3035A	G679S	LBD/Exon 8	50% of WT[a] luciferase activity	Ruiz et al., 2001
4 bp deletion	Splice site deletion	Intron 6	50% reduction in GR numbers; hypercortisolism	Karl et al., 1993
A2054T	D641V	LBD	No *in vitro* CAT[b] activity; significantly reduced affinity for glucocorticoids	Hurley et al., 1991
C to T	D677D	LBD/Exon 8	No effect; silent	Koper et al., 1997
G2317A	V729I	LBD/Exon 9	4-fold reduction in mutant GR activity	Malchoff et al., 1993
T2373G	I747M	LBD	Decreased affinity for glucocorticoids, and GR transactivation	Kino et al., 2002a; Vottero et al., 2002
A2391T	L753F	LBD	Increased glucocorticoid-GR dissociation	Palmer et al., 1991; Ashrof and Thompson, 1993
T to C	N766N	LBD/Exon 9	No effect; silent	Koper et al., 1997
2439A	Insertion	LBD/Exon 9	Frameshift mutation; decreased numbers of GR; association with Lupus nephritis	Jiang et al., 2001

[a]WT = wild type or "normal" GR

[b]CAT = chloramphenical acetyltransferase reporter gene

genes are polymorphic, often resulting in amino acid changes that cause reduced glucocorticoid binding and (or) decreased expression of cellular GR in affected patients.

Another GR domain of interest from glucocorticoid-resistant patients is the DBD. Although DNA binding by GR is not essential for regulation of some glucocorticoid-responsive genes (Tjian and Maniatis, 1994; Tsai and O'Malley, 1994; Scheinman et al., 1995), it is necessary for GR regulation of many other genes. Mutations in this region not only influence GR's ability to interact with DNA, but also affect transactivation from τ2. Site-directed mutagenesis studies have identified numerous amino acid residues that, when mutated, abrogate GR binding to DNA and resultant transactivation (Oro, et al., 1988; Schena et al., 1989). In 1993, Zandi et al. characterized induced GR mutants (H451N/S459G) that actually caused increased DNA binding capacity. Another interesting mutation found in the rat GR DBD was a lysine to alanine switch at residue 461 (Starr et al., 1996). This change resulted in a GR τ2 variant that replaced normal AP-1 dependent transrepression with τ2 transactivation (Meyer et al., 1997). Based on these studies, patients with glucocorticoid resistance were also tested for the presence of natural mutations in the DBD-encoding region of the GR gene (**Table 1**). In one study, a G-to-C mutation at bp 1549 was identified but did not associate with glucocorticoid resistance in the patients tested (Koper et al., 1997). More recently, a G to A mutation resulting in a R477H amino acid change was identified in GR's DBD, but also was not associated with glucocorticoid-resistant phenotypes (Ruiz et al., 2001). So, to date, the bulk of mutations affecting the biological function of GR's DBD have resulted from mutagenesis studies and are not naturally occurring. Whereas naturally occurring mutations in the LBD often abrogate the activity of GR, mutagenesis of the DBD has displayed a variety of effects that include all

possible variations in transactivation and transrepression activity of τ2.

Recently, mutations have also been identified in the promoter region of hGR genes. One such mutation alters the recognition site for the BclI restriction endonuclease in the promoter region of the GR gene (Panarelli et al., 1998; **Table 2**). The precise location of this mutation is not yet known, but the mutation is statistically associated with clinical glucocorticoid resistance in the forms of abdominal obesity, insulin resistance, and elevated blood pressure (Rosmond et al., 2000). Another mutation was identified within the hGR gene promoter using the restriction enzyme, Tth111I (Rosmond et al., 2001). The subjects tested in this study had significantly increased circulating glucocorticoid concentrations but no other overt physical, endocrine, or metabolic disorders (Rosmond et al., 2001). Although the consequences of the presence of such mutations in the promoter regions of GR genes have not been fully elucidated, it is possible that they may determine the density of cytoplasmic GRs.

In summary, it is clear that numerous biologically relevant mutations exist in the promoter, DBD-encoding, and LBD-encoding regions of human GR genes. These molecular genetic variations lead to changes in the number, structure, and (or) function of mutant GRs, some of which lead to discernable health and metabolic disorders in humans. While it is quite possible that such mutations could also affect cellular gene expression during glucocorticoid challenge, the patients harboring these forms of mutant GR appear to compensate for the glucocorticoid resistance by increasing the secretion of glucocorticoids from the adrenal cortex. It is this elevation in basal levels of blood glucocorticoids that alert doctors to the presence of a glucocorticoid-resistance disease; however, effects of mutations in the GR gene promoter, LBD and DBD on gene expression per se are not clear. Because GR regulation of gene expression is primarily through the τ1 domain, this domain would be an interesting

Table 2. Naturally Occurring Mutations in the First Transactivation Domain of Human GR.

Several naturally occurring mutations have been identified in exon 2 of primate GR genes, the exon that encodes the first transactivation domain (τ1) of the GR protein. However, most of these mutations have not been associated with significant changes in GR phenotype or human health. The left-hand columns of this table indicate the nucleotide and amino acid positions of the listed mutations. Also shown are the GR functional domain and exons effected, the resulting GR phenotypes, and the references that document these changes.

Table 2. Naturally-Occurring Mutations in the First Transactivation Domain of Human GR.

Nucleotide	Amino Acid	Domain/Exon	GR Phenotype	Reference
Multiple	Multiple	τ1/Exon 2	Structure changes in Marmoset	Brandon et al., 1991
BclI site		Intron 1/Exon 2	Associated with hyperinsulinemia in obese women	Weaver et al., 1992
BclI site		Intron 1/Exon 2	Associated with increased abdominal fat	Buemann et al., 1997
BclI site		Intron 1/Exon 2	Associated with vasoconstriction; varied numbers of GR	Panarelli et al., 1998
G198A	E22E	τ1/Exon 2	No detectable phenotype; silent	Feng et al., 2000; Koper et al., 1997
G200A	R23K	τ1/Exon 2	No detectable phenotype	de Lange et al., 1997; Koper et al., 1997
C219G	F29L	τ1/Exon 2	No detectable phenotype	Encio and Detera-Wadleigh et al., 1991
C466T	L112F	τ1/Exon 2	No detectable phenotype	IBID
G829A	D233N	τ1/Exon 2	No detectable phenotype	IBID
G1011A	K293K	τ1/Exon 2	No Phenotype; silent	Feng et al., 2000
1189T	Insertion	τ1/Exon 2	Truncated GR protein	Karl et al., 1996a
G1220A	N363S	τ1/Exon 2	No detectable phenotype	Karl et al., 1993
G1220A	N363S	τ1/Exon 2	May be associated with increased sensitivity to glucocorticoids	Huizenga et al., 1998
G1220A	N363S	τ1/Exon 2	No association with adrenal androgen excess	Kahsar-Miller et al., 2000
G1220A	N363S	τ1/Exon 2	Associated with obesity and glucocorticoid-sensitivity	Rosmond et al., 2001
G1220A	N353S	τ1/Exon 2	Associated with trunkal obesity	Dobson et al., 2001

region to study biologically relevant mutations that cause changes in gene expression and thus phenotypic changes during stress responses of humans and other species. Studies outlined in the subsequent sections and in **Table 2** indicate that the τ1 domain of hGR is indeed polymorphic.

D. Mutations in the τ1-Encoding Region of Human GR Genes

Transactivation domains enhance one or more rate-limiting step(s) in the assembly of the preinitiation complex by interacting directly or indirectly with transcription factors or co-factors. The first transactivation domain of GR is composed of 185 amino acids (between residues 77 and 262 of hGR cDNA) with a core 41 amino acid region necessary for transactivation or transrepression activity (Dahlman-Wright et al., 1994). GR τ1 interacts with members of the basal transcriptional machinery, as well as numerous transcriptional co-factor (see **Section D*iii***, above). For this reason the importance of GR's τ1 is its involvement in every aspect of target gene regulation by glucocorticoid hormones.

Naturally occurring and site-directed mutants of human GR τ1 have been identified that display varying effects on transcriptional regulation by GR. Naturally occurring GR τ1 mutants that have been identified all stem from point mutations harbored in exon 2 of human GR genes (Karl et al., 1993; Koper et al., 1997; Huizenga et al., 1998; etc. **Table 2**). Two such mutations with a small separation between them were identified in the N-terminal region of τ1 (Koper et al., 1997). The first G to A change at bp 198 was silent (E22E) while the second G to A mutation at bp 200 changed an arginine residue to a lysine (R23K; Koper et al., 1997). Other mutations have been identified that changed amino acid sequence but led to no discernable changes in phenotype (**Table 2**). An additional mutation was identified as a G to A change at bp 1220

in exon 2 of hGR genes, resulting in an asparagine to serine change at amino acid residue 363 (N363S; Karl et al., 1993). None of these mutations were found to be associated with phenotypes characteristic of glucocorticoid-resistance (Koper et al., 1997). However, the N363S mutation was further investigated for possible association with cellular sensitivity to glucocorticoids (Huizenga et al., 1998). Changes in insulin and cortisol levels following glucocorticoid treatment suggested that patients harboring the N363S mutation tended toward increased glucocorticoid sensitivity, but values for these variables were not significantly different from normal patients with "wild type" τ1. Additionally, there was no direct association between the mutant GR τ1 domains and degree of transactivation of a reporter gene *in vitro* (Huizenga et al., 1998). More recently, several studies have analyzed the N363S mutation for possible associations with adrenal androgen excess (Kahsar-Miller et al., 2000), glucocorticoid-sensitivity (Rosmond et al., 2001), and central obesity as a factor of coronary disease (Dobson et al., 2001). Only central obesity in a group of Dutch men was associated in any way with this mutant form of τ1 (Dobson et al., 2001). This work on the N363S GR mutant suggests that τ1 may be able to tolerate multiple mutations without dramatically altering its transactivation activity. Certainly, the role of GR as the key regulator of glucocorticoid-responsive gene expression would suggest that τ1 be necessarily tolerant to mutations. However, site directed mutagenesis studies do not support this finding. In marked contrast to the naturally occurring mutations so far identified in hGR τ1, site directed mutagenesis of τ1 has identified important residues that effect significant changes in the transactivation activity of this domain (**Table 3**). Most of these studies have focused on the τ1 core region. In the first of such studies, mutagenesis of four proline residues, or combination of residues (at amino acid positions 194, 197, 220, 223), reduced the

transactivation potential of τ1 between 50 and 80% compared to wild type τ1 (Dahlman-Wright and McEwan, 1996; **Table 3**). These residues were selected for study because they flank the putative α-helices of τ1, structures which may be partially responsible for τ1 interactions with other proteins. As expected, mutagenesis affected the stability and function of the τ1 core. In a subsequent study, Almlof et al. (1997) mutated nearly every amino acid in the τ1 core in order to identify residues critical for τ1 function. The core was divided into four regions for site-directed mutagenesis; helices 1, 2, 3, and a loop region. Mutations that resulted in reductions of helix 1 activity were found primarily at three hydrophobic amino acid residues; F191, I193, and D196 (see **Appendix B** for classification of amino acid residues). Replacement of these hydrophobic residues with either alanine or an acidic residue resulted in up to 80% reduction in the transactivation of a reporter gene. Mutation of the two residues, I193 and D196, displayed increases in transactivation of up to 151% (Almlof et al., 1997). Within the putative loop region of τ1, the amino acid residue W213 displayed the most dramatic reduction of 70%, but substitutions with other hydrophobic residues (W213F and W213Y) restored transcriptional ability. Single substitutions within helix 2 affected transcription to a mild degree, with multiple substitutions of both L224 and L225 showing the most significant down-regulation of transcription (64-72%). One cysteine residue (C223G,R) also showed rather significant reductions in transactivation upon mutagenesis, causing speculation that this residue may be involved in formation of disulfide bridges. Double mutations in helix 3 displayed the greatest reductions in transcriptional activity (60-89%). The exception to this finding was amino acid L236, which displayed 83% reduction in activity when it was the only mutated amino acid in helix 3 (Almlof et al., 1997). The effect of this single mutation is interesting in light of the fact that GR τ1 has been shown to

Table 3. Site-Directed Mutagenesis of GR's First Transactivation Domain Reveals Key Amino Acid Residues Required for Transcriptional Regulation by the Receptor Molecule.

Site-directed mutation of residues within the first transactivation domain (τ1) region of GR, including in the τ1 core, resulted in several significant changes to GR transactivation activity. Summarizing across the table, mutations to multiple amino acid residues were required to substantially disrupt τ1 activity, with the exception of residues 196, 213, and 236, which when mutated on their own resulted in low τ1 activity. Additionally, mutations to the synergy control motif (SCM) resulted in significant increases in τ1 activity. The left-hand column describes the amino acid targeted for mutation and the actual mutation induced. The next column lists the putative structures disrupted by the mutations. The middle column indicates the transactivation activity of affected GR molecules as percentages of wild type (WT) GR activity. The right hand column lists the references that documented these phenomena.

†Amino acid residue numbers are based on human τ1 in Dahlman-Wright and McEwan, 1996 and Almlof et al., 1997, and rat τ1 for Iniguez-Lluhi et al., 1997 and Iniguez-Lluhi and Pearce, 2000.

‡SCM is the synergy control motif

Table 3. Site-Directed Mutagenesis of GR's First Transactivation Domain Reveals Key Amino Acid Residues Required for Transcriptional Regulation by the Receptor Molecule.

Amino Acid Mutated†	Region of τ1 Mutated	Transactivation of a Reporter Gene as Compared to WT GR	Reference
L194P & L197P	Helix 1	20%	Dahlman-Wright and McEwan, 1996
I220P & N223P	Helix 2	50%	IBID
L194P, L197P, I220P, N223P	Helix 1 & 2	15%	IBID
F191E or F191D	Helix 1	29%	Almlof et al., 1997
I193F	Helix 1	151%	IBID
D196Y	Helix 1	28%	IBID
W213R	Loop	30%	IBID
W213Y	Loop	117%	IBID
E221F	Helix 2	288%	IBID
L225F	Helix 2	174%	IBID
L224V & L225V	Helix 2	28%	IBID
F235L & L236V	Helix 3	11%	IBID
F235V & L236I	Helix 3	30%	IBID
L236V	Helix 3	17%	IBID
E238K & N240D	Helix 3	40%	IBID
G239R & N240D	Helix 3	24%	IBID
E219K	Helix 2	120%	Iniguez-Lluhi, et al., 1997
F220L	Helix 2	95%	IBID
W234R	Helix 3	80%	IBID
E219K & F220L	Helix 2	40%	IBID
E219K & W234R	Helix 2 & 3	50%	IBID
F220L & W234R	Helix 2 & 3	40%	IBID
K298E	SCM‡	6-fold Increase	Iniguez-Lluhi and Pearce, 2000
K313E	SCM	5-fold Increase	IBID
K298E & K313E	SCM	12-fold Increase	IBID

tolerate single mutations much better than multiple mutations (Iniguez-Lluhi, et al., 1997). In fact, it has been theorized that τ1 has pleomorphic activation "surfaces", which interact directly with transcriptional co-factors to regulate transcription. The Almlof study above indicated that outside of helix 1, single point mutations in τ1 are not as important to transcriptional activity as multiple mutations. The "surface" theory combined with the unstructured nature of τ1 at neutrophil pH would explain why it is able to tolerate single mutations much better than multiple mutations. Moreover, helix 1 of the τ1 core is most likely located at the surface of the receptor protein and may be accessible for interactions with target proteins (McEwan et al., 1994). If true, mutations in helix 1 would be expected to have a significant impact on τ1 function. One amino acid within the putative loop structure, W213, appears to be critical for this interaction surface and would explain the dramatic effect when mutated (Almlof et al., 1997).

In another mutagenesis study, the full τ1 region of rat GR (rGR) was used for mutagenesis work (Iniguez-Lluhi et al., 1997). In rGR, the τ1 region is located between amino acid residues 108 and 317 and, like hGR, is comprised of a large percentage of acidic residues (Tasset et al., 1990). In the Iniguez-Lluhi et al (1997) study, rGR τ1 domains harboring multiple mutations (rather than point mutations) were generated and then fused to the DBD and LBD of rGR and expressed in mammalian cells along with a luciferase reporter gene. No specific cluster of mutations in the τ1 region regulating luciferase gene activity were generated, but an inverse relationship was observed between overall transcriptional activity of mutant τ1s and the number of amino acids changed (Iniguez-Lluhi et al., 1997). The more residues mutated in rGR τ1, the greater the reduction in luciferase gene transcription. Several strongly affected mutants had an altered tryptophan (W234) residue in common (**Table**

3). Interestingly, mutation of a tryptophan residue (W213) in a homologous region of hGR was also found to significantly affect transcription of the luciferase reporter gene (Almlof et al., 1997). Both studies would indicate that this tryptophan residue is of particular importance to transcriptional regulation mediated by GR τ1. This work also indicates that individual amino acid mutations within τ1 may have little effect on transcription regulation of target genes by GR, whereas clusters of two or three critical mutations may cause dramatic down-regulation of transcriptional activity. This finding argues in favor of GR τ1 possessing surfaces that function to regulate gene activation or repression, where multiple rather than one or two residues makes up a contact area for target co-factors. This notion of contact surfaces in τ1 would also be in line with the known flexibility of this domain (described in **Section D*iii***), and implies that τ1 selectively exposes or uses different regulatory surfaces depending on the context of the specific gene promoter affected and the cellular proteins involved in GR's regulation of the gene. If true, it is easy to envision that single mutations in τ1 are better tolerated than clusters of mutations, which may modify an entire protein-binding surface of the domain. In a subsequent study of rGR, Iniguez-Lluhi and Pearce (2000) identified a specific motif within the rGR τ1 region which, when mutated, dramatically increased the transactivation of τ1. This motif, determined as (I/V)KXE, where either an isoleucine or valine is followed by a lysine, any amino acid residue, and a glutamic acid residue, appears twice within a 25 amino acid stretch and is highly conserved across the hormone receptor superfamily (Iniguez-Lluhi and Pearce, 2000). Mutation of the first lysine (to glutamic acid) resulted in a 6-fold increase in rGR τ1 activity (**Table 3**), while the mutation of only the second conserved lysine (within 25 amino acids) resulted in a 5-fold increase in τ1 activity and mutation of both lysines resulted in a 12-fold increase in rGR τ1 transactivation of a

reporter gene (**Table 3**). Due to the apparent synergistic increase in rGR activity, they called this motif [(I/V)KXE-X_{12}-(I/V)KXE; where X represents any amino acid residue] the synergy control motif (SCM) and hypothesized that this motif is responsible for controlling GR from run-away transactivation of target genes. Additional studies are required to investigate the possibility that naturally occurring mutation clusters exist in the τ1 region, including the SCM, of GR. This possibility is supported by the fact that τ1 lies within the larger and variable immunogenic domain of the GR molecule (Okret et al., 1982; Gametchu and Harrison, 1984; Harmon et al., 1984; Hollenberg et al., 1985).

Transcriptional regulation of glucocorticoid-responsive genes by GR is of particular interest in the bovine species due to the deleterious effects of stress-related increases in blood glucocorticoid concentrations on animal health and productivity (Filion et al., 1984; Mitchell et al., 1988; Nanda et al., 1990; Minton et al., 1994; Burton et al., 1995; Grandin, 1997; Kehrli et al., 1999; Preisler et al., 2000a,b; Weber et al., 2001; Burton et al., 2001). The glucocorticoid surge at parturition has been documented to induce immunosuppression, which is heritable (Detilleux et al., 1994). Additionally, bulls challenged with exogenous glucocorticoids display significant additive genetic variation, and modest to high heritability estimates, in a variety of immune traits (**Table 4**) including several neutrophil functions, lymphocyte blastogenesis traits, and the percentages of circulating T cell subsets (Tempelman et al., 2002; Abdel-Azim et al., in review; Burton et al., in review; Kelm et al., in review). Identification of biologically-relevant mutations within τ1 of bovine GR would not only allow for a better understanding of variation in the regulation of animal sensitivity to stress in high producing cattle, but would also provide important information to aid future biomedical studies on stress-related disease susceptibility. This was the goal of the current dissertation research.

Table 4. Selected Immune Traits and Their Heritability Estimates In Holstein Bulls Challenged with Exogenous Glucocorticoid.

Significant additive genetic variation has been observed in numerous immune traits of Holstein bulls following exogenous glucocorticoid challenge. Many of these traits harbor modest to significant heritability estimates. The left-hand column lists immune traits displaying significant genetic variation, with the remaining columns listing the range of heritability estimates and the references which documented the heritable trait.

Table 4. Selected Immune Traits and Their Heritability Estimates in Holstein Bulls Challenged with Exogenous Glucocorticoid.

Immune Trait Displaying Genetic Variation	Heritability Estimate	Reference
Percentage of circulating B cells	0.138 to 0.203‡	Burton et al., in review
Percentage of circulating eosinophils	0.10 to 0.58†	Kelm et al., in review
Percentage of circulating T cells that express the CD4 accessory molecule	0.23 to 0.391‡	Burton et al., in review
Percentage of circulating T cells that express the CD8 accessory molecule	0.06 to 0.391‡	Burton et al., in review
Percentage of circulating T cells expressing the γδ T cell receptor	0.272 to 0.628‡	Burton et al., in review
Percentage of mononuclear cells expressing MHC II	0.22 to 0.308	Burton et al., in review
Expression of MHC I on mononuclear cells	0.221 to 0.287	Burton et al., in review
Expression of MHC II on mononuclear cells	0.21 to 0.561‡	Burton et al., in review
Spontaneous lymphocyte blastogenesis	0.40 to 0.56†	Kelm et al., in review
Lymphocyte blastogenesis following stimulation with conconavalin A	0.10 to 0.45†	Kelm et al., in review
Expression of CD18 on circulating neutrophils	0.10 to 0.56‡	Tempelman et al., 2002
Random migration of neutrophils	0.30 to 0.32†	Kelm et al., in review
Antibody-dependent neutrophil killing	0.31 to 0.47†	Kelm et al., in review
Neutrophil oxidative metabolism and oxidation of membranes	0.13 to 0.52†	Kelm et al., in review
Neutrophil generation of superoxide anions	0.14 to 0.37†	Kelm et al., in review
Neutrophil myeloperoxidase-catalyzed activity	0.24 to 0.29†	Kelm et al., in review
Neutrophil phagocytosis of *Staphylococcus aureus*	0.18 to 0.39†	Kelm et al., in review

‡Heritability estimate is dependent on day relative to glucocorticoid administration

†Heritability estimate is dependent on week relative to glucocorticoid administration

CHAPTER 4. IDENTIFICATION OF MUTATIONS IN THE τ1-ENCODING REGION OF BOVINE GR GENES

A. Introduction

Glucocorticoids have far-reaching effects in animals that include the suppression of inflammation (Almawi et al., 1996; Barnes 1998; Newton 2000), induction of gluconeogenesis (Cole et al., 1995; Hanson and Reshef, 1997), modulation of blood pressure (Whitworth et al., 2001; Mitchell and Webb, 2002), and promotion of cellular differentiation and development of various organs and tissues (Cole et al., 1993; Brewer et al., 2002), and regulation of behavior (Takahasi, 1996; Korte 2001). The end result of a glucocorticoid challenge is regulation of hormone-responsive gene expression in target cells (Bamberger et al., 1996). The receptor for glucocorticoids, GR, is composed of four main functional domains. These are the ligand binding (LBD), DNA binding (DBD), first transactivation (τ1), and second transactivation (τ2) domains (Hollenberg et al., 1985; Giguere et al., 1986). In particular, τ1 plays a critical and direct role in regulation of gene expression by recruiting basal transcriptional machinery and other important regulatory proteins to the promoters of glucocorticoid-responsive genes (Ford et al., 1997; Hittelman et al., 1999; McEwan 2000). GR uses numerous direct (DNA-binding) and indirect (protein-protein interaction) mechanisms to effect activation or repression of target genes in glucocorticoid-responsive cells. Thus, the τ1 domain plays a central role in GR's regulation of gene expression and is a good candidate for polymorphism studies.

As with other members of the steroid receptor superfamily, GR's individual functional domains are transcribed from specific exons. GR's τ1 is encoded by the second exon, called the GR2 locus. In humans, τ1-encoding GR2 has

been shown to be polymorphic (de Lange et al., 1997; Koper et al., 1997; Huizenga et al., 1998; Dobson et al., 2001; see **Table 3**). Additionally, specific mutations in the GR2 locus of human and rat have been studied for transcriptional regulatory effects using mutagenesis. These studies have demonstrated that multiple mutations in a small region of GR2 have a greater effect on GR transactivation activity than any single mutation (Almlof et al., 1997), raising the possibility that there are functional surfaces in $\tau 1$ that are disrupted by mutagenesis of two or more amino acid residues (Iniguez-Lluhi et al., 1997). Amino acid changing mutations within GR2 could have far-reaching effects on glucocorticoid-responsive gene expression. In fact, additive genetic variation has been identified in bovine leukocyte traits known to be sensitive to glucocorticoids (Tempelman et al., 2002; Abdel-Azim et al., in review; Burton et al., in review, Kelm et al., in review; **Table 4**), indicating that the manner in which leukocytes respond to glucocorticoids is inherited in cattle. These variable sensitivities to glucocorticoid hormones are likely due to polymorphism in numerous pathway genes, including GR itself. If true, the study of polymorphism in $\tau 1$-encoding GR2 would be logical.

The hypothesis of this study was that the bovine GR2 locus is polymorphic. The objectives were to isolate genomic DNA from six cattle breeds, amplify the GR2 locus from genomic DNA, identify the presence of mutations within GR2, characterize corresponding amino acid changes, and predict if the identified mutations may effect the tertiary protein structure of the $\tau 1$ region of bovine GR.

B. Materials and Methods

i. Genomic DNA from Six Cattle Breeds.

Whole blood was collected from 50 Angus, 40 Polled Hereford, and 140 Holstein cattle from the Michigan State University Beef and Dairy Research and Teaching farms (**Appendix C**). Whole blood was also collected from 62 Brown Swiss cattle (Mashek Farm, Iowa), semen samples for 6 Brahman were generously donated by Dr. Melvin Pagan (Puerto Rico), and DNAs were obtained from semen of 10 Jersey bulls (generously donated by NorthStar Select-Sire, Lansing, MI; **Appendix C**). Genomic DNA was isolated from either leukocytes or sperm from these samples.

a. Isolation of Genomic DNA from blood.

Whole blood was drawn into 6 ml vacutainers containing the anti-coagulant acid-citrate dextrose. Blood was transferred to sterile 50 ml conical tubes (Fisher Scientific, Hanover Park, IL) and erythrocytes lysed by addition of a 0.5 x volume of cold sterile hypotonic lysis solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, at pH 7.3) and gentle inversion for 1.5 minutes. Then a 2 x volume of sterile ice-cold hypertonic solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, 0.43 M NaCl, at pH 7.3) was added to restore isotonicity. The mixture was then centrifuged at 1100 x g at room temperature (RT) for 5 minutes and the supernatant discarded. The remaining leukocyte pellet was washed with 6 ml of sterile phosphate buffered saline (PBS; 0.13 M NaCl, 2.68 mM KCl, 4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, at pH 7.4), followed by centrifugation at 1100 x g at RT for 5 minutes and one wash with 3 ml of sterile PBS. After centrifugation (as previously described), the supernatant was discarded and the leukocyte pellet flash frozen at –80°C for 5 minutes to lyse the cells. Cells were thawed on ice for 15 minutes and refrozen at –80°C for an additional 5 minutes to ensure cell lysis. Following thaw, 25 µl of 10% dodecyl sodium

sulfate (SDS), 5 µl of 20 mg/ml Proteinase K (Roche, Indianapolis, IN), and 400 µl of proteinase K buffer (50 mM Kcl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.5% Tween 20) were added to each sample to finish cell lysis and degrade proteins, and incubated at 55°C for 2 hours. The Proteinase K enzyme was then heat inactivated at 94°C for 10 minutes followed by incubation on ice for 1.5 hours. Next, 1.5 ml of sterile PBS was added, the samples vortexed, and centrifuged at 2500 x g for 10 minutes at RT. The supernatants containing genomic DNA were then removed into sterile 15 ml polypropylene tubes (Fisher Scientific, Hanover Park, IL) and DNA precipitated by the addition of 300 µl of 3 M Na acetate (pH 5.2) and 4 ml of ice-cold 100% ethanol. Precipitated DNA was transferred to sterile 1.5 ml microfuge tubes and micro-centrifuged at 15,000 x g for 10 minutes at 4°C. Supernatants were then decanted and the DNA pellet washed by the addition of 1 ml of ice-cold 70% ethanol. The ethanol was decanted and the DNA pellet dried resuspended in milli-Q water to 100 ng/µl until use in the polymerase chain reaction (PCR).

b. Isolation of Genomic DNA from semen.

Semen straws were stored at –80°C until use for isolation of genomic DNA. At that time, semen was thawed and dispensed into a series of sterile 50 ml polypropylene conical tubes (Fisher; Hanover Park, IL), after which 350 µl of 30% STE [0.1M NaCl, 0.05M Tris base, 1 mM ethylenediaminetetraacetic acid (EDTA)], 55 µl of 20% SDS, 25 µl of 4.3% 1M di-thiothreotal (DTT; Sigma, St. Louis, MO), and 150 µl of 26% Proteinase K (20 mg/ml; Roche, Indianapolis, IN) were added. The mixture was vortexed, allowed to incubate at 60°C for 1 hour, and then twice the volume of Tris base-EDTA was added and mixed thoroughly. Twice the volume of phenol and chloroform (1:1 ratio) was then added and mixed gently at RT for 10 minutes. The solution was centrifuged at 2,000 x g for 10 minutes at RT and the aqueous layers containing

genomic DNA transferred to new sterile tubes. Equal volumes of a solution containing chloroform:isoamyl alcohol (24:1 ratio) were then added to the aqueous layer and mixed for 10 minutes at RT. Tubes were centrifuged for 10 minutes at 2,000 x g at RT and the aqueous layers again removed and placed into sterile 15 ml conical tubes. DNA was precipitated by the addition of $1/10^{th}$ the volume of 3 M sodium acetate (pH 5.2) and 2 x volumes of ice-cold 100% ethanol and the tubes rotated gently until DNA precipitates appeared. The DNA was removed into sterile 1.5 ml microfuge tubes, washed once with ice-cold 70% ethanol, suspended to 200 ng/µl in sterile milli-Q water, and stored at 4°C until use in PCR.

ii. PCR Amplification of GR2 Loci.

GR2 loci were amplified from genomic DNA samples using PCR. For efficiency of GR2 polymorphism screening, a series of 200 ng/µl aliquots of DNA for 8 animals per aliquot from 5 of the 6 breeds (discluding Brahman) were pooled and the pooled samples PCR-amplified. Primers for the PCR reaction were designed based on DNA sequence for hGR exon 2 and were:

Forward primer 5'-GGGACTGTATATGGGAGAGAC-3'

Reverse primer 5'-GGACTTTGAACTTCTCTGCTCGATC-3'

These primers encompassed the τ1 core-encoding domain and most of its 5' and 3' flanking DNA in hGR exon 2, with an anticipated PCR amplicon size of 903 bp. Bovine GR2 was then amplified using the pooled genomic DNA samples previously described. To perform PCR, 200 ng of genomic DNA/pooled sample was combined with a PCR master mix containing 10 mM of deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP; Invitrogen, Carlsbad, CA), 60 mM of Tris-SO_4 (pH 9.1), 18 mM of $(NH_4)_2SO_4$, and1.2 mM Mg_2SO_4. Then, 12.5 mM each of the forward and reverse primers and 2 µl of ELONGASE Enzyme mix [20 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 1

mM DTT, stabilizers, 50% (v/v) glycerol; Invitrogen, Carlsbad, CA] were added to the reaction mixtures for total PCR reaction volumes per sample of 50 µl. Ingredients were combined in sterile polypropylene PCR tubes and heated at 94°C for 2 minutes to totally denature the genomic DNA, followed by 25 cycles of 94°C for 1 minute (denaturing), 56°C for 2 minutes (annealing), and 72°C for 1 minute (elongation), using a Robocycler PCR machine (Stratagene, La Jolla, CA). A final 72°C elongation step was applied for 10 minutes. Aliquots (~5 µl) of the resulting PCR amplicons were then checked for size and single bands on 1.2% agarose checking gels with ethidium bromide staining and the remaining sample stored in milli-Q water at 4°C until GR2 polymorphism screening was performed.

iii. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) Screening of GR2 Amplicons.

The PCR-RFLP technique was used along with 11 restriction endonucleases [AluI, BfaI, BstUI, DpnII, HaeIII, HhaI, MseI, MspI, NlaIII, RsaI, TaqaI (all from New England Biolabs, Beverly, MA)] for preliminary screening of GR2 polymorphism. In this protocol, 2.5 µl of PCR amplicons/sample were combined with 1 U of the respective restriction endonucleases and appropriate buffers that were provided with the enzymes. Reactions were allowed to incubate at 37°C for 12 hours and were then resolved on 2.5/4% stacked, nondenaturing polyacrylamide gels. For polyacrylamide gel electrophoresis (PAGE), a 4% gel solution [80% milli-Q water, 10% 10 x tris-boric acid-EDTA (TBE), 10% of a 40% (19:1) acrylamide:acrylamide bis solution; Bio-Rad, Hercules, CA] was mixed and placed under vacuum for 20 minutes (degassing), during which time the Mini-Protein II electrophoresis chamber (Bio-Rad, Hercules, CA) was assembled. Following degassing, 0.10 x of 10% ammonium persulfate and 0.015 x of N,N,N',N'-Tetramethyl-1,2-Ethanediamine (TEMED; Bio-

Rad; Hercules, CA) were added to the gel solution, which was rapidly pipetted into the Mini-Protein II gel rigs. The unpolymerized gels were covered with water and allowed to polymerize for 40 minutes at RT, after which time the water was removed and a second, 2.5% stacking gel [84% milli-Q water, 10% 10 x TBE, 6% of a 40% (19:1) acrylamide:acrylamide bis solution; Bio-Rad, Hercules, CA] was added. Gels were allowed to polymerize for an additional 40 minutes at RT. They were then covered with 1 x TBE and subjected to electrophoresis at 4°C. The digested GR2 amplicons were then combined with 4 µl of sample loading buffer and pipetted into individual lanes of the gels. The Amplisize molecular mass ruler (Bio-Rad, Hercules, CA) was added to one lane of each gel. Gels were electrophoresed at 70 volts for 4 hours at 4°C and PCR-RFLP banding patterns visualized using ethidium bromide staining and photographed on a UV light source (GelDoc 2000; Bio-Rad, Hercules, CA).

For partial confirmation of differences in pooled samples revealed by PCR-RFLP, DNAs from individual animals making up the DNA pools were PCR amplified in the GR2 locus and digested for PCR-RFLP analysis as described above. The individual samples were amplified using the protocol outlined in **Section *II*** above, digested using the restriction endonuclease(s) that revealed clear differences in the pooled DNA samples across or within breed, and analyzed by electrophoresis and ethidium bromide staining for differences in banding patterns (as described above).

Iv. Molecular Cloning of GR2 Genes.

To elucidate the precise mutations contributing to GR2 polymorphism, GR2 amplicons from five animals of each breed (including Brahman) were cloned and the DNA inserts sequenced. To do this, individual GR2 amplicons were purified using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) and suspended in milli-Q water to a final concentration of 200

ng/µl. These GR2 amplicons were then combined with 2 x of T4 DNA ligase buffer, 50 ng of pGEM-T Easy vector, and 3 U of T4 DNA ligase (all from Promega, Madison, WI) for molecular cloning of individual GR2 alleles. Ligation reactions were incubated overnight at 4°C and aliquots (2 µl) combined with 50 µl of 1 x 10^8 cfu/mg high efficiency competent JM109 *Escherichia coli* cells, per the manufacturers protocol (Promega, Madison, WI). Bacterial suspensions were incubated at 4°C for 20 minutes, heat shocked at 42°C for 45 seconds, and cold shocked (4°C) for 2 minutes. Room temperature SOC media (2.0 g tryptone, 0.5 g yeast extract, 1ml 1M NaCl, 0.25 ml 1M KCl, 0.5 ml 1M $MgCl_2$-$6H_2O$, 0.5 ml 1M $MgSO_4$-$7H_2O$, 2 ml 1M glucose at pH 7.0) was added for a total volume of 1 ml. Bacteria were then incubated at 37°C for 60 minutes and 50 µl of the cell suspension spread on Luria agar plates containing 100 mg/ml of ampicillin, 50 mg/ml of X-gal dissolved in 5-bromo-4-chloro-3-indolyl-b-D-galactoside, and 0.1M of isopropylthiogalactoside (IPTG, Sigma, St. Louis, MO). Plates were incubated at 37°C for 12-16 hours and a blue-white colony screening conducted. Where possible, nine white colonies per animal were individually picked and grown in 5 ml of Luria broth for an additional 12-16 hours. Where 9 colonies could not be identified, as many white colonies as were available were picked and grown as described. Aliquots of each colony were used to verify the presence of plasmid containing the inserted GR2 DNA (GR2 insert). To do this, one microliter of cells for each clone was combined with the EcoRI restriction endonuclease and its buffer (Invitrogen, Carlsbad, CA) and incubated at 37°C for 1 hour to release the cloned GR2 inserts, which were visualized on 1.2% agarose checking gels using ethidium bromide staining.

v. DNA Sequence analysis of Cloned GR2 Inserts.

DNA sequencing of cloned GR2 alleles for each animal was performed on 3 sets of isolated plasmids, two that were purified from two separate clones per animal (and thus representing individual GR2 alleles) and one from the pool of 9 plasmids per animal (thus representing both GR2 alleles for elucidation of heterozygotes). The DNA sequencing reactions were prepared using 200 ng of plasmid DNA and the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits (with AmpliTaq DNA polymerase; Applied Biosystem, Foster City, CA), for 15 cycles of 96°C for 10 seconds, 55°C for 5 seconds, 70°C for 60 seconds. This was followed by 15 cycles at 96°C for 10 seconds, and 70°C for 60 seconds. Sequencing reactions were concentrated by the addition of 0.1 x of 3M Na-acetate (pH 5.2) and 2 x of RT 95% ethanol, incubated at 4°C for 15 minutes, centrifuged at 4°C and 15,000 x g for 20 minutes, rinsed with 1 x of RT 70% ethanol, and the labeled DNA dried in a speed-vacuum (Savant Speed Vac Plus, ThermoSavant, Holbrook, NY).

The sequencing reactions were then electrophoresed on 4.75% denaturing polyacrylamide gels (40% 19:1 acrylamide:acrylamide bis, sterile water, urea) with the addition of 10 x TBE, 0.10 x of 10% ammonium persulfate, and 0.015 x of TEMED (Bio-Rad; Hercules, CA). Gels were allowed to polymerize for 2 hours, at which point 4 µl of sequencing loading buffer [1:5 ratio of blue dextran and EDTA in sterile water with AmberLite resins (Roche, Indianapolis, IN) in formamide] was added to the samples. Samples were vortexed and heat-denatured at 90°C for 3 minutes. Sample mixtures were immediately chilled on ice and loaded into individual lanes of the sequencing gel. All cloned GR2 inserts in the individual plasmids and pooled plasmids were sequenced in both the forward and reverse directions, using forward and reverse M13 and M13 primers (Applied Biosystem, Foster City, CA). Forward and reverse sequenc-

ing typically yielded 500 to 550 bp of usable GR2 sequence, with 100 to 150 bp of overlapping sequence for later alignment of full GR2s.

vi. Analysis of GR2 Mutations at the DNA and Predicted Amino Acid Sequence Levels.

DNA sequences were analyzed using the Genetics Computer Group (GCG; Madison, WI) software. Following reformatting ('chopup' and 'reformat' commands) of the forward and reverse sequences, they were joined using the 'assemble' command. Multiple alignment files were generated using the 'pile-up' and 'pretty' commands for identification of polymorphism between alleles and a consensus sequence, which was the sequence of nucleotides that occurred with the greatest frequency across the population of test animals. Multiple sequence alignment files for DNA and predicted amino acid sequences were imported into an Excel spreadsheet (Microsoft Office v. x, Redmond, WA) for generation of graphs depicting position and frequency of identified mutations.

vii. Visualization of GR2 Mutations Using Three-Dimensional Protein Modeling.

Following translation of DNA sequences into predicted amino acid sequences, the most divergent predicted protein sequences from the Holstein breed were submitted to the FOLD-DOE server at UCLA (http://fold.doe-mbi.ucla.edu). Predictions of α-helix, β-pleated sheet, loop, and turn structures were thus obtained. The FOLD-DOE server was then used in conjunction with Insight II modeling software (Molecular Simulations Incorporated; San Diego, CA) to homology model the GR protein structures for divergent alleles from each breed. A crystallized protein structure from chrysanthemum (pectate lyase) with close secondary protein structural homology to GR2 was identified by the FOLD-DOE server for homology modeling. This protein has been

identified by other researchers as having a secondary protein structure closely related to that of GR2 (Iniguez-Lluhi, personal communication). The amino acids of pectate lyase were changed to reflect the residue sequence of GR2, for the selected alleles, using Insight II software for visualization as a three-dimensional protein model. The GR2 models were then compared for structural differences resulting from GR2 amino acid changing polymorphism.

C. Results

Images in this dissertation are presented in color

i. PCR Amplification of Bovine GR2 and PCR-RFLP Analysis.

Due to the fact that the PCR primers used to amplify bovine GR2 DNA were designed from human sequence (**Figure 8a**), there was no guarantee that bovine GR2 could be amplified. However, the primers produced single bands in all animals across the breeds (**Figure 8b**) and, upon DNA sequence analysis, these were shown to be 92% homologous to the corresponding DNA sequence of hGR2 (Weber and Burton, unpublished; **Figure 8c**). However, PCR amplicons were 915 bp in length instead of the expected 903 bp, due to

Figure 8a. Design of Primers to PCR Amplify Bovine GR2.

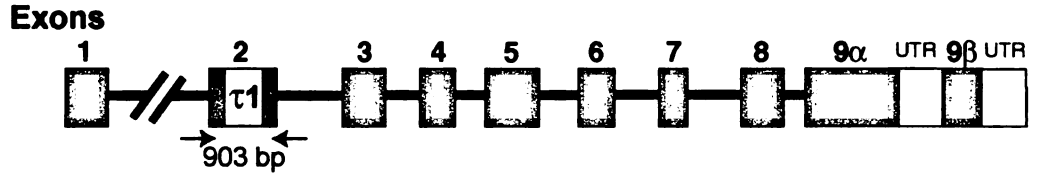


Figure 8a. Design of Primers to PCR Amplify Bovine GR2.

DNA sequence for exon 2 of the human GR gene (Hollenberg et al., 1985; Giguere et al., 1986) was used to design primers (see methods) for PCR amplification of the bovine GR2 locus. The primers used for this purpose were based on human GR2 sequence and were predicted to amplify a 903 bp fragment of bovine GR2 that included the τ1-encoding domain.

Figure 8b. PCR Amplification of Bovine GR2 Genes.

Primers designed from human GR2 were used to amplify bovine GR2. In this figure, agarose checking gels show a series of single bands from the PCR amplification of bovine GR2 using the primers shown in **Figure 8a** and genomic DNA from representative DNA pools from 8 animals of six cattle breeds as template. Lanes M are the Amplisize DNA sizing marker; lanes N are negative controls (no DNA template added during PCR). In panel (***i***) the checking gel shows representative GR2 bands for the Angus (lanes 1 and 2), and Hereford (lanes 3 and 4) breeds, while in panel (***ii***), the checking gel shows representative GR2 bands for the Holstein (lanes 5, 6, and 7), Brown Swiss (lanes 8 and 9), and Jersey (lanes 10 and 11) breeds. The check gel in panel (***iii***) shows representative GR2 bands for the Brahman breed (lanes 12 and 13). These gels show that the primers designed from human GR2 were able to amplify bands of the correct approximate size from bovine genomic DNA.

Figure 8b. Bovine GR2 Loci Amplified by PCR.

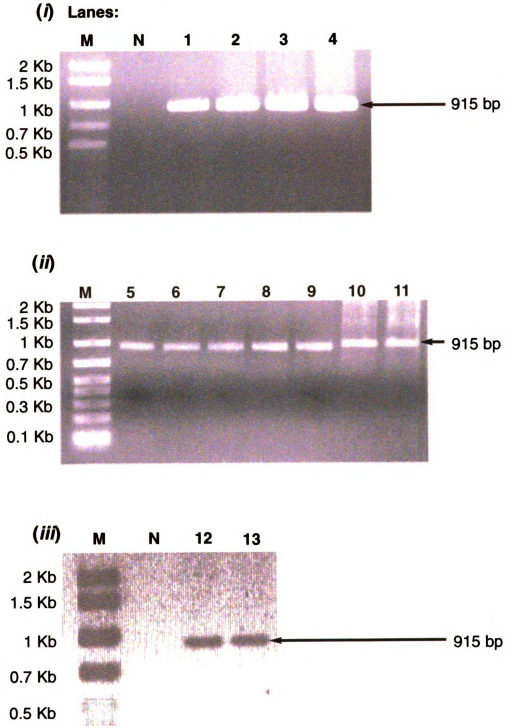


Figure 8c. DNA Sequence of the Bovine GR2 Locus.

This figure shows alignment and comparison of GR2 DNA sequences between one Holstein (bovine) and human (GenBank accession #BC015610.1). Primer sequence is shown in bold capital letters. This sequence alignment shows that bovine GR2 includes a 12-nucleotide insertion (bold lowercase letters) within the τ1 core-encoding region of GR2 (highlighted by a gray box) not found in human GR2 DNA sequence. The bovine GR2 amplicons (shown in **Figure 8b**) are therefore 915 bp rather than the 903 bp predicted from human GR2 sequence. Overall, this bovine GR2 DNA sequence was 92% homologous to human GR2 sequence.

Figure 8c. DNA Sequence of the Bovine GR2 Locus.

```
        1                                                                                                   90
bovine  GGGACTGTAT ATGGGAGAGA CAGAGACAAA AGTGATGGGA AATGACCTGG GATTCCCACA GCAGGGCCAA ATCAGCCTTT CCTCTGGGGA
human   ---------- ---------- ----A----- ---------- ---------- ---------- ---------- ---------- ----G-----
       91                                                                                                  180
bovine  AACAGACTTT CGTCTTCTGG AAGAAAGCAT CGCAAACCTC AATAGGTCAA CCAGTGTTCC AGAGAACCCC AAGAATTCGG CATCCACTGC
human   ---------A AAG---T--- ---------- T--------- --------G- ---------- ---------- ----G---A- ----------
       181                                                                                                 270
bovine  TGTTTCTGCT GCCCCCACAG AGAAGGAGTT TCCAAAAACT CACTCTGATG TGTCTTCAGA ACAGCAGAAT TTGAAAGGCC AGAAGGGCAG
human   ---G------ ---------- ---------- ---------- ---------- -A-------- ------AC-- -----G---- ---CT----C
       271                                                                                                 360
bovine  TAACGGGGGT AATATGAAGT TGTATACCAC AGACCAAAGC ACCTTTGACA TTTggaggaa aaagcTCCAG GATTTGGAGT TTTCTTCTGG
human   C-----T--C ---G----A- ---------- ---------- ---------- ---              -G--- ---------- ----------
       361                                                                                                 450
bovine  GTCCCCAAGT AAAGAGACAA GTGAGAGTCC TTGGAGCTCA GACCTCTTGA TAGATGAAAA CTGTTTGCTT TCTCCTTTGG CAGGAGAGGA
human   -------G-- --------G- A--------- ------A--- -----G---- ---------- ---------- ------C--- -G-----A--
       451                                                                                                 540
bovine  TGATCCATTC CTTCTGGAAG GAAGCTCGAA TGAGGACTGT AAGCCTCTTG TTTTACCGGA CACTAAGCCT AAAATTAAAG ATAATGGAGA
human   C---T----- ---T------ ---A------ ---------C --------CA ---------- ------A--C --------G- ----------
       541                                                                                                 630
bovine  TTTGATCTTA CCAAGTCCTA GCAGTGTGCC ACTGCCCCAA GTGAAAACAG AAAAAGAAGA TTTTATCGAA CTCTGCACCC CTGGGGTAAT
human   -C--G-T--G T----C--C- -T-A---AG- ---------- ---------- ---------- ---C------ ---------- ----------
       631                                                                                                 720
bovine  TAAGCAGGAG AAACTGGGCC CAGTTTATTG TCAGGCCAGC TTTTCTGGGG CCAATATAAT TGGTAATAAA ATGTCTGCCA TTTCTGTTCA
human   ------A--- ---------A -------C-- ------A--- ---C----A- -A-------- ---------- ---------- ----------
       721                                                                                                 810
bovine  CGGTGTGAGT ACCTCTGGGG GCCAGATGTA CCACTACGAC ATGAATACAG CATCCCTTTC TCAACAGCAG GATCAGAAAC CTATTTTTAA
human   T--------- --------A- -A-------- ------T--- ---------- ---------- ---------- --------G- ----------
       811                                                                                                 900
bovine  TGTCATTCCA CCAATTCCTG TCGGTTCAGA AAATTGGAAT AGATGCCAAG GGTCTGGAGA TGATAACTTG ACTTCCTTGG GGACTTTGAA
human   ---------- --------C- -T-----C-- ---------- --G------- -A-------- ---C------ -----TC--- -----C----
       901          915
bovine  CTTCTCTGGT CGATC
human   ----C----- ---A
```

Figure 8d. Predicted Protein Sequence of the Bovine GR τ1 Region.

```
        1                                                            60
bovine  GLYMGETETK VMGNDLGFPQ QGQISLSSGE TDFRLLEESI ANLNRSTSVP ENPKNSASTA
 human  ---------- ---------- ---------- --LK------ ---------- ----S-----

       61                                                           120
bovine  VSAAPTEKEF PKTHSDVSSE QQNLKGQKGS NGGNMKLYTT DQSTFDIwrk klQDLEFSSG
 human  ---------- ---------- --H----T-T ----V----- -------     ---------

      121                                                           180
bovine  SPSKETSESP WSSDLLIDEN CLLSPLAGED DPFLLEGSSN EDCKPLVLPD TKPKIKDNGD
 human  --G---N--- -R-------- ---------- -S-----N-- ------I--- ----------

      181                                                           240
bovine  LILPSPSSVP LPQVKTEKED FIELCTPGVI KQEKLGPVYC QASFSGANII GNKMSAISVH
 human  -V-S---N-T ---------- ---------- ------T--- ----P----- ----------

      241                                                           300
bovine  GVSTSGGQMY HYDMNTASLS QQQDQKPIFN VIPPIPVGSE NWNRCQGSGD DNLTSLGTLN
 human  ---------- ---------- ---------- ---------- ---------- ----------

        304
bovine  FSGR
 human  -P--
```

Figure 8d. Predicted Protein Sequence of the Bovine GR τ1 Region.

Comparison of predicted amino acid sequences encoded by GR2 between one Holstein (bovine) and human (translated from GenBank accession #BC015610.1). Dashes in the human sequence represent amino acids that are conserved between bovine and human GR2. As was predicted from GR2 DNA sequence in **Figure 8c**, amino acid sequence alignment shows that the bovine sequence has a 4 amino acid insertion (bold lowercase letters) within the core of τ1 (highlighted by a gray box) that was not present in human τ1. Overall, the bovine amino acid sequence of bovine τ1 was 92.6% homologous to the human τ1 region.

the insertion of 12 nucleotides near the 5' end of the τ1 core-encoding DNA that are not found in hGR2 (**Figure 8c**).

Following successful amplification of GR2 from pooled DNAs, amplicons were subjected to digestion with a battery of 11 restriction endonucleases. Digestion products were then electrophoresed and visualized by ethidium bromide staining for changes in banding patterns resulting from the mutation of various restriction endonuclease recognition sites. Differences in banding patterns were observed following digestion with three enzymes; TaqI, HaeIII, and MspI (**Figure 9a, b, c**). Digestion with TaqI resulted in banding patterns that tended to reveal differences between, rather than within, breeds of cattle (**Figure 9a**), while banding pattern differences following digestion with HaeIII were exclusive to the Holstein breed (**Figure 9b**). In addition, MspI digestions revealed banding patterns in Holsteins with varying degrees of band intensity (**Figure 9c**). Differences in the intensity of ethidium bromide staining in DNA pools indicate variable amounts of DNA resident within affected bands. As PCR amplicons from 8 animals were represented in each PCR-RFLP sample, variation in staining intensity between pools implied differences in the banding patterns of individual DNAs making up those samples. Changes in PCR-RFLP banding patterns were observed using TaqI, HaeIII, and MspI, implying possible polymorphism in bovine GR2 genes. In order to verify differences observed between pooled samples, individual DNAs from a selected pool (H3) were analyzed using PCR-RFLP for identification of differences between GR2 in individual animal's DNAs. Individual DNAs from Holsteins digested with the MspI restriction endonuclease demonstrated several different banding patterns (**Figure 9c**), which were represented in the pooled sample from these DNAs (**Figure 9c, lane 3**). These findings imply changes to restriction endonuclease

Figure 9a. TaqI PCR-RFLPs Between DNA Pools of Five Cattle Breeds.

Following PCR amplification of bovine GR2 from genomic DNAs pooled within a breed, amplicons were subjected to digestion with restriction endonucleases and electrophoresed on polyacrylamide gels. Possible differences in banding patterns (PCR-RFLPs) were visualized by ethidium bromide staining. Digestion of GR2 amplicons with the restriction endonuclease TaqI revealed differences in banding patterns between breeds thought of as dairy versus beef. Lanes M are the DNA sizing ladder, lanes U are GR2 amplicons from one Holstein cow that were left uncut, and lanes C are digested (cut) GR2 amplicons from the same cow. In Panel (*i*) the gel picture depicts banding patterns from Holstein (lanes 1, 2, and 3), and Brown Swiss (lanes 4, 5, and 6) pools, while in panel (*ii*) the gel picture depicts banding patterns for Angus (lanes 7 and 8) and Hereford (lanes 9 and 10) pools, as well as Jersey pools (lanes 11 and 12). This figure shows differences between the banding patterns for dairy (Holstein, Brown Swiss, and Jersey) versus beef(Angus and Hereford) breeds.

Figure 9a. TaqI PCR-RFLPs Between DNA Pools of Five Cattle Breeds.

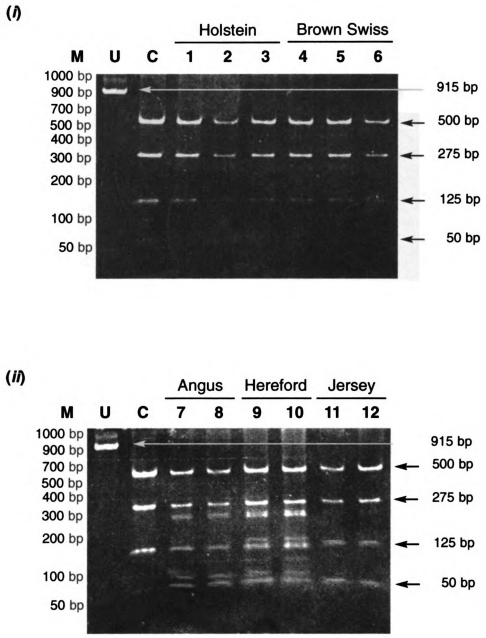


Figure 9b. HaeIII PCR-RFLPs for Holstein Cattle DNA Pools.

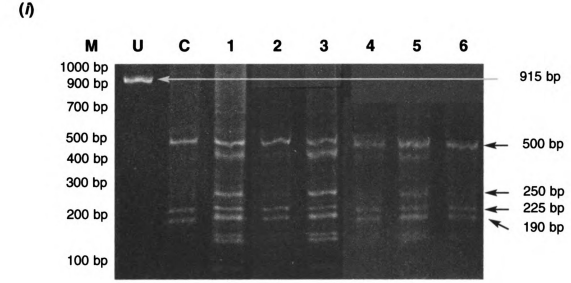


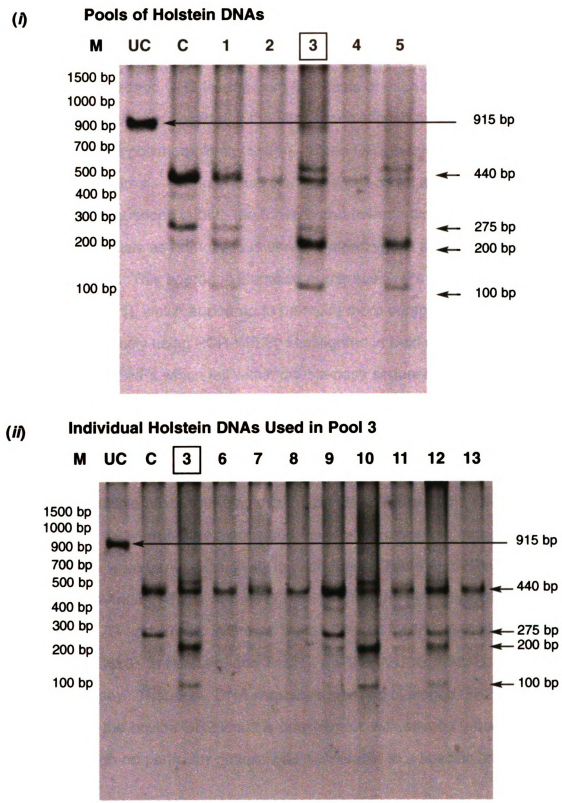
Figure 9b. HaeIII PCR-RFLPs for Holstein Cattle DNA Pools.

Digestion of GR2 amplicons using the restriction endonuclease HaeIII revealed differences within the Holstein breeds. Lane M is the DNA sizing ladder, lane U is a GR2 amplicon from one cow that was left uncut, lane C is the digested (cut) GR2 amplicon from the same cow. This gel picture shows banding patterns from Holstein pool 4 (lane 1), pool 7 (lane 2), pool 8 (lane 3), pool 10 (lane 4), pool 12 (lane 5), and pool 14 (lane 6). As shown, there appears to be differences in banding patterns between Holstein DNA pools following digestion with HaeIII.

Figure 9c. MspI PCR-RFLPs in DNA from Holstein Pools and Individual Animals within a Pool.

Digestion of GR2 amplicons using the restriction endonuclease MspI revealed polymorphism in the Holstein breed. Lanes M are the DNA sizing ladder, lanes U are uncut GR2 amplicons from one cow, and lanes C are digested (cut) GR2 amplicons from the same cow. In panel ***(i)*** the gel shows differences in banding patterns between Holstein pool 1 (lane 1), pool 2 (lane 2), pool 3 (lane 3), pool 4 (lane 4), and pool 5 (lane 5). Holstein pool 3 (boxed lane 3) was particularly interesting due to differences in banding patterns and variation in band intensities. GR2 was amplified from the eight individual animals making up Holstein pool 3 and subjected to PCR-RFLP, as shown in pane (ii). In panel **(ii)** the gel shows differences between Holsteins H2538 (lane 6), H3245 (lane 7), H3093 (lane 8), H3145 (lane 9), H3052 (lane 10), H2887 (lane 11), H2783 (lane 12), and H2622 (lane 13), the 8 animals constituting pool 3. Differences between banding patterns from individual Holstein GR2 amplicons digested with MspI may partially explain variation in Holstein pool 3 band intensities.

Figure 9c. MspI PCR-RFLPs in DNA from Holstein Pools and Individual Animals within a Pool.



recognition sites in Holsteins and the other breeds and therefore the presence of GR2 polymorphism.

II. DNA Sequence Analysis of Bovine GR2 Genes.

In order to elucidate the nature of possible GR2 polymorphism observed by PCR-RFLP, cloned GR2 inserts from 5 animals of each of the six cattle breeds were DNA sequenced and the sequences aligned for identification of single nucleotide polymorphisms (SNPs). Three GR2 sequences were generated for each animal. Two individual clones as well as an aliquot of all clones (pools) were sequenced in both the forward and reverse directions for identification of individuals as homozygous versus heterozygous at each SNP position (**Figure 10**). This approach identified numerous SNPs in bovine GR2 genes (**Figure 11**), which appeared to be much more complex in nature than observed previously using PCR-RFLP. Highlighted in bold red letters of **Figure 11a** are SNPs which fall within double-pass sequence while SNPs falling to the left and right of the red letters were generated from single-pass sequence. SNPs tended to cluster in the 5' and 3' sequences flanking the τ1 core-encoding DNA of GR2 (**Figure 11a**), and several of these SNP were present as multiple allelic forms across the breeds (**Figure 11b**). Of the animals studied, 19 were heterozygous and 4 were homozygous. Interestingly, several SNPs were found exclusively in each breed (**Table 5**); SNPs G346A and T813C in Angus, A520T and A617T in Hereford, A545G and C578A in Brahman, C77G and A786C in Brown Swiss, C441G, T454C, A480T, A492T, A529C, and C820T in Holstein, and T147C, A472G, A510G, A531G, and T565G in Jersey. Therefore, DNA sequence analysis of cloned GR2 alleles revealed that the bovine GR2 locus is polymorphic with several breed-specific SNPs, although no particular pattern was attributable to a specific breed.

Figure 10. Identification of Heterozygosity in GR2 via Analysis of Pooled and Individual Cloned GR2 Alleles.

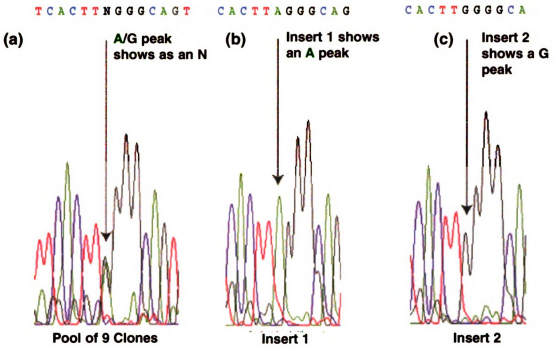


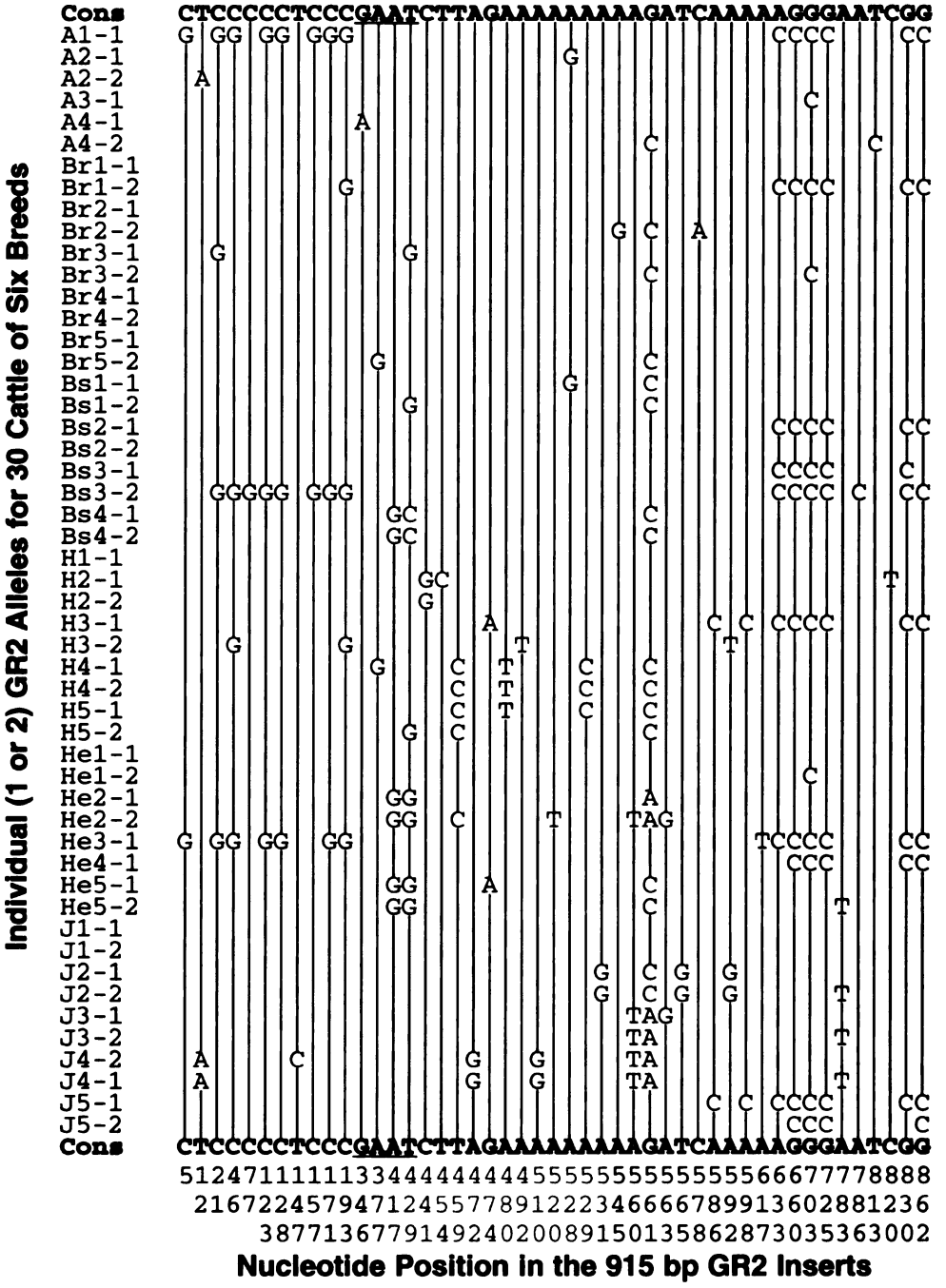
Figure 10. Identification of Heterozygosity in GR2 via Analysis of Pooled and Individual Cloned GR2 Alleles.

To identify whether an animal was heterozygous or homozygous at each nucleotide, two cloned GR2 inserts and a pool of all cloned inserts per animal were DNA sequenced. Heterozygosity at a nucleotide position would be observed as an ambiguous base pair or 'N' in the sequence. In this example, the 'N' (in **panel a**) is an overlay of 'G' and 'A' peaks. GR2 insert 1 **(b)** contains an 'A' at the same nucleotide position, while GR2 insert 2 **(c)** contains a 'G'. This would confirm that the animal is indeed heterozygous at this nucleotide position, and thus that a SNP exists in this position.

Figure 11a. Summary of Mutations in GR2 Loci Across Six Cattle Breeds.

Following sequencing, alignments of GR2 alleles for 30 animals revealed 47 unique SNP locations in the 915 bp bovine GR2. In this figure, the vertical axis lists the animals' identification numbers (see **Appendix C** for coding; A1, A2, A3 etc.) along with the allele (1 or 2) for a particular row of DNA sequence. The consensus GR2 DNA sequence (**Cons**) generated by GCG is shown at the top and bottom of the figure. The horizontal axis at the bottom of the figure shows the nucleotide positions in the 915 bp GR2 fragments where SNPs were identified. Letters interrupting vertical lines are the actual changed nucleotides (relative to consensus). Four SNP locations (346, 377, 417, 429) fell within the $\tau 1$ core-encoding region of GR2 (underlined in the consensus sequence). Highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 11a. Summary of Mutations in the GR2 Locus Across Six Cattle Breeds.



Key:

A = Angus, Br = Brahman, Bs = Brown Swiss, H = Holstein, He = Hereford, J = Jersey, Cons = Consensus sequence

Figure 11b. Summary of SNPs in GR2 Loci Across Six Cattle Breeds.

In this figure, allelic SNPs observed in the 915 bp GR2 loci from 5 animals in each of 6 cattle breeds are reported as the percentage of all alleles sequenced.. The Y-axis shows the percentage of allele differences detected and the X-axis shows the nucleotide position of these allele changes. Hatched bars at nucleotide positions 346, 377, 417, and 429 show SNPs found within the τ1 core-encoding DNA. Solid bars to the left and right of the hatched bars represent SNPs 5' and 3' (respectively) of DNA that encodes the τ1 core.

Figure 11b. Summary of SNPs in GR2 Loci Across Six Cattle Breeds.

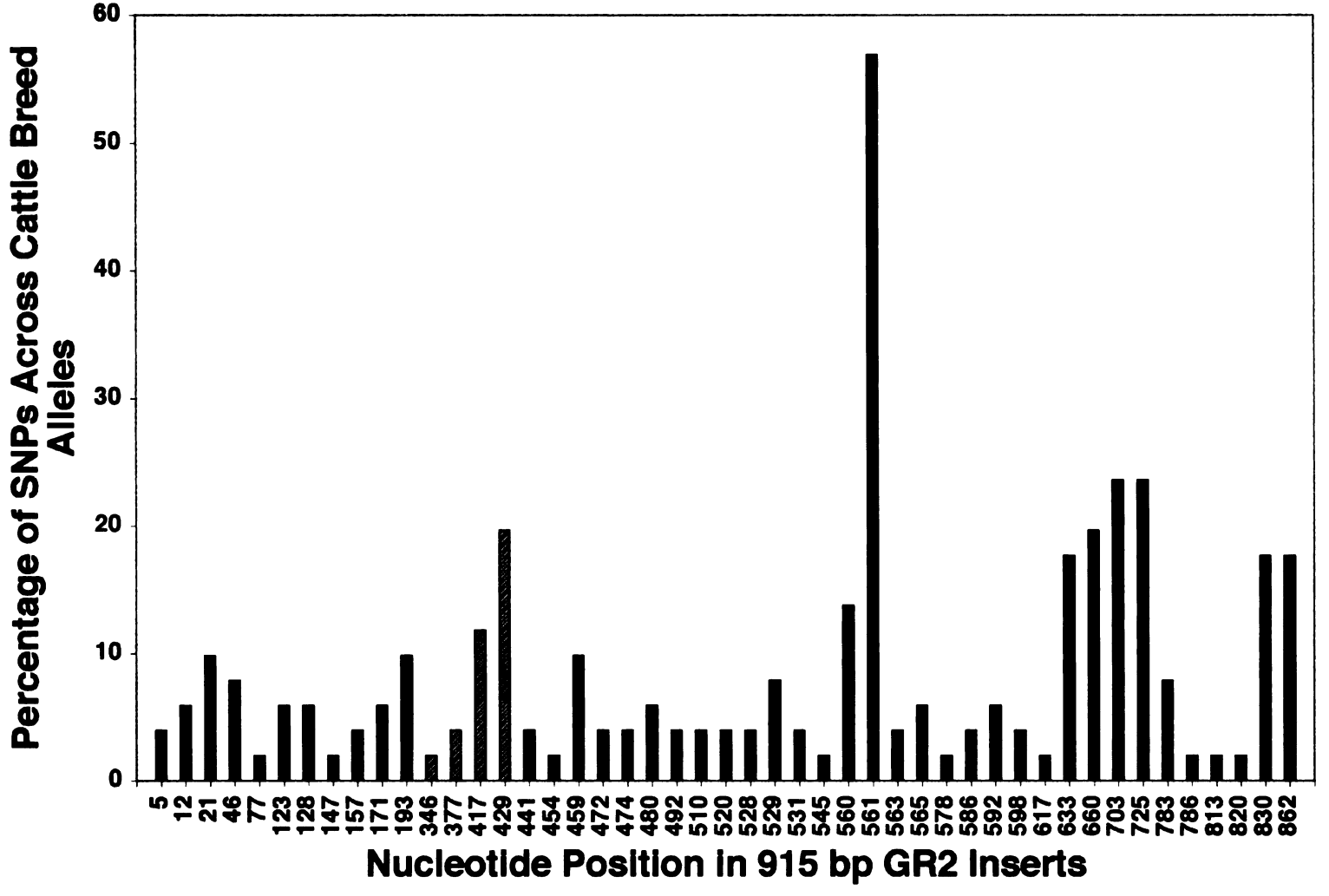


Table 5. Table of GR2 SNPs Identified Within Each of the Cattle Breeds.

This table details each of the 47 SNP locations identified across the cattle breeds and elucidates which SNPs were harbored by which breed. The left-hand column lists the nucleotide position of the SNP and the remaining 6 columns indicate which breed harbored the SNP. Several SNPs appear exclusively in each breed (in bold).

Table 5. Table of GR2 SNPs* Identified Within Each of the Cattle Breeds.

Position of SNP	Angus	Hereford	Brahman	Brown Swiss	Holstein	Jersey
5	C5G	C5G				
12	T12A					T12A
21	C21G	C21G	C21G	C21G		
46	C46G	C46G		C46G		
77				**C77G**		
123	C123G	C123G		C123G		
128	C128G	C128G		C128G		
147						**T147C**
157	C157G	C157G		C157G		
171	C171G	C171G		C171G		
193	C193G	C193G	C193G	C193G	C193G	
346	**G346A**					
377			A377G		A377G	
417		A417G	A417G	A417G	A417G	
429		T429G	T429G	T429G	T429G	
441					**C441G**	
454					**T454C**	
459		T459C			T459C	
472						**A472G**
474		G474A			G474A	
480					**A480T**	
492					**A492T**	
510						**A510G**
520		**A520T**				
528	A528G			A528G		
529					**A529C**	
531						**A531G**
545			**A545G**			
560		A560T				A560T
561	G561C	G561C,A	G561C	G561C	G561C	G561C,A
563		A563G				A563G
565						**T565G**
578			**C578A**			
586					A586C	A586C
592					A592T	A592G
598					A598C	A598C
617		**A617T**				
633	A633C	A633C	A633C	A633C	A633C	A633C
660	G660C	G660C	G660C	G660C	G660C	G660C
703	G703C	G703C	G703C	G703C	G703C	G703C
725	G725C	G725C	G725C	G725C	G725C	G725C
783		A783T				A783T

*Differences are coded as the consensus nucleotide followed by the nucleotide position and the changed nucleotide, or SNP.

Table 5. (Cont'd)

Position of SNP	Angus	Hereford	Brahman	Brown Swiss	Holstein	Jersey
786				**A786C**		
813	**T813C**					
820					**C820T**	
830	G830G	G830G	G830G	G830G	G830G	G830G
862	G862C	G862C	G862C	G862C	G862C	G862C

*Differences are coded as the consensus nucleotide followed by the nucleotide position and the changed nucleotide, or SNP.

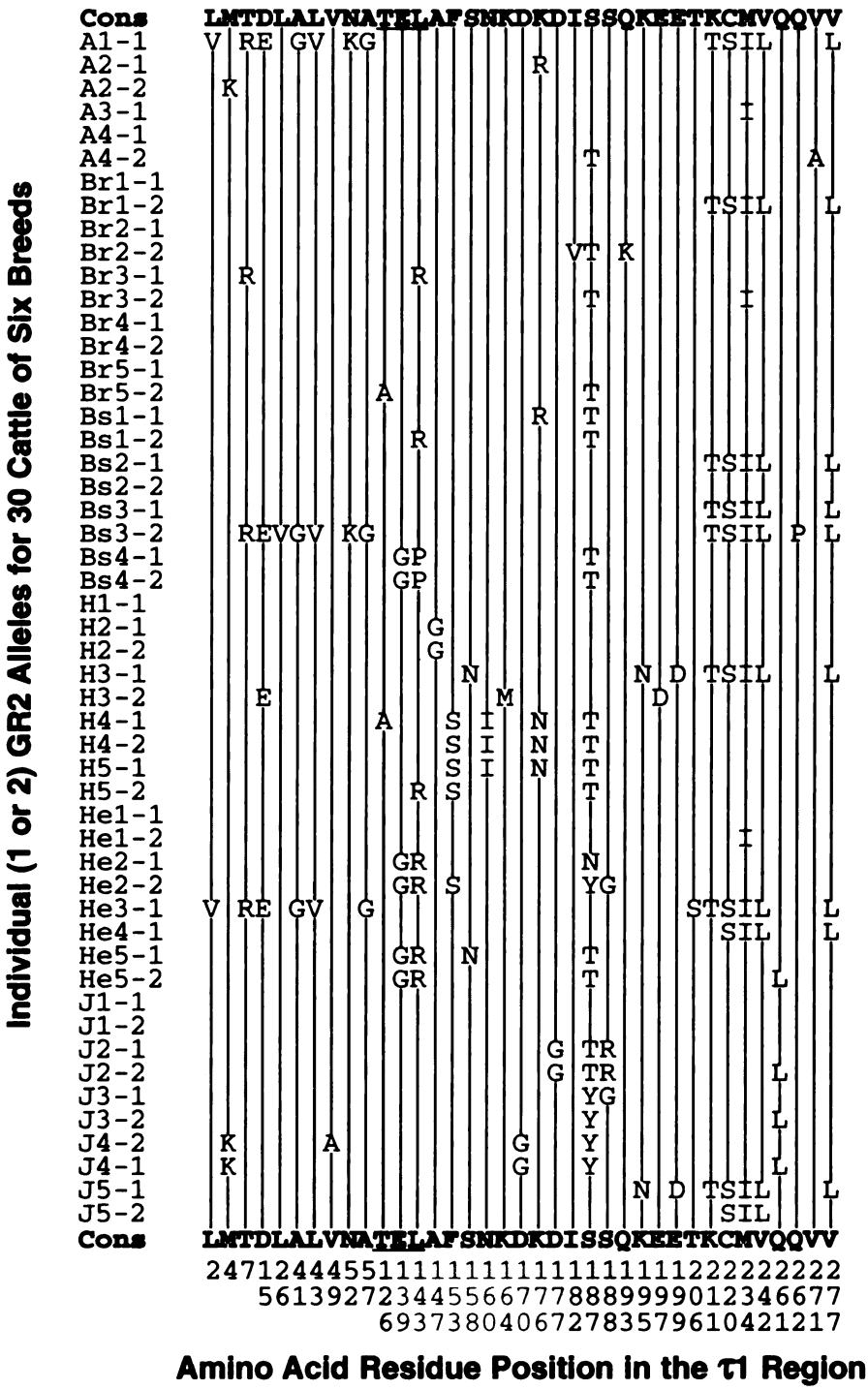
iii. Amino Acid Sequences Predicted from GR2 DNA Sequences.

To determine whether identified GR2 SNPs alter τ1 region predicted protein sequences, DNA sequences were translated into predicted amino acid sequences. As expected from the large number of GR2 SNPs identified, multiple amino acid substitutions were detected in the predicted τ1 region peptides. It is known that some amino acid substitutions are more disruptive to protein secondary and tertiary structure than others. Historically, these changes were categorized as conservative or non-conservative amino acid changes, such as T7R which represents a neutral to basic (respectively) change, or F153S which represents a nonpolar to polar change (**Figure 12a**, see **Appendix B** for residue classifications). Changes at amino acid positions 195 and 211 (highlighted by an astrerix in **Figure 12b**) are located within a synergy control motif of GR2 which enhances transcription 6 to 12 fold when mutated (Iniguez-Lluhi and Pearce, 2000). Changes indicated in bold red letters in **Figure 12a** are those generated by double-pass DNA sequence, while changes on either side are generated from single-pass DNA sequence. Several predicted amino acid changes were located within the τ1 core (residues 101 to 145; underlined in **Figure 12a** and identified by hatched bars in **Figure 12b**), however, the majority of amino acid changes clustered in the amino-terminal and carboxy-terminal regions (**Figure 12a**) of the predicted protein sequences (**Figure 12b**). Therefore, it is possible that amino acid changes resulting from SNPs in GR2 genes could cause changes to the structure of the τ1 region and possibly GR's ability to regulate glucocorticoid-responsive gene expression.

Figure 12a. Summary of Changes in the Predicted Amino Acid Sequences of GR2-Encoding τ1 Regions Across Six Cattle Breeds.

Thirty-seven amino acid residue locations were predicted to contain substitutions encoded by SNPs in the GR2 loci shown in **Figure 11**. In this figure, the vertical axis lists the animals' identification numbers (eg. A1, A2, A3, etc.) along with the allele (1 or 2) shown for a particular row of amino acid sequence. The consensus sequence (**Cons**) identified by GCG is shown at the top and bottom of the panel. The horizontal axis at the bottom of the panel shows the amino acid residue position within the predicted peptide sequence where amino acid substitutions occurred due to GR2 SNPs. Letters interrupting vertical lines are the actual changed residues (relative to consensus). Three substitutions occurred within the τ1 core (126, 139, and 143, underlined in the consensus sequence), predicted to lie between residues 101 and 146. Bold red letters indicate amino acid changes which fall within double-pass sequence, while changes on the left and right are changes generated from single-pass sequence.

Figure 12a. Summary of Changes in the Predicted Amino Acid Sequences of GR2-Encoding τ1 Regions Across Six Cattle Breeds.



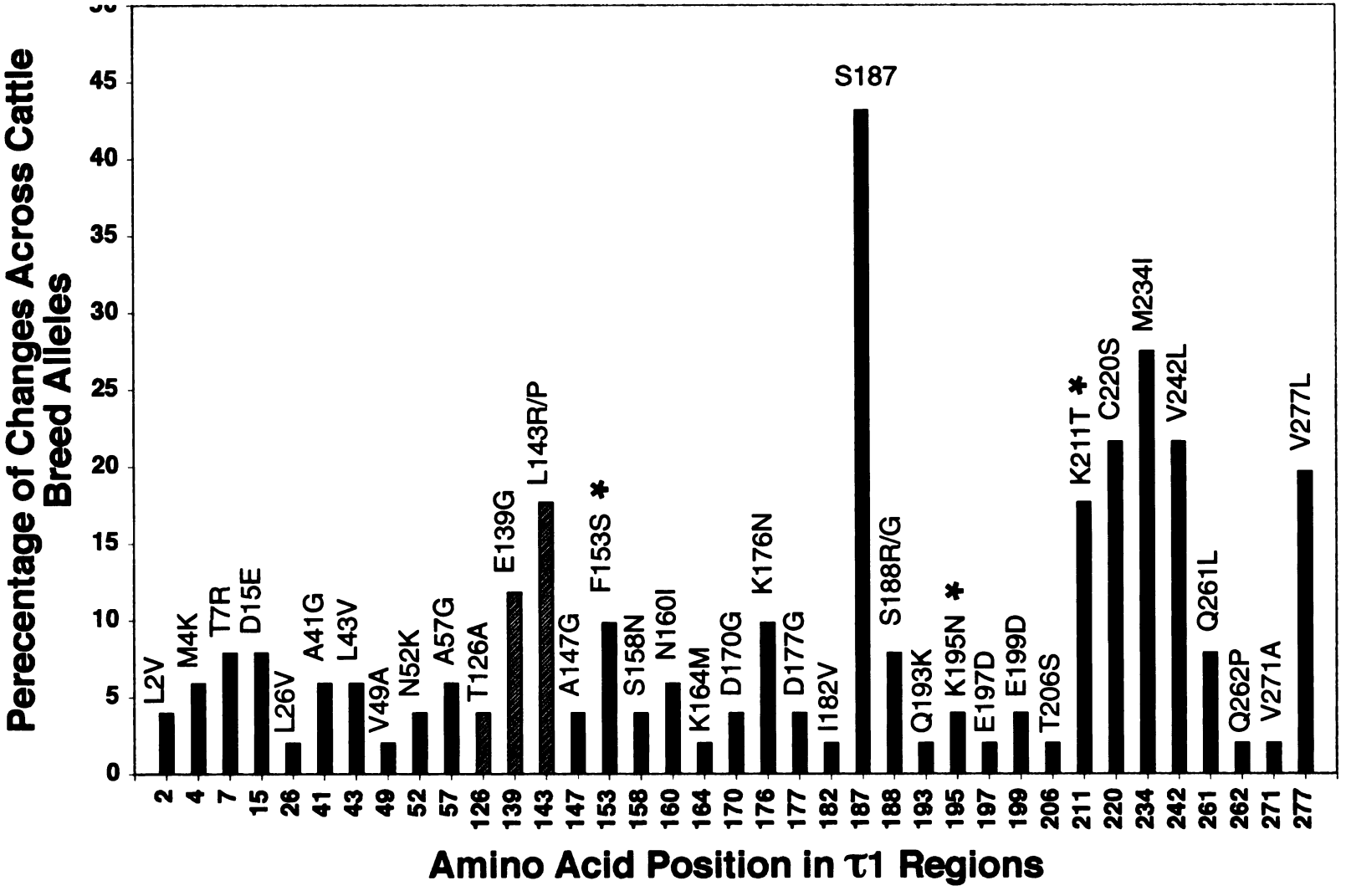
Key:

A = Angus, Br = Brahman, Bs = Brown Swiss, H = Holstein, He = Hereford, J = Jersey, Cons = Consensus sequence

Figure 12b. Summary of Changes in the Predicted Amino Acids of GR2-Encoding τ1 Regions Across Six Cattle Breeds.

This figure shows allelic amino acid residue substitutions predicted from GR2 SNPs in **Figure 11**. The Y-axis shows the percentage of allele differences detected and the X-axis shows the residue positions of these allele changes. Hatched bars at residue positions 126, 139, and 143 show changes found within the τ1 core. Solid bars to the left and right represent changes in the amino- and carboxy-terminal regions flanking the τ1 core. Changes highlighted by an asterix are those which lie within a synergy control motif and enhance transcription by GR when mutated.

Figure 12b. Summary of Changes in the Predicted Amino Acids of GR2-Encoding τ1 Regions Across Six Cattle Breeds



vi. Three-Dimensional Homology Modeling of Mutant τ1 Regions

Three-dimensional homology protein modeling is a powerful method for visualizing the effect of amino acid changing mutations on tertiary protein structure (Kuhn et al., 1990; Sternberg, 1996). Modeling entails identifying changes in key protein structures, such as α-helices and β-pleated sheets, and is based on the availability of crystal structures for highly homologous (or the same) molecules (Rice and Eisenberg, 1997). Since GR's τ1 region has not been crystallized, a protein having a secondary structure closely related to that of the bovine τ1 region was identified and subsequently used in the modeling process. For this work, the crystallized protein was pectate lyase (**Figure 13**).

Secondary structural predictions for both human and bovine τ1 regions were determined. As predicted by secondary structural analysis (FOLD-DOE server; Rice and Eisenberg, 1997), the τ1 regions of both human and bovine GR are predicted to possess fourteen β-pleated sheets and numerous loops, as well as four α-helices, one of which is predicted to lie within the τ1 core (highlighted by gray boxes in **Figures 14a** and **b**). The four additional amino acid residues observed within bovine τ1 core (**Figure 14a**) results in an elongated α-helix when compared to the human τ1 core (**Figure 14b**). In bovine τ1, several SNPs previously shown to change amino acid sequences (**Figure 12a**) are also predicted to change secondary protein structure, shown in parentheses above the amino acid sequence in **Figure 14b**. These might be expected to alter tertiary protein structure (as viewed by homology modeling). Within the Holstein breed, homology modeling did predict some structural changes to β-sheets and loops in τ1 regions of two animals with divergent τ1 predicted amino acid sequences (**Figure 15**). In portions of the model, alterations in orientation and length of β-sheets and loops can be observed. Four areas containing notable changes in predicted structure are highlighted by cir-

Figure 13. X-Ray Crystallized Tertiary Protein Structure for Pectate Lyase.

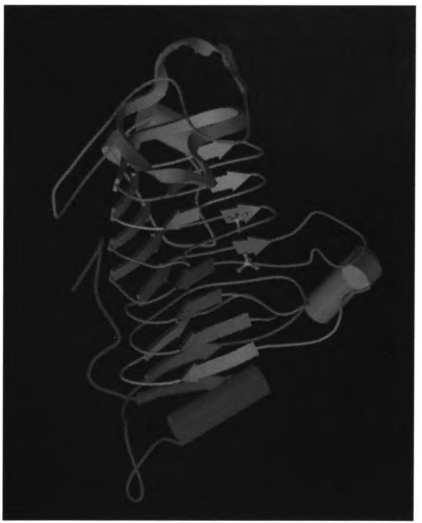


Figure 13. X-Ray Crystallized Tertiary Protein Structure for Pectate Lyase.

The protein pectate lyase (NDB Accession #1AIR; Lietzke et al., 1996) was identified as the crystallized protein with closest homology to bovine GR2-encoding $\tau 1$ based on predicted secondary protein structure (confidence value of 2.7). Therefore, this protein was used in the subsequent homology modeling of mutant bovine $\tau 1$ regions. Pectate lyase has been identified to contain 4 α-helices and 17 β-sheets. Loops and turns are found throughout the tertiary structure.

Figure 14a. Secondary Structural Prediction of the Human GR τ1 Region.

In order to determine differences in predicted protein structure between human and bovine τ1 regions, hGR amino acid sequence corresponding to that of the bovine τ1 region was submitted for prediction of secondary protein structure. In this figure, the amino acid sequence is listed across the top of each row with the secondary structural prediction below. Predictions of α-helices are shown as cylinders, β-pleated sheets as arrows, and loops and turns as ribbons and lines (respectively). The τ1 region of human GR is predicted to contain 4 α-helices, 14 β-pleated sheets, and numerous loops and turns. The τ1 core is highlighted by a gray box.

Figure 14a. Secondary Structural Prediction of the Human GR τ1 Region.

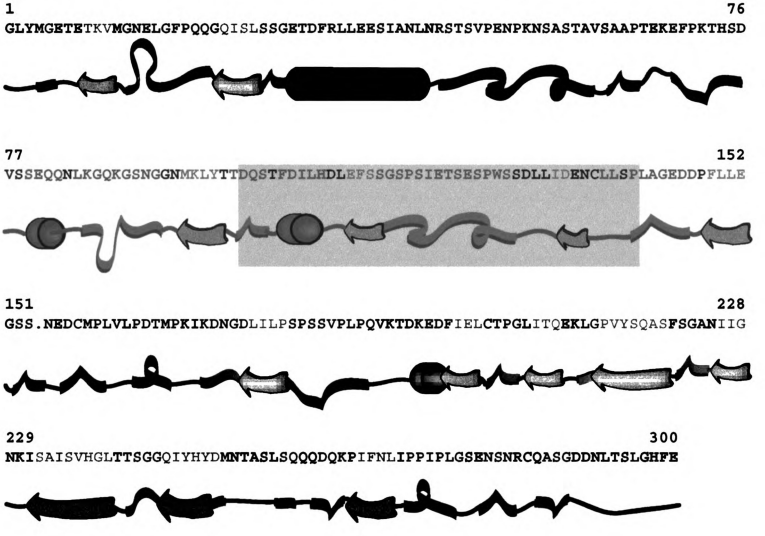


Figure 14b. Secondary Structural Prediction of the Bovine GR τ1 Region.

Following identification of changes in predicted amino acid sequences for polymorphic τ1, these sequences were submitted for prediction of secondary protein structure. In this figure, the amino acid sequence (from allele H1-1 in **Figures 11a** and **12a**) is listed across the top of each row with the secondary structural prediction below. Below the amino acid sequence predictions of α-helices are shown as cylinders, β-pleated sheets as arrows, and loops and turns as ribbons and lines (respectively). Like human GR τ1, the τ1 region of bovine GR is also predicted to contain 4 α-helices, 14 β-sheets, and numerous loops and turns. One α-helix and 2 β-sheets are predicted to lie within the τ1 core (highlighted by a gray box), although the α-helix is longer by 4 amino acid residues than that predicted for human (**Figure 14a**). Changes in predicted amino acid sequence between the second allele modeled (H3-1) are shown in parenthesis above the amino acid sequence for H1-1, and tend to affect β-sheets and loops more than α-helices.

Figure 14b. Secondary Structural Prediction of the Bovine GR τ1 Region.

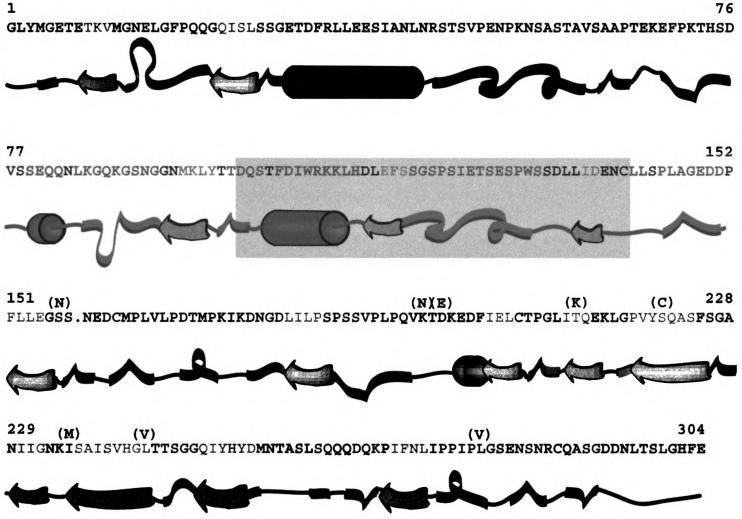
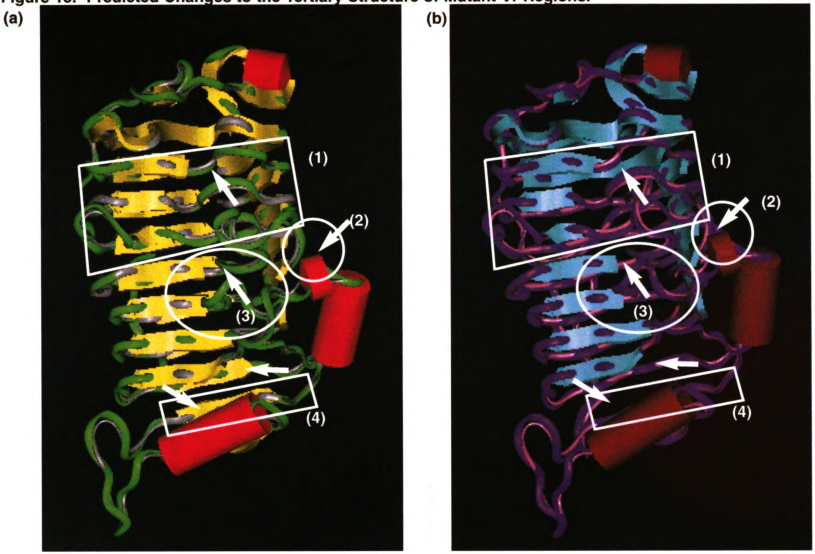


Figure 15. Predicted Changes to the Tertiary Structure of Mutant τ1 Regions.

Pectate lyase was used to homology model bovine GR τ1 regions. Two Holstein alleles, divergent in their predicted amino acid sequences (H1-1 and H3-1; **Figure 12a**), were modeled by replacing the amino acid residues of pectate lyase with those predicted for bovine τ1 regions. Computer generated models of the alleles are shown in this figure, with predicted changes in tertiary protein structure highlighted by circles, boxes, and arrows. Although no α-helices were altered, several β-sheets and loops were predicted to be different between the two structures. Most noteworthy are four areas (1 through 4) depicting changes in the lengths of β-sheets (area 1 and 3), as well as changes in the orientation of loops (area 2) and disruption of other β-sheets (area 4). This figure implies that some amino acid changing GR2 SNPs may alter the tertiary structure of GR's τ1 region.

Figure 15. Predicted Changes to the Tertiary Structure of Mutant τ1 Regions.



cles and boxes (**Figure 15**). Such changes in protein structure may be expected to influence the ability of bovine GR molecules to bind transcriptional coactivators and regulate transcription of glucocorticoid-responsive genes (Vottero et al., 2002).

D. Discussion

The ability to PCR amplify the τ1-encoding region of bovine GR genes (GR2 locus) allowed for the investigation of naturally-occurring polymorphism in populations of animals. Polymorphism was tentatively identified in GR2 using PCR-RFLP. However, since restriction endonucleases are insensitive to SNPs falling outside of their specific recognition sequences, a more rigorous investigation of GR2 polymorphism was warranted. Therefore, differences in PCR-RFLP banding patterns were used to generate preliminary data for a wider polymorphism search via DNA sequence analysis. In total, DNA from 28 individual animals was sequenced. Sequencing revealed 47 unique nucleotide positions harboring SNPs, 37 of which were predicted to change amino acid sequences. Of interest are 2 changes located within a synergy control motif (amino acid positions 195 and 211), and which enhance transcription by GR from 6 to 12 fold when mutated (Iniguez-Lluhi and Pearce, 2000). In addition, several SNPs were found to be exclusive to specific cattle breeds (bolded in **Table 5**), which may be partially responsible for breed-specific responses to stress.

The complexity of mutations identified in the GR2 loci of 6 cattle breeds by DNA sequence analysis rivals that of the bovine MHC II (DRB3) locus (van Eijk et al., 1992). The role of the highly variable DRB3 locus is to give rise to MHC II antigen binding grooves capable of binding and presenting numerous antigenic peptides to CD4 T cells that ultimately help trigger adaptive immune responses (Tonegawa, 1988). Similarly, the role of GR as a broad-spectrum

transcription factor requires that τ1 interact with numerous transcriptional co-regulators and proteins of the basal transcription machinery. Variability of the τ1 regions of GR that directly interact with transcriptional regulators may cause subtle differences in individuals and breeds in the expression of glucocorticoid-responsive genes, and thus of "stress phenotypes". The GR2 SNPs identified in this study, especially those SNPs that changed amino acids leading to alterations in the tertiary protein structure of τ1, may contribute to such variability in gene expression during stress.

It has been documented that changes in 2 or 3 amino acids within a stretch of τ1's protein sequence may have a greater impact on the overall function of this transactivation domain than single amino acid changes (Almlof et al., 1997). Iniguez-Lluhi et al (1997) postulated that sequential amino acid residues form active surfaces within and surrounding the τ1 core, surfaces that presumably provide contact sites for the multitude of transcriptional co-regulators that GR interact with. If true, the multiple mutations identified in this study could be more likely to disrupt such surfaces and affect τ1's transactivation function than any single mutation. Indeed, the GR2 SNPs were predicted to change several amino acids in the bovine τ1 region which, upon homology modeling did appear to influence β-sheet and loop structures that could be perceived as surfaces. However, the tertiary protein modeling approach did not show such gross structural changes in most of the alleles modeled (see **Appendix D** for examples), suggesting that other changes to the τ1 region (ie. charge) may have occurred instead. That said, the usefulness of homology modeling in this study was limited by the lack of a crystallized structure for GR's

τ1. This is a particularly difficult problem since the τ1 region of GR is unstructured until it interacts with other transcriptional proteins (Sigler, 1988; Yamamoto et al., 1998). Thus, even if a crystal structure for τ1 were available, it might represent only one of many possible structures for this key region of GR. Accordingly, other methods of assessing the possible biological significance of GR2 polymorphism in cattle are required. In the subsequent chapters this is done by estimating statistical associations between GR2 SNPs and heritable leukocyte and mammary health traits that are sensitive to GC.

E. Conclusions

The bovine GR2 locus, which encodes the τ1 region of the GR protein, is polymorphic in 6 breeds of cattle. Sequencing of GR2 genes revealed changes at 47 unique nucleotide positions, several of which occurred across breeds. Additionally, several SNPs were found to be breed-specific, including 1 found exclusively within beef breeds (Angus and Hereford), which will allow for exploration of QTL in a breed-specific fashion. Multiple SNPs identified in GR2 were observed to change τ1 amino acid sequences and may therefore affect τ1's charge, hydrophobicity or hydrophilicity, hydrogen bonding, or tertiary protein structure. Protein modeling did not reveal significant changes in tertiary protein structure, however, this approach precluded for modeling of charge and bonding. Given our findings, it would be useful to develop a rapid PCR-based genotyping assay for future research in determining association between GR2 polymorphism and traits of biologic or economic importance to the cattle industry.

CHAPTER 5. MUTATIONS IN EXON 2 OF BOVINE GLUCOCORTICOID RECEPTOR GENES ASSOCIATE WITH LEUKOCYTE SENSITIVITY TO GLUCOCORTICOIDS

A. Introduction

Suppression of the immune system following adrenal release of glucocorticoids has been extensively studied. The steroids effect profound changes in both the innate and adaptive branches of the bovine immune system (Kehrli et al., 1999). Changes include repressed expression of important trafficking proteins on neutrophils, antigen presentation molecules on monocytes and B cells, reduced numbers of circulating γδ T cells, and changed proportions of circulating B cells, CD4 T cells, and CD8 T cells (Ashwell et al. 2000; Burton and Erskine, 2003).

Of particular concern in cattle and other livestock species are glucocorticoid-induced immune cell changes that lead to a reduced ability of leukocytes to migrate and recognize invading pathogens in infected tissue, thereby leaving animals vulnerable to infection and clinical disease. Natural immunosuppression in dairy cows is striking following parturition (Detilleux et al., 1995; Burton and Erskine, 2003), during which time glucocorticoid concentrations are 7 to 10 times higher than basal concentrations (Preisler et al., 2000a; Weber et al., 2001). Parturient immunosuppression can be mimicked by treating cattle with the synthetic glucocorticoid, dexamethasone, which also causes reduced expression of CD62L and CD18 adhesion molecules in neutrophils (Burton et al., 1995; Burton and Kehrli, 1995; Weber et al., 2001), reduced synthesis of numerous proinflammatory cytokines (Almawi et al., 1996; Nonnecke et al., 1997) and tissue receptors for these molecules (Cronstein et al., 1992; Yu et al., 1997), reduced numbers of circulating γδ T cells (Burton and Kehrli, 1996; Anderson et al., 1999), reduced expression of MHC II molecules on antigen

presenting cells (Burton and Kehrli, 1996), and reduced lymphocyte responsiveness to mitogens and antigens *in vitro* (Oldham and Howard, 1992; Nonnecke et al., 1997).

Milk production traits have also been shown to be responsive to glucocorticoids, and are observed as reductions in milk yield following stressful husbandry events (Bruckmaier et al., 1993) and a reduction in production of milk proteins β-casein, α-lactalbumin, and whey associated protein (Reichardt et al., 2001; Houdebine et al., 1985). Milk somatic cell score (SCS), an indicator trait of mammary gland health, is a measurement of the number of somatic cells in milk, the majority of which in infected glands are blood-derived neutrophils (Paape et al., 2000; Riollet et al., 2000). Neutrophil adhesion molecules are profoundly down-regulated following glucocorticoid challenge (Burton et al., 1995) presenting the possibility of transiently reduced migration of phagocytic neutrophils into infected mammary tissue and increased mastitis susceptibility (Burton and Kehrli, 1995). Therefore, the sensitivity of leukocytes and mammary cells to glucocorticoids partly determines health and productivity of dairy cows.

The cellular receptor that mediates glucocorticoid effects is called the glucocorticoid receptor, or GR. It is expressed in bovine neutrophils (Preisler et al., 2000a; Weber et al., 2001) and mononuclear leukocytes (Preisler et al., 2000b) as well as in cells of most other tissues (Bamberger et al., 1996). GR is a ligand-activated transcription factor that regulates expression of glucocorticoid-responsive genes (Bamberger et al., 1996). Upon glucocorticoid binding, cytosolic GR becomes activated to shed its accessory molecules, dimerizes with another hormone-activated GR, and translocates into the cell's nucleus (Tsai and O'Malley, 1994). Once in the nucleus, GR interacts in various direct and indirect ways with regulatory DNA in and around glucocorticoid-sensitive

genes to regulate their transcription (Tsai and O'Malley 1994; Bamberger et al., 1996; McEwan 2000). GR is suspected to directly regulate gene expression of adhesion molecules such as CD62L on neutrophils (Weber et al., 2001), multiple cytokine genes (Gessani et al., 1988; Almawi et al., 1996; Brack et al., 1997; Nonnecke et al., 1997), cytokine receptors on multiple cell types (Almawi et al., 1996; Yu et al., 1997; Galon et al., 2002), and antigen presentation proteins (Burton and Kehrli, 1996; Jabara et al., 2001; Galon et al., 2002). Genetic variation in some of these glucocorticoid-sensitive traits has been observed in parturient dairy cows (Detilleux et al., 1995a,b) and dairy bulls used as breeding stock following exogenous treatment with dexamethasone (Tempelman et al., 2002; J.L. Burton in review; Kelm et al., in review). In fact, dexamethasone-induced immunosuppression in these bulls has recently been shown to be predictive of health status in their daughters, especially of mammary gland health indicated by milk SCS (G.A. Abdel-Azim in review). Therefore, genetic variation in infectious disease susceptibility in cattle may be mediated in part by genetic variation in the sensitivity of leukocytes to glucocorticoids (Tempelman et al., 2002).

The goal of the current study was to determine if GR genes of Holstein dairy cattle contain mutations that could partly explain genetic variation in leukocyte sensitivity to glucocorticoid challenge and relevant milk production traits. We focused our polymorphism search in GR gene exon 2 (GR2 locus) because this locus contains DNA sequence that encodes the τ1 region of GR proteins (Hollenberg et al., 1985; Giguere et al., 1996). The τ1 domain directly interacts with proteins of the basal transcription machinery and other transcription regulatory proteins (Ford et al., 1997; Hittelman et al., 1999) and is thus key to a cell's sensitivity to glucocorticoids. The same Holstein bulls already determined to exhibit genetic variation in neutrophil and mononuclear leuko-

cyte sensitivities to glucocorticoid challenge *in vivo* (Tempelman et al., 2002; J.L. Burton in preparation) was the test population selected for this study. Our main objectives were to: (1) obtain DNA sequences for GR2 genes of 40 previously phenotyped Holstein bulls; (2) identify GR2 polymorphism; (3) determine if GR2 polymorphism translated into amino acid sequence polymorphism; and (4) estimate statistical associations between GR2 polymorphisms and heritable traits of leukocyte sensitivity to glucocorticoids and milk production genetic merit of the test bulls.

B Methods

i. Animals

DNA from 40 Holstein bulls with known leukocyte phenotypes in response to glucocorticoid (dexamethasone) challenge was available for this study (see **Appendix E**). The leukocyte traits included in this study are listed in **Table 6** and were: neutrophil expression of CD18; mononuclear leukocyte expression of MHC II; percentages of blood CD4 and CD8 T cells; and the percentage of blood B cells. All traits were measured flow cytometrically before, during, and after dexamethasone was administered to the bulls (as described in Tempelman et al., 2002) using antibodies and immunostaining protocols reported by Burton and Kehrli (1995; 1996).

ii. Isolation of Genomic DNA

Holstein bull genomic DNA was obtained from ACD-anticoagulated blood samples donated for this study by Genex, Cooperative Resources International (formerly 21st Century Genetics, Shawano, WI). Extraction of genomic DNA from leukocyte pellets was performed by adaptation of a method described in Lahiri et al. (1992). In brief, non-DNA cellular components were precipitated and removed by addition of saturated sodium chloride and resulting DNA pre-

cipitated by addition of 100% ethanol. To lyse leukocytes for this DNA extraction, approximately equal volumes of blood (5 mL) and solution A (10 mM Tris-HCl at pH 7.6, 10 mM KCl, 10 mM MgCl2, 2 mM EDTA in milli-Q water) were added to 15 ml conical tubes, followed by addition of 100 mL of 2.0% Triton X-100 (Invitrogen, Carlsbad, CA) and mixed by inversion. Cellular debris and nuclei were separated by centrifugation at 1000 x g for 10 minutes at room temperature and the supernatants removed. The resulting nuclei pellets were suspended in the original volume of solution A, followed by a repeat of the centrifugation and supernatant removal steps. For lysis of nuclear pellets, 0.8 µl of solution 2 (10 mM Tris-HCl at pH 7.6, 10 mM KCl; 10 mM $MgCl_2$, 400 mM NaCl, 2 mM EDTA in milli-Q water) and 50 µl of 10% SDS were added and vortexed to suspend the pellet. This was followed by incubation at 55°C for 10 minutes, addition of 300 µl of 6 M NaCl, and mixing to precipitate proteins. Contents were transferred to 1.5 ml microcentrifuge tubes and proteins removed by centrifugation at 12,000 x g for 5 minutes. Supernatants containing genomic DNA were transferred to fresh sterile tubes and DNA strands precipitated by the addition of 2 volumes of 100% ethanol at room temperature. Precipitated DNA strands were removed with a glass pipette and transferred into tubes containing 1 ml of ice-cold 70% ethanol. Tubes were centrifuged at 12000 x g for 5 minutes at 4°C. DNA pellets were dried and suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA at pH 8.0 and incubated at 65°C for 15 minutes. Genomic DNA was stored at 4°C until use in PCR to amplify the GR2 loci.

iii. PCR Amplification of GR2

The GR2 locus was amplified from bovine genomic DNA using PCR as described in **Chapter 4, Section B*ii***. Briefly, primers were designed from human GR2 gene sequence to include the τ1 core-encoding DNA and most of its 5' and 3' flanking DNA. GR2 was PCR amplified using genomic DNA from

the 40 Holstein bulls and amplicons visualized on 1.2% agarose checking gels with ethidium bromide staining. PCR products were stored in milli-Q water at 4°C until molecular cloning.

iv. Molecular Cloning of GR2

PCR amplicons of GR2 were purified using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) and suspended in milli-Q water to a final concentration of 200 ng/µl. These GR2 fragments were then ligated and cloned as described in **Chapter 4, Section B*iv***. Aliquots of each selected clone were used in PCR to verify the presence of cloned GR2 inserts. To do this, 1 µl of the cells for each clone were pipetted into wells of a sterile 96-well plate (Fisher Scientific, Hanover Park, IL), which were incubated at 94°C for 10 minutes to lyse the cells. Then, 48 µl of PCR master mix comprised of 10 mM dNTPs, 5 µl PCR mix [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 25 mM $MgCl^{+2}$, 12.5 mM each of forward primer (5'-AGTCCTTG-GAGCTCAGA-3') and reverse primer (5'-GTTTATTGTCAGGCAGC-3'), 36.5 µl MQ water, and 1 µl of Taq Polymerase (Invitrogen, Carlsbad, CA) were added to each well and the plate was subjected to 94°C for 10 minutes to denature the genomic DNA, and 25 cycles of 94°C for 30 seconds of denaturing, 53°C for 30 seconds of annealing, and 72°C for 1 minute of elongation in a MJ100 PCR machine (MJ Research, Reno, NV), with a final 72°C elongation for 10 minutes. PCR reactions were visualized for single bands on 1.0% agarose checking gels following electrophoresis using ethidium bromide staining to confirm the presence of the GR2 inserts. Clones that were positive for GR2 inserts in the PCR were next used for DNA sequencing.

v. GR2 DNA Sequence Analysis

Two of the picked clones per bull were selected for GR2 DNA sequencing. Additionally, aliquots of all picked clones were pooled for each bull and

sequenced for identification of heterozygotes. Sequencing reactions were prepared for sequencing by aliquotting 250 µl of appropriated bacterial cells into wells of 96-well plates (Fisher Scientific). The plates were shipped on dry-ice to Genome Therapeutics (Waltham, MA) for DNA sequencing of the cloned GR2 inserts in both the forward and reverse directions using M13 forward and M13 reverse primers to prime the reactions.

Each sequencing reaction resulted in approximately 600 bp of usable GR2 sequence in both the forward and reverse directions from the cloned 915 bp GR2 inserts, resulting in 300 bp of overlapping sequence. Electropherograms for pooled clones were analyzed for heterozygosity by the identification of peaks representing individual nucleotides that were overlaid (as shown in **Figure 10** from **Chapter 4**). Pooled and individual clone sequences were aligned and used for comparison and subsequent polymorphism identification.

vi. Polymorphism Identification

GR2 sequences were analyzed using GCG (Madison, WI) software. Following reformatting ('chopup' and 'reformat' commands) of the forward and reverse sequences, they were joined using the 'assemble' command. Multiple alignment files were generated using the 'pileup' and 'pretty' commands for generation of a consensus GR2 sequence (based on most frequently occurring nucleotide at each nucleotide position within the population of test bulls). Polymorphism was demonstrated when a nucleotide at a particular position in GR2 was different from consensus. The multiple sequence alignment files were then imported into an Excel spreadsheet (Microsoft Office v. x, Redmond, WA) for generation of graphs depicting position and frequency of identified SNPs.

vii. Defining GR2 Polymorphism at the Amino Acid Sequence Level

DNA sequences for each allele of each bull were translated into predicted

amino acid sequences using GCG's "translate" command and multiple alignment sequences generated using the 'pileup' and 'pretty' commands for generation of a consensus sequence and identification of amino acid changes as described above for DNA sequences. Once again, multiple sequence alignment files of predicted amino acid sequences were imported into an Excel spreadsheet and graphs generated as a way of visualizing the frequency and position of amino acid changes relative to consensus.

viii. Study of GR2 Associations with Glucocorticoid-Responsive Leukocyte and Milk Production Traits

Several leukocyte traits exhibited significant additive genetic variation during recovery from dexamethasone challenge *in vivo* (Tempelman et al., 2002; Burton et al., in review), and these were used for analyses of GR2 SNP-trait associations. Heritability estimates for these recovery traits were modest to high, ranging from 0.06 to 0.56 (**Table 6**). The actual traits used for the association analyses were neutrophil expression of CD18, mononuclear leukocyte expression of MHC II, percentages of CD4 and CD8 T cells in blood, and the percentage of blood B lymphocytes. In addition, bulls' genetic merit (as PTA) for milk yield, milk protein yield, and milk SCS was obtained from the AIPL-USDA web site (http://www.aipl.arsusda.gov/). SAS was used to determine the best linear unbiased prediction (BLUP) of linear recovery for each of these traits. GR2 SNP or amino acid-trait associations were conducted in SAS using one-way analysis of variation (ANOVA). Results were plotted as 'SNP position' against the '*P*-value' from the F-statistic, with a line of significance added across the plots where $P = 0.05$. The SNPs falling on or below this line were considered significantly associated with traits.

Table 6. Summary Table Depicting the Leukocyte and Milk Production Traits Studied, Including the Acronyms Used for Each Trait and Their Physiological Relevance.

Leukocyte and milk production traits are listed in the left-hand column. The leukocyte traits were chosen because they exhibited significant genetic variation in recovery from dexamethasone challenge in this group of 40 test bulls (Tempelman et al., 2002; Burton et al., in preparation). Acronyms used in this study for each trait are listed in the middle column along with an arrow indicating leukocyte traits which were down-regulated following glucocorticoid administration. The physiological relevance and heritability estimates of each studied trait are briefly summarized in the right-hand columns of the table.

Table 6. Summary Table Depicting the Leukocyte and Milk Production Traits Studied.

Leukocyte or Milk Production Trait	Acronym	Physiological Relevance	Estimated Heritability
Percentage of circulating T cells that express the CD4 accessory molecule	↓% CD4	Helper T cells; induction of inflammatory, cell-mediated, and humoral immune responses following recognition of foreign antigen presented in the context of MHC II molecules on antigen presenting cells	0.23 to 0.391[a] depending on day relative to glucocorticoid administration
Percentage of circulating T cells that express the CD8 accessory molecule	↓% CD8	Cytotoxic T cells; kill altered host cells presenting foreign antigen in the context of MHC I molecules	0.06 to 0.391[a] depending on day of glucocorticoid administration
Percentage of circulating B cells	↓% B cells	Antibody production upon activation by antigen and CD4 T cells	~0.151[a]
Expression of MHC II on mononuclear cells	↓MHC II	Presentation of antigen to CD4 T cells	0.21 to 0.561[a] depending on day of glucocorticoid administration
Expression of CD18 on circulating neutrophils	↓CD18	Adhesion molecules used by blood neutrophils for migration into infected tissue.	0.10 to 0.542[b] depending on day relative to glucocorticoid administration
Predicted transmitting ability for milk yield	Milk PTA	Estimation of genetic merit for milk yield in daughters	0.133[c]
Predicted transmitting ability for milk protein yield	Milk Protein PTA	Estimation of genetic merit for milk protein yield in daughters	0.09[c]
Predicted transmitting ability for milk somatic cell score	Milk SCS	Estimation of genetic merit for milk somatic cell counts in daughters	0.17[d]

[a]Burton et al. (in review)
[b]Tempelman et al. (2002)
[c]De Groot et al. (2002)
[d]Cranford and Pearson (2001)

C. Results

Images in this dissertation are presented in color

i. Cloning and Sequencing of Bovine τ1-Encoding GR2.

A population of 40 Holstein bulls, exhibiting significant additive genetic variation in several leukocyte traits in response to glucocorticoids (Tempelman et al., 2002; Burton et al., in preparation), were used to identify polymorphism in the GR2 locus. The full-length bovine GR cDNA has yet to be sequenced, therefore human GR cDNA sequence was used to design primers that would be expected to amplify a 903 nucleotide amplicon of bovine GR2 using PCR. These primers were designed to include DNA sequence encoding the τ1 core as well as flanking DNA regions (**Figure 8a**). **Figure 16** shows that the primers successfully amplified GR2 from bovine genomic DNA (six representative bulls shown). Subsequent sequence analysis of the GR2 amplicon from a randomly selected Holstein cow, followed by BLASTn analysis, showed that bovine GR2 contained 12 nucleotides in the τ1 core-encoding region that are not present in human GR2 (GenBank accession #BC01-5610.1; **Figure 8c**).

Figure 16. Holstein Bull GR2 Amplicons.

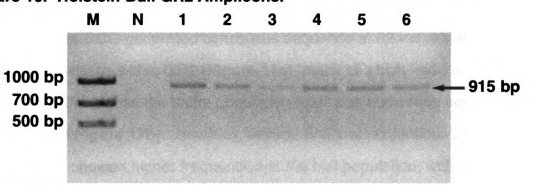


Figure 16. Holstein Bull GR2 Amplicons.

A 1.2% agarose checking gel showing a series of single bands following PCR amplification of bovine GR2 from six representative Holstein bulls. The PCR products were 915 bp in length. Lane M is the Amplisize DNA sizing marker; lane N is a negative control (no DNA template added during PCR); lanes 1-6 are single 915 bp PCR products amplified from genomic DNA templates of six Holstein bulls.

Thus, our PCR fragment was 915 bp in length rather than the 903 bp we expected based on human sequence. Overall, bovine GR2 DNA sequence was highly homologous (92%) to corresponding human GR2 sequence (**Figure 8c**). When GR2 DNA sequence from the Holstein cow was translated into predicted amino acids, the peptide was 92.6% homologous to human predicted amino acid sequence (**Figure 8d**). Thus we have demonstrated that the bovine GR2 locus is relatively homologous to human GR2, and translates into a homologous predicted amino acid sequence.

ii. Identification of GR2 Single Nucleotide Polymorphisms.

In order to elucidate individual alleles for each bull, PCR-amplified GR2 DNA amplicons were cloned into the pGEM T-Easy vector and propagated in JM109 *Escherichia coli* competent cells. Several individual clones per bull, each containing a single allele, were selected and pooled for DNA sequence analyses (as described in **Chapter 4**). The Genetics Computer Group software was then used to generate sequence files for each of 76 successfully sequenced alleles (4 bulls had unacceptable sequence for one allele each), which were then compared for identification of SNPs. We observed differences from the GCG-generated consensus sequence at 52 individual nucleotide positions within GR2 (**Figure 17a**), many of which had frequencies of 1.3% or 2.6% across the entire population (e.g., see nucleotide positions 24, 62, 77, etc. in **Figure 17b**). However, several SNPs at nucleotides 28, 289, 571, and 617 showed higher frequencies in the bull population, with a discernable "SNP cluster" lying between nucleotides 780 and 801 (**Figure 17b**). In **Figure 17b**, the hatched bars at positions 346 and 415 represent SNPs found within the τ1 core-encoding region of GR2. All other solid bars to the left and right of these hatched bars represent SNPs found in the 5' and 3' (respectively) flanking DNA of GR exon 2 that surrounds the τ1 core-encoding region.

Seventeen SNPs occurred in the 5' flanking area and 33 SNPs occurred in the 3' flanking DNA.

iii. Identification of Changes in Predicted Amino Acid Sequences.

GR2 alleles were further analyzed by GCG for potential influences of SNPs on predicted amino acid sequences. This analysis revealed that 29 amino acid residue positions were affected by SNPs in the GR2 locus (**Figure 18a**). These predicted peptide sequences showed mostly single amino acid changes (**Figure 18b**), including 11 that were considered conservative and 19 that were considered non-conservative. One amino acid position (residue 234) contained both conservative and non-conservative changes (M234V, M234T), depending on the bull (**Figure 18a** and **18b**). Additionally, there were four amino acid changes involving proline substitutions (L35P, Q193P, P207I, P267H; **Figure 18b**), which could cause steric hindrance in the peptide regions of GR that flank τ1. Of interest is a change at amino acid position 195, which falls within a synergy control motif of GR and which has been found to enhance transcription by 9 fold when mutated (Iniguez-Lluhi and Pearce, 2000). It was interesting to note that neither of the two SNP locations occurring in the actual τ1 core-encoding region (nucleotide positions 346 and 415 in **Figure 17**) translated into amino acid changes in the τ1 core itself, which lies between residues 101 and 146 in the predicted amino acid sequences. In total, 29 unique τ1 region amino acid sequences were identified in this Holstein bull population. Therefore, many SNPs in bovine GR2 are predicted to change amino acid sequence, which could result in changes to the protein structure and charge, possibly affecting the transcription regulatory functions of GR. The τ1 core itself did not contain any predicted amino acid residue changes in this group of Holstein bulls, suggesting that this critical functional domain of bovine GR is relatively stable and intolerant to change.

Figure 17a. Summary of Single Nucleotide Polymorphisms (SNPs) Observed in the GR2 Locus of 40 Holstein Bulls.

Fifty-two SNP locations were identified in the 915 bp bovine GR2 PCR amplicons that were cloned for DNA sequencing of individual alleles. In the figure, the vertical axis lists the bulls' identification numbers, from least to greatest, (eg. 4536, 4591, 4628, etc.) along with the allele (1 or 2) for a particular row of DNA sequence. The consensus GR2 DNA sequence (**Cons**) generated by GCG is shown at the top and bottom of the figure. The horizontal axis at the bottom of the figure shows the nucleotide positions within the original 915-bp GR2 fragments where SNPs were identified. Letters interrupting vertical lines are the actual changed nucleotides (relative to consensus). Two SNP locations (346, 415) fell within the $\tau 1$ core-encoding region of GR2 and are underlined in the consensus sequences. Highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 17a. Summary of Single Nucleotide Polymorphisms (SNPs) Observed in the GR2 Locus of 40 Holstein Bulls.

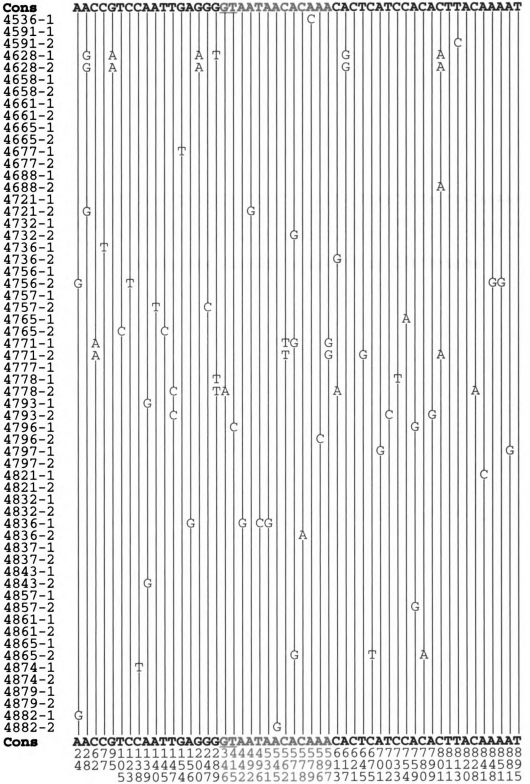
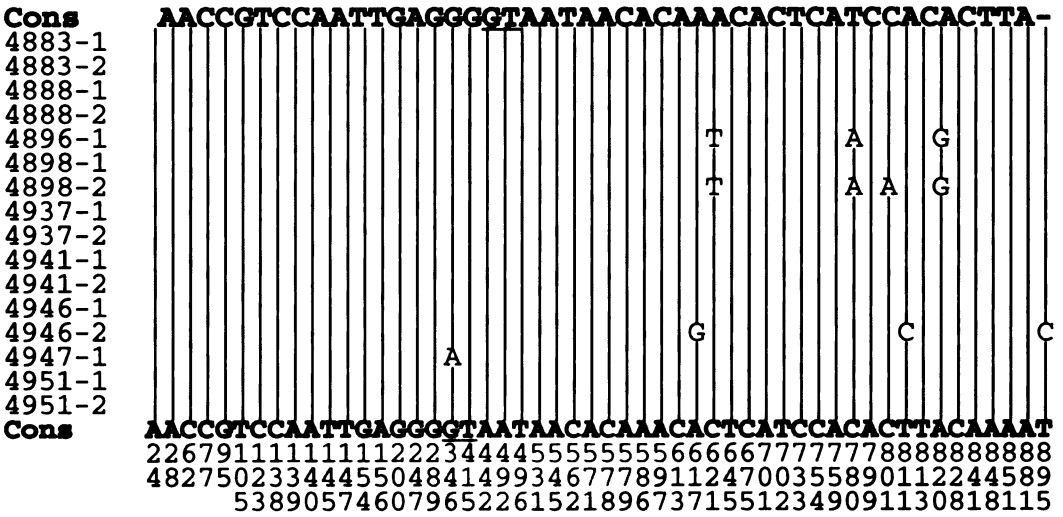


Figure 17a. (cont'd)



Nucleotide Position in the 915 bp GR2 Inserts

Figure 17b. Summary of GR2 SNP Frequencies in a Population of 40 Holstein Bulls.

Frequencies of allelic SNPs observed in the 915 bp GR2 loci of 40 Holstein bulls expressed as the percentage of all alleles studied. The Y-axis shows the percentage of allele differences detected and the X-axis shows the nucleotide position of these alleles. The hatched bars at nucleotides 346 and 415 show SNPs found within the τ1 core-encoding DNA of GR2. The solid bars to the left and right of the hatched bars shows SNPs found in the 5' and 3' (respectively) DNA flanking the τ1 core-encoding region. This figure shows that the majority of identified SNPs occurred in low frequencies of 1.3% and 2.6%. However, SNPs at locations 28, 289, 571, 617, 780, and 801 occurred in higher frequency (3.9% to 6.6%).

Figure 17b. Summary of GR2 SNP Frequencies in a Population of 40 Holstein Bulls.

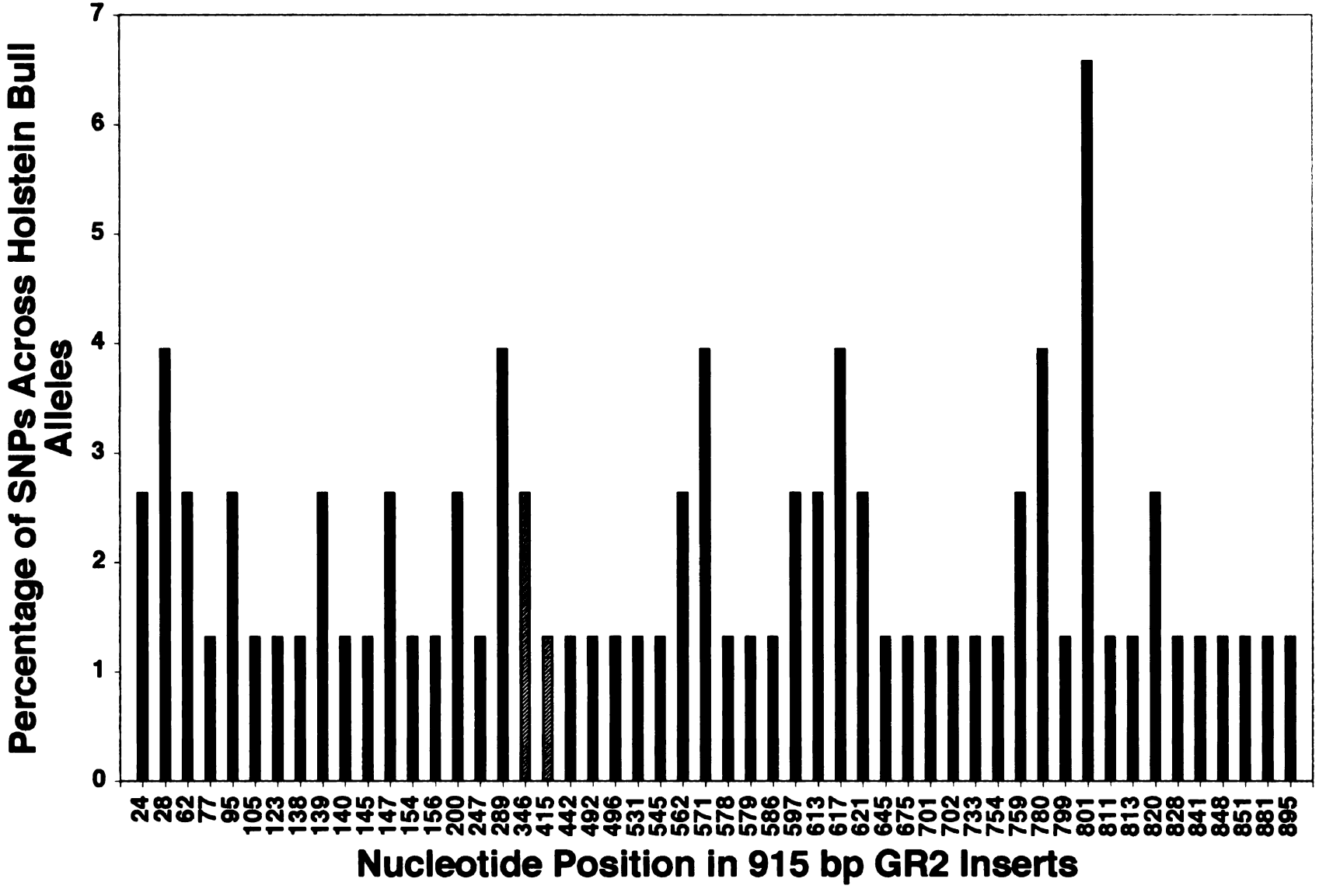


Figure 18a. Summary of Polymorphism Observed in the Predicted Amino Acid Sequences for the τ1 Region of GR in a Population of 40 Holstein Bulls.

Twenty-nine amino acid residue locations were predicted to contain substitutions encoded by SNPs in the GR2 loci shown in Figure 16. In this figure, the vertical axes in both panels list the bulls' identification numbers, from least to greatest, (eg. 4536, 4591, 4628, etc.) along with the allele (1 or 2) shown for a particular row of amino acid sequence. The consensus sequence (**Cons**) identified by GCG is shown at the top and bottom of each panel. The horizontal axes at the bottom of the panels show the amino acid residue positions within the full τ1 predicted peptide sequence where amino acid substitutions occurred due to GR2 SNPs. Letters interrupting vertical lines are the actual changed residues (relative to consensus). No amino acid substitutions occurred within the actual τ1 core, which was predicted to lie between residues 101 and 146. As before, highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 18a. Summary of Polymorphism Observed in the Predicted Amino Acid Sequences for the τ1 Region of GR in a Population of 40 Holstein Bulls.

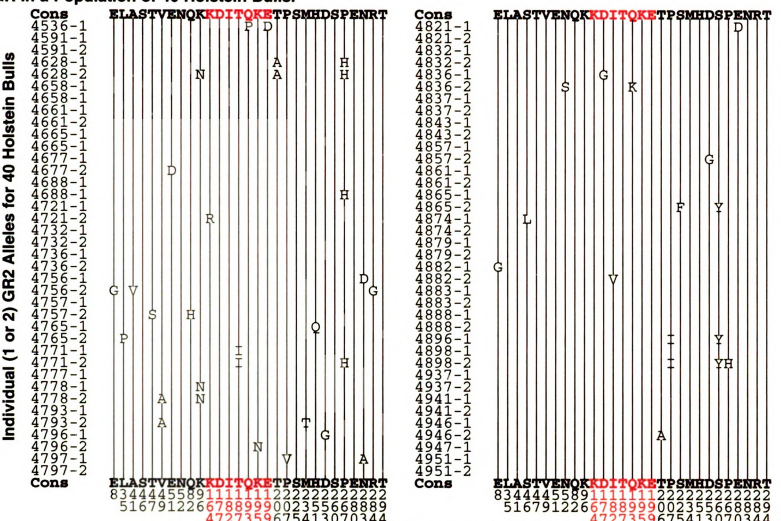
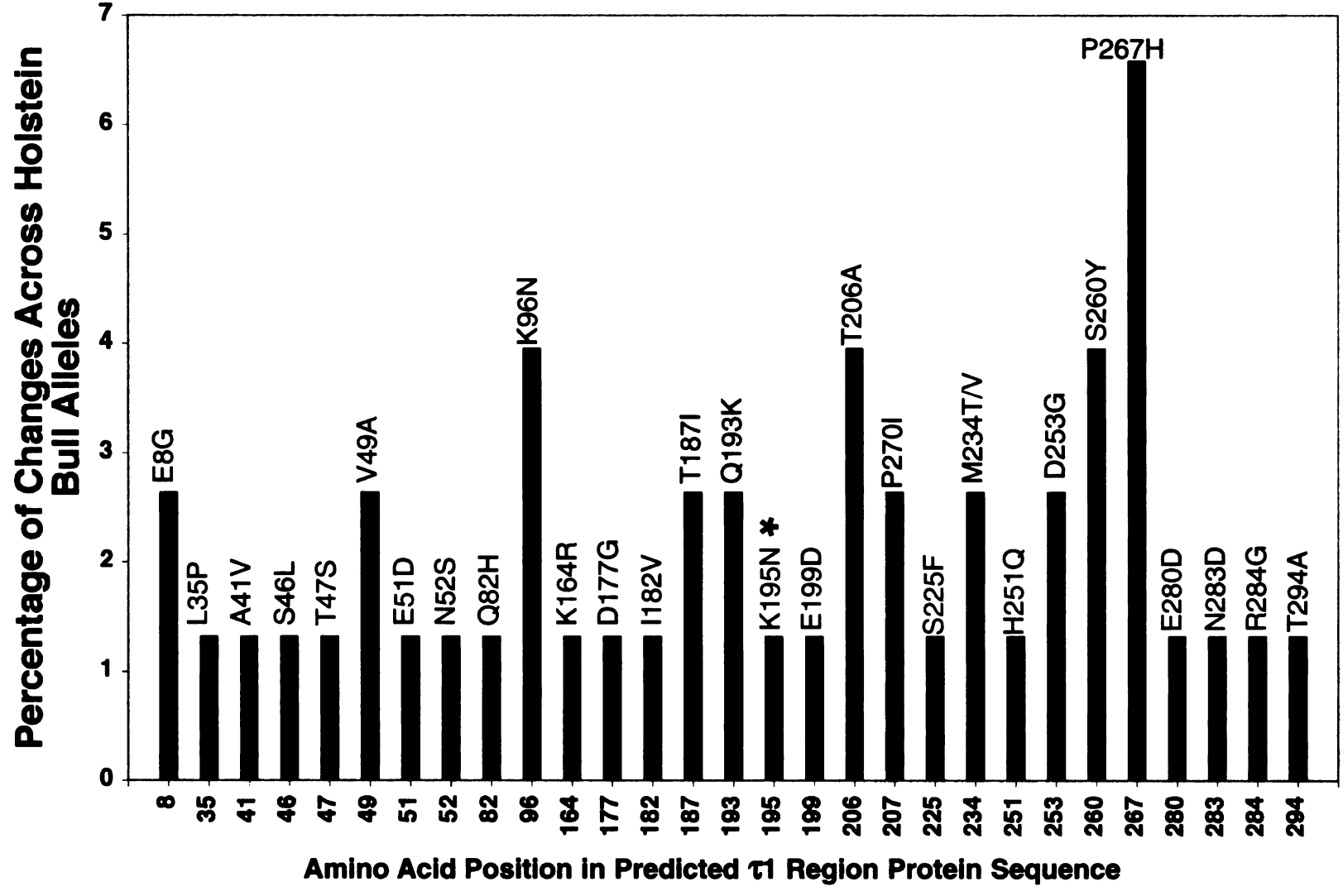


Figure 18b. Summary of the Frequencies of Changes in the Predicted Amino Acids of GR τ1 Regions in 40 Holstein Bulls.

This figure shows frequencies of allelic amino acid residue substitutions predicted from GR2 SNPs in Figure 17 and expressed as percentages of all alleles studied. The Y-axis shows the percentage of allele differences detected and the X-axis shows the residue position of these allele changes. Actual amino acid substitutions at each residue location are given above each bar. The asterix above amino acid position 195 identifies this change as falling within a synergy control motif. This figure shows that the most frequently occurring amino acid substitutions occurred at residues 96 (3.9%), 206 (3.9%), 260 (3.9%), and 267 (6.6%).

Figure 18b. Summary of the Frequencies of Changes in the Predicted Amino Acids of GR τ1 Regions in 40 Holstein Bulls.



iv. Associations of Identified GR2 SNPs with Glucocorticoid-Responsive Traits.

In order to estimate potential associations between GR2 polymorphism and inherited glucocorticoid-sensitive leukocyte traits or milk production potential in the 40 test bulls, the MIXED procedure of Statistical Analysis System (SAS; Cary, NC) was used to perform one-way analysis of variance (ANOVA), which was used to statistically associate individual SNPs with the battery of traits shown in **Table 6**. These included 6 glucocorticoid-sensitive leukocyte traits [percentages of circulating T lymphocytes expressing CD4 (% CD4) and CD8 (% CD8); percentage of circulating B lymphocytes (% B cells); neutrophil expression of the adhesion molecule CD18 (CD18 expression); and mononuclear leukocyte expression of MHC II molecules (MHC II expression)]. Each of these leukocyte traits responded to glucocorticoid challenge in the test bulls and possessed significant additive genetic variation in the recovery phase following termination of the steroid treatments (Tempelman et al., 2002; Burton et al., in review; **Table 6**). Therefore, the recovery phase of these leukocyte traits were used in the analyses of SNP-phenotype associations. Genetic merit (predicted transmitting abilities, or PTA) for the milk production traits were obtained for each bull from a web site housed at the Animal Improvement Programs Laboratory of the United States Department of Agriculture (AIPL-USDA, http://www.aipl.arsusda.gov/). The PTAs studied included milk yield, milk protein yield, and milk SCS (**Table 6**). These traits of genetic merit (PTAs) were weighted against their reliability estimates (the number of daughter records which were used to generate the milk production PTAs) before SNP-trait associations were estimated.

Results of the association analyses are shown in the panels of **Figure 19**, which presents a series of trait plots where the x-axes are the nucleotide posi-

tions of identified GR2 SNPs and the y-axes are the *P*-values from F-tests performed during the one-way ANOVAs. A total of 13 unique SNP positions were identified as associating significantly with the listed traits at $P \leq 0.05$ (**Table 7**). In **Figures 19a**, **b**, and **c**, SNPs at positions 24, 77, 95, 105, 145, 200, 617, 754, and 813 significantly ($P \leq 0.05$) associated with % CD4 (**Figure 19a**), % B cells (**Figure 19b**), and MHC II (**Figure 19c**). Additionally, several SNPs were found to associate significantly with multiple traits. These SNPs are highlighted by solid black data points in the panels of **Figure 19**. For example three SNPs at nucleotides 621, 780, and 820 associated significantly ($P \leq 0.05$) with MHC II (**Figure 19c**) and CD18 (**Figure 19d**), and a SNP at position 139 significantly associated with both CD18 MFI (**Figure 19d**) and % CD8 (**Figure 19e**). Summarizing across **Figures 19a** through **19e**, SNPs at nucleotide positions 139, 621, 780, and 820 in GR2 may be important to GR function because each influenced the phenotype of at least two glucocorticoid-sensitive leukocyte traits. It was noteworthy that neither of the two SNPs occurring within the τ1-encoding DNA, at positions 346 and 415, were associated with any analyzed traits. This may have been due to the fact that these SNPs did not change amino acids (**Figures 17** and **18**).

SNPs at GR2 nucleotide positions 621, 780, and 820 were also shown to associate ($P \leq 0.05$) with a bull's predicted transmitting ability for milk yield (**Figure 19f**), but not milk protein or milk somatic cell score PTAs. Interestingly, these SNPs also influenced MHC II expression (**Figure 19c**) and CD18 expression (**Figure 19d**; **Table 7**), two traits that are critical for early inflammatory responses that clear intramammary infections in dairy cows.

Following translation of GR2 DNA sequences into amino acid changes, these changes were analyzed to determine whether changes to the GR protein would also associate with traits of immune dysfunction. Changes at amino

Figure 19. Associations Between Individual GR2 SNPs and a Variety of Glucocorticoid-Responsive Leukocyte Traits and Milk Production PTAs.

In each panel, *P*-values, on a $\log_{10}$ scale, from one-way ANOVAs of SNP-trait analyses are given on the y-axes, with a horizontal black line representing significance at $P \leq 0.05$. The x-axes in each panel show nucleotide positions within the GR2 locus where SNPs were identified. SNPs that associated with more than one trait in panels **(a)** through **(f)** are indicated with solid black circles (●), SNPs that associated with only the trait in a given panel are indicated by hatched circles (⊘), and SNPs that were not associated with any measured trait are indicated by open circles (○). **(a)** % CD4 T cells; **(b)** % B cells; **(c)** mononuclear leukocyte expression of MHC II; **(d)** neutrophil CD18 expression; **(e)** % CD8 T cells; **(f)** milk yield PTA, **(g)** milk protein yield PTA, and **(h)** milk SCS PTA. SNPs at nucleotide positions 139, 621, 780, and 820 all associated significantly ($P \leq 0.05$) with multiple leukocyte traits (listed above) as well as with milk yield (nucleotides 621, 780, 820).

Figure 19a. % CD4 Cells

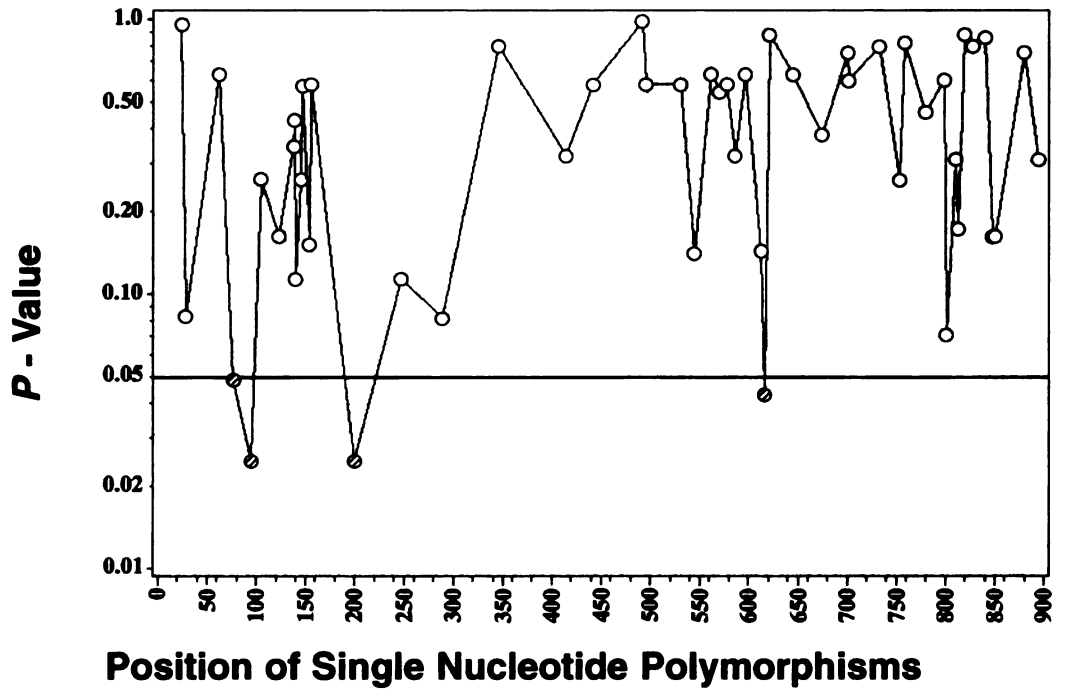
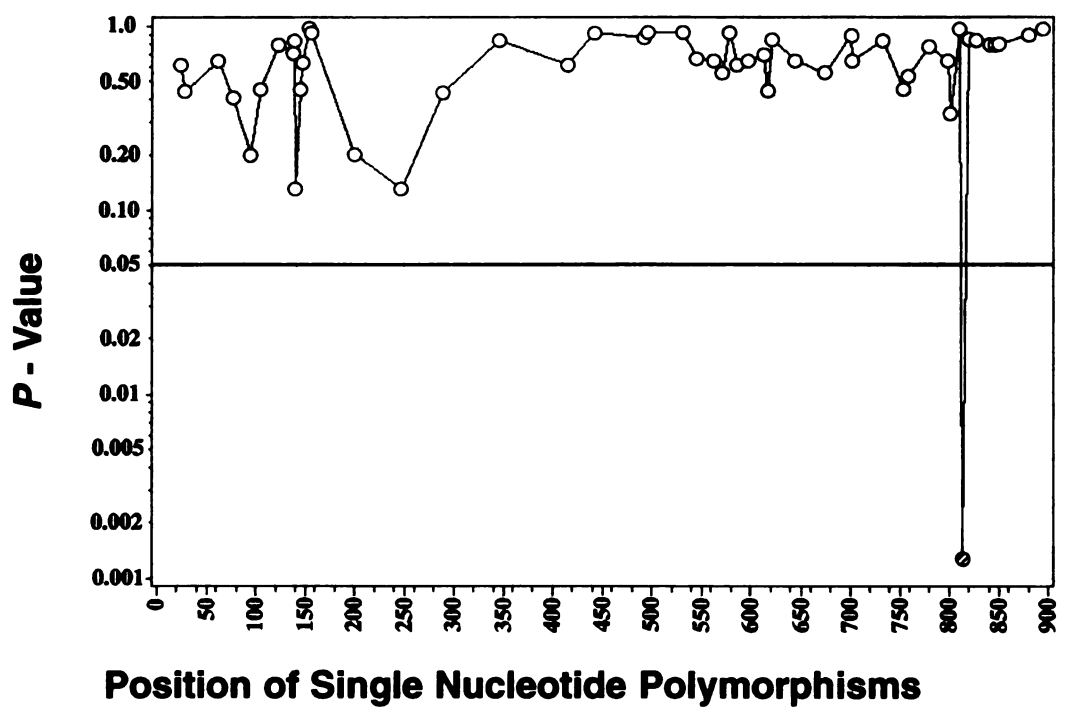


Figure 19b. % B Cells



⊘ = Associates with only this trait

● = Associates with 2+ traits

○ = No association with any traits

Figure 19c. MHC II

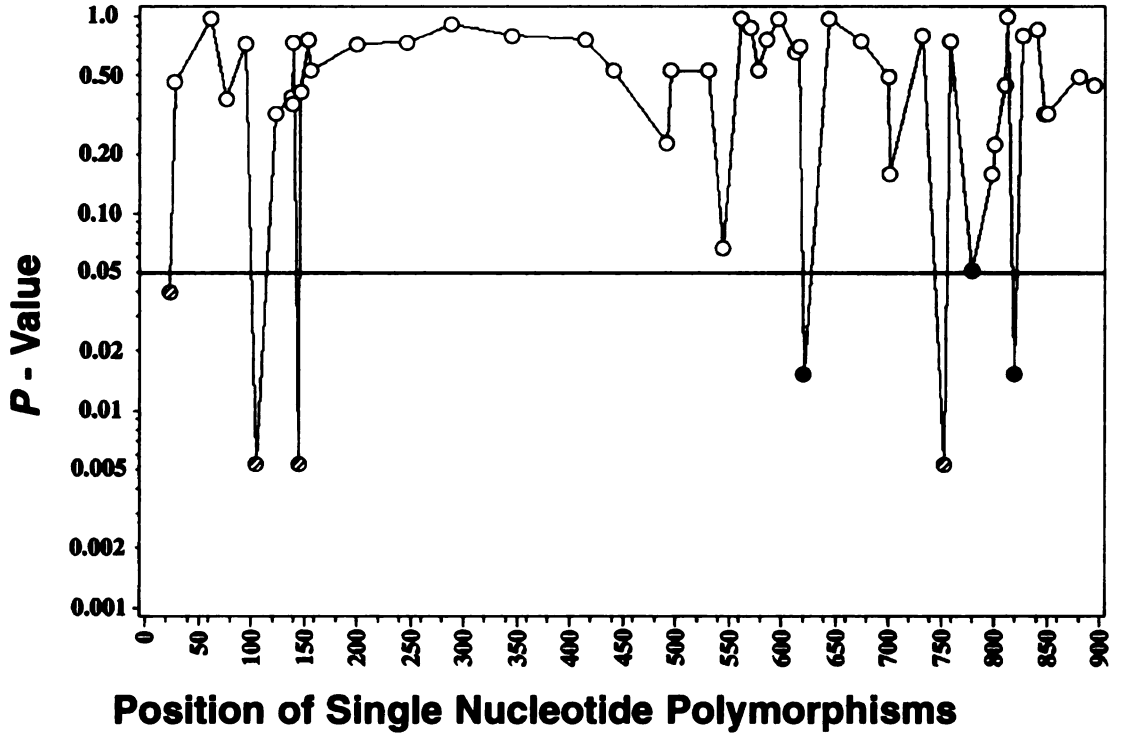
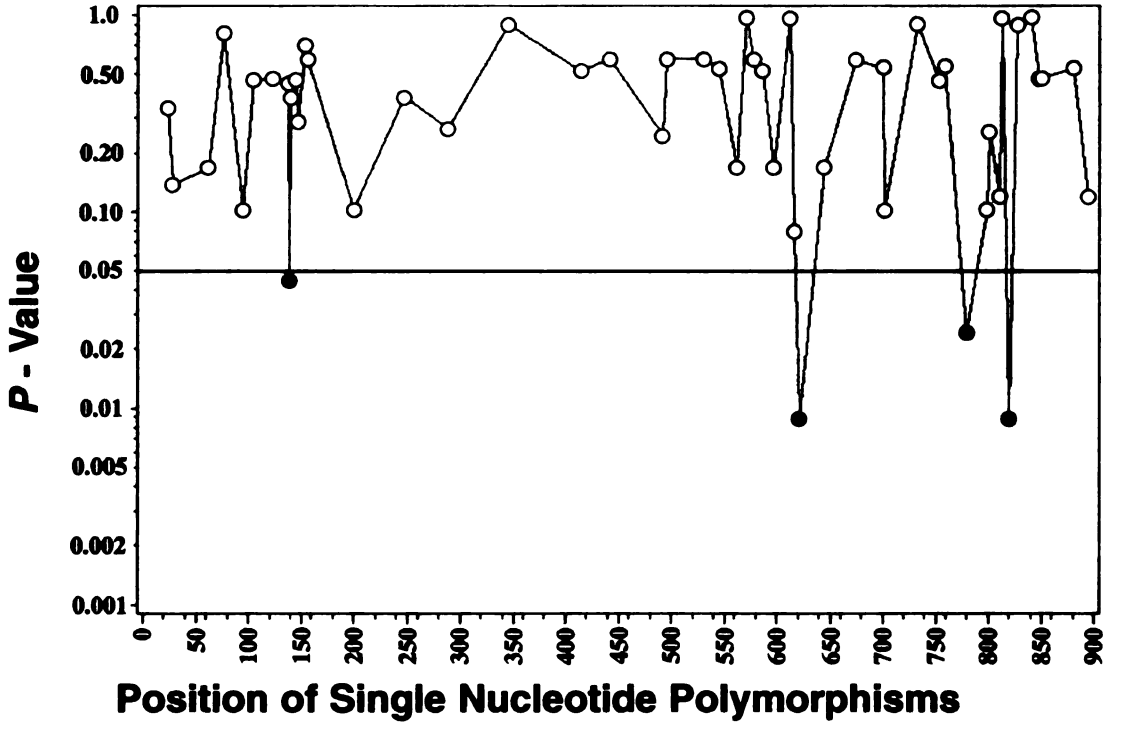


Figure 19d. CD18



⊘ = Associates with only this trait

● = Associates with 2+ traits

○ = No association with any traits

Figure 19e. % CD8 Cells

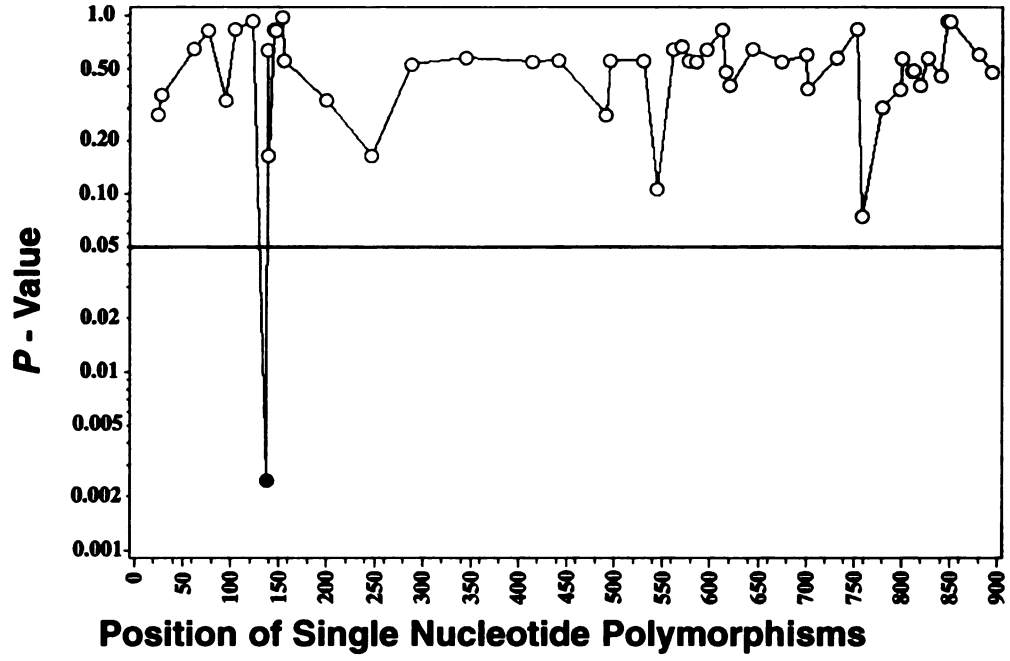
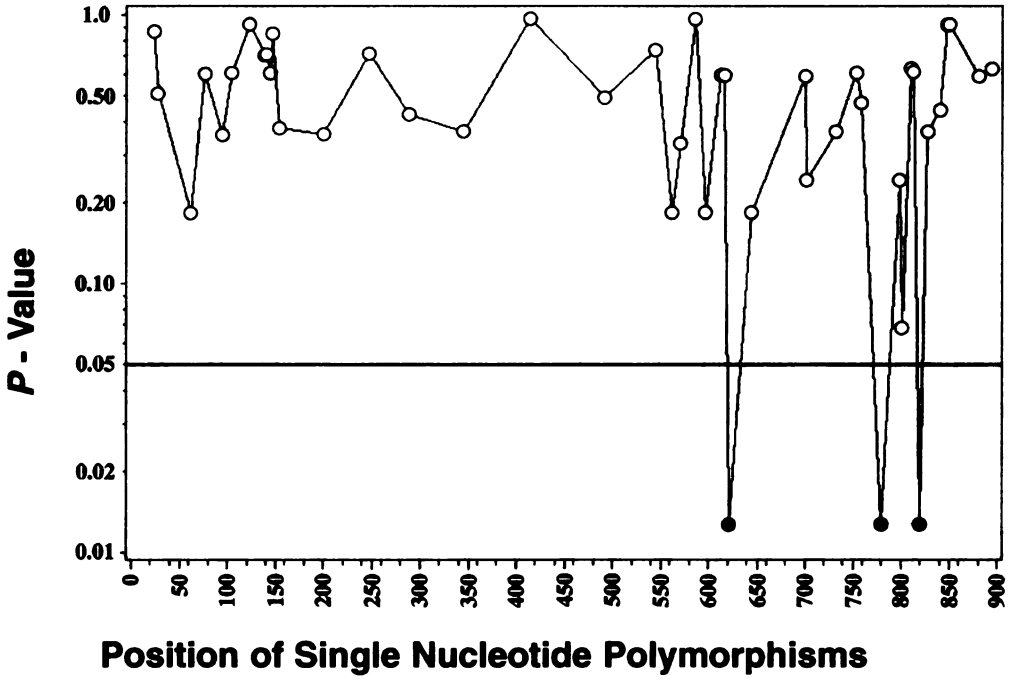


Figure 19f. Milk PTA



⊘ = Associates with only this trait

● = Associates with 2+ traits

○ = No association with any traits

Figure 19g. Milk Protein PTA

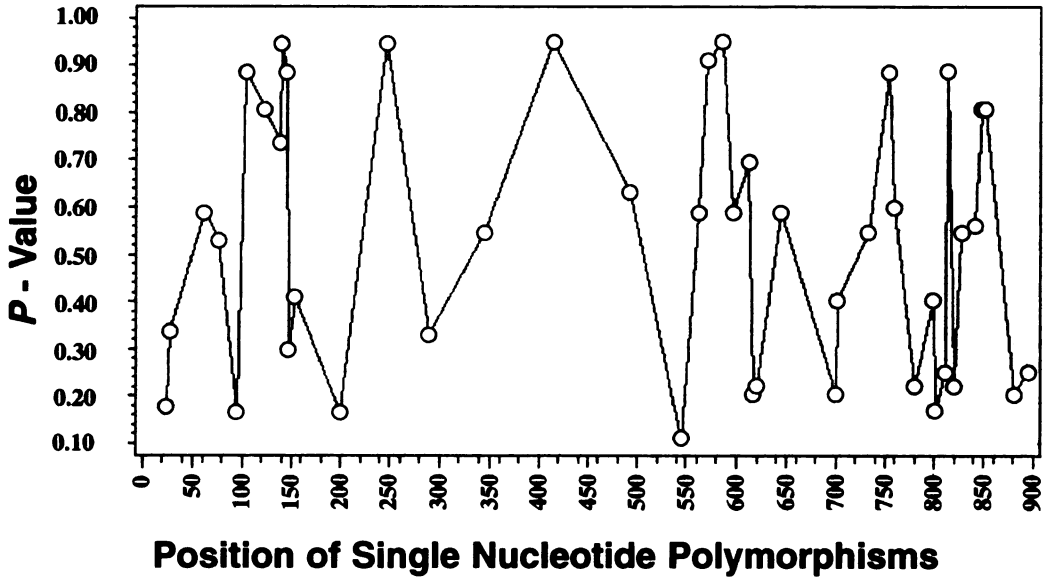
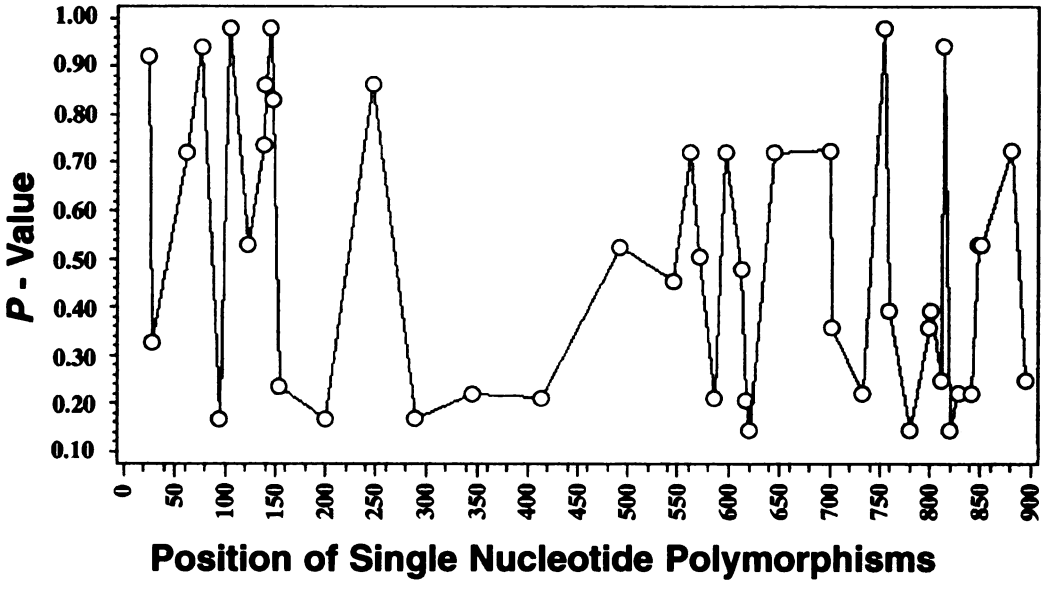


Figure 19h. Milk SCS PTA



= Associates with only this trait

= Associates with 2+ traits

= No association with any traits

Table 7. Summary of Significant Associations Between GR2 SNPs and Leukocyte and Milk Production Traits.

GR2 SNPs are coded as the consensus nucleotide (left) followed by the nucleotide position and the changed nucleotide that made up the SNP (right). The left-hand column depicts the GR2 nucleotide positions harboring SNPs and the traits used for association analyses are listed across the top. Nucleotide changes from the consensus GR2 sequence found to be associated with a trait are listed under that trait. Associations that were significant ($P \leq 0.05$), or were highly significant ($P \leq 0.0005$) are indicated by superscripts "a" and "b" respectively. Fourteen SNPs were significantly associated with traits at $P \leq 0.05$. Interestingly, bulls containing SNPs significantly associating with % CD4 T cells had higher percentages of CD4 T cells than the remaining bulls.

Table 7. Summary of Significant Associations Between GR2 SNPs and Leukocyte and Milk Production Traits.

POSITION OF SNP	% CD4 T Cells	CD 18 Expression	MHC II Expression	% B Cells	% CD8 T Cells	Milk Yield PTA
24			A24G[a]			
77	C77T[a]					
95	G95A[a]					
105			T105C[b]			
139		A139G[a]			A139G[b]	
145			T145C[b]			
200	G200A[a]					
545						
617	A617G[a]					
621		C621T[b]	C621T[b]			C621T[b]
754			C754A[b]			
759						
780		C780A[a]	C780A[a]			C780A[b]
813				T813C[b]		
820		A820G[b]	A820G[b]			A820G[b]

[a] Significant at $P \leq 0.05$
b Highly significant at $P \leq 0.0005$

Table 8. Summary of Significant Associations Between GR2 Amino Acid Changes and Leukocyte and Milk Production Traits.

Polymorphisms are coded as the consensus amino acid (left) followed by the amino acid position and the amino acid change that was encoded by the GR2 SNP (right). The left-hand column depicts the GR2 amino acid positions harboring changes and the traits used for association analyses are listed across the top. Changes from the consensus GR2 amino acid sequence found to be associated with a trait are listed under that trait. Five amino acid changes were significantly associated with traits at $P \leq 0.05$.

Table 8. Summary of Significant Associations Between GR2 Amino Acid Changes and Leukocyte and Milk Production Traits.

POSITION OF Amino Acid Change	% CD4 T Cells	CD 18 Expression	MHC II Expression	% CD8 T Cells	Milk Yield PTA
8			E8G		
35			L35P		
46				S46L	
206	T206A	T206A	T206A		T206A
260		S260Y	S260Y		S260Y

acid positions 8, 35, 46, 206, and 260 all associated significantly ($P \leq 0.05$) with MHC II expression, % CD8, % CD4, CD18 expression, and milk yield PTA within this group of Holstein bulls (**Table 8**), indicating that changes to the GR protein may indeed effect immune responses to stressful stimuli. Therefore, GR2 SNPs identified in this study associate significantly with both inherited glucocorticoid-sensitive leukocyte traits and milk yield PTA, suggesting that these may be potential candidate molecular genetic markers for health and productivity in dairy cows, particularly as 5 of these SNPs translated into amino acid changes which also associated significantly with altered immune function.

D. Discussion

A population of 40 Holstein bulls with documented additive genetic variation in leukocyte traits known to be responsive to glucocorticoids were used for GR gene polymorphism identification. A PCR-amplified region of the GR2 locus of 915 bp in length was found to be 92% and 93% homologous to the corresponding GR2 DNA and protein sequences in humans. We also found that the GR2 locus in Holsteins contained SNPs in 52 locations, more than half of which translated into conservative and (or) non-conservative changes in predicted amino acid sequences in and around the $\tau 1$ core of bovine GR. The amino acid change at position 195 is interesting since it falls within a GR2 synergy control motif, *in vitro* mutation of which resulted in a 9 fold enhancement of transcription by human GR (Iniguez-Lluhi and Pearce, 2000). Association of GR2 SNPs with traits of dexamethasone-induced leukocyte changes highlighted key mutations (at nucleotide positions 139, 621, 780, 820) that related to glucocorticoid sensitivity of a bull's leukocytes. Interestingly, the same mutations at nucleotide positions 621, 780, 820 associated significantly with a bull's PTAs for milk yield. Milk protein and.milk SCS PTAs only tended toward an

association ($P \leq 0.20$) with any GR2 SNP. Therefore, we have not only found that the GR2 locus is polymorphic in Holsteins, but also that some of these polymorphisms are predicted to change protein sequence and to associate with heritable glucocorticoid-responsive leukocyte traits and genetic merit for milk production.

Although this is the first study of polymorphism in bovine GR genes, biomedical literature is abundant in which protein-coding regions of human GR genes have been studied for mutations suspected to be associated with disease phenotypes such as primary glucocorticoid resistance (Malchoff et al., 1993), Cushing's syndrome (Karl et al., 1996a,b), increased sensitivity to glucocorticoids (Huizenga et al., 1998), high blood pressure (Koper et al., 1997), and obesity (Dobson et al., 2001; **Tables 1** and **2**). Some work has even identified point mutations in the human GR2 locus (reviewed in de Lange et al., 1997), but these had no apparent associations with the specific diseases or conditions under study. In contrast, site-directed mutagenesis of the human GR2 locus has revealed key amino acids that are critical for GR's transcription regulation of target gene expression (Almlof et al., 1997; **Table 3**). In that work it was noted that clusters of 2 or 3 mutations had significantly more impact on subsequent GR regulation of glucocorticoid-responsive gene expression than any single mutation had on its own. Additionally, such mutagenesis studies in the human GR2 locus revealed what appeared to be different "surfaces" of the $\tau 1$ domain that either activated or repressed transcription of glucocorticoid-sensitive genes (Iniguez-Lluhi et al., 1997). It was also noted that several amino acid substitutions within these surfaces were required to significantly alter the transcription regulatory functions of GR's $\tau 1$ domain (Iniguez-Lluhi et al., 1997). In the current study we have identified several clusters of naturally occurring mutations in bovine GR2 that change predicted amino acid

sequences and also related to multiple leukocyte and milk production phenotypes with known sensitivity to glucocorticoids. Together with findings from site-directed mutagenesis of human GR2 (Almlof et al., 1997; Iniguez-Lluhi et al., 1997), it is possible that the SNP clusters we have identified in bovine GR2 genes changes one or more "activation surfaces" of bovine GR proteins, thus explaining associations with the traits we observed. However, analyses utilizing the association of multiple SNPs with leukocyte traits need to be performed in order to begin determining whether GR τ1 surfaces are disrupted by GR2 amino acid changing SNPs. If true, these SNPs may be useful in identifying bulls who may transmit beneficial "stress resistance" phenotypes to their daughters, which may in turn benefit health and milk production in these cows.

CHAPTER 6. ASSOCIATIONS BETWEEN MILK PRODUCTION TRAITS AND GLUCOCORTICOID RECEPTOR GENE POLYMORPHISM IN AMERICAN JERSEY BULLS

A. Introduction

Inflammation of the mammary gland, or mastitis, affects milk-producing mammals and is responsible for significant monetary losses for the US dairy industry (DeGraves and Fetrow, 1993). Within the bovine mammary gland the primary immune cells that fight invading pathogens are blood-derived neutrophils (Paape et al., 2000; Kehrli and Harp, 2001), which cause the characteristic increase in milk somatic cell counts during mastitis. Milk somatic cell counts are averaged over a cow's lactations and put on a $\log_{10}$ scale to determine her somatic cell score (SCS; Schutz and Powell, 2003), which is used by veterinarians and producers as an indicator of mastitis for management decisions related to improved mammary gland health. One such decision which is utilized currently is genetic selection, where bulls with PTAs for low milk SCS can be selected to produce daughters with decreased milk somatic cells. However, milk SCS may not be the optimal trait for genetic selection against mastitis because its heritability is moderate (0.10 to 0.14; Detilleux 2002) and it is influenced by many environmental factors in addition to the presence of intramammary infection. As a result, researchers have studied a variety of immune traits as possible indicators of mastitis susceptibility and resistance. One such group of traits are leukocyte sensitivities to glucocorticoid hormones because these steroids achieve high concentration in blood around parturition and other stress periods when cows are the most susceptible to mastitis (Burton and Erskine, 2003).

Previous studies have demonstrated that when cattle experience signifi-

cantly increased levels of circulating glucocorticoids, either during parturition (Preisler et al., 2000a) or exogenous administration of the hormone (Burton et al., 1995), their blood neutrophils display marked decreases in expression of the adhesion molecules CD62L and CD18 (Lee and Kehrli, 1998; Weber et al., 2001) and reduced respiratory burst activity (Detilleux et al., 1994), rendering the leukocytes dysfunctional in their migration and bactericidal activities and leaving the mammary gland vulnerable to colonization by opportunistic bacteria (Burton and Kehrli, 1995; Shuster et al., 1996). Glucocorticoids have also been shown to affect various phenotypes of lymphocytes and monocytes/macrophages involved in inflammatory and adaptive immune responses (Burton and Kehrli, 1996; Nonnecke et al., 1997; Yu et al., 1998), all of which may contribute to mastitis susceptibility in hormone-treated and parturient dairy cows (Burton and Erskine, 2003).

The effects of glucocorticoids on leukocytes are mediated by homologous cytosolic receptors called glucocorticoid receptors or GR. GR is a ligand-activated transcriptional regulator of glucocorticoid-sensitive genes (Bamberger 1996; Tsai and O'Malley, 1994). To affect gene expression, hormone activated GR form homodimers that readily translocate into the nucleus. Once in the nucleus, GR interacts with other transcription regulatory proteins through its τ1 domain to affect expression of glucocorticoid-responsive genes through both direct and indirect mechanisms (Bamberger et al., 1996; Ford et al., 1997).

Recently, our laboratory has shown that bovine leukocyte sensitivities to glucocorticoid challenge possess additive genetic variation (Tempelman et al., 2002; Burton et al., in review). These findings implicated GR as possibly contributing to genetic variation in leukocyte dysfunctions that correlate with mastitis susceptibility in parturient and hormone-treated cows. We substantiated this possibility by showing that the τ1-encoding region of bovine GR genes

(called the GR2 locus) is polymorphic in six cattle breeds (**Chapter 4**) and in Holstein bulls (**Chapter 5**), and that certain key amino acid-changing GR2 mutations associated statistically with a bull's neutrophil and lymphocyte sensitivities to glucocorticoid challenge and his PTA for milk yield (Jacob et al., in preparation; see **Figure 19** in **Chapter 5**). Therefore, GR2 polymorphism may explain some of the genetic variation in mammary gland immunity against mastitis, including milk SCS.

In the current study we sought to determine whether the GR2 locus is also polymorphic in a population of Jersey bulls selected for high versus low milk SCS PTA, and if certain GR2 mutations associate significantly with a bull's PTA for milk SCS, milk yield, and (or) milk protein yield. We identified a group of 20 Jersey bulls with milk SCS PTAs ≥ 2 standard deviations above (n = 11) or below (n = 9) the mean SCS for all of their contemporaries and used genomic DNA from sperm of these animals to confirm that the GR2 locus is polymorphic and that one key mutation associated with the milk production traits studied.

B. Materials and Methods

i. Identification of Jersey Bulls with High Versus Low Milk SCS PTA

Twenty Jersey bulls were identified for this study based on milk SCS PTA. As shown in **Table 9**, 11 of the bulls had milk SCS PTA ≥ 2 standard deviations from the mean SCS PTA (3.27) of their contemporaries and were labeled HSCS1, HSCS2, ..., HSCS11. The remaining 9 bulls had milk SCS PTAs ≤ 2 standard deviations from the contemporary mean (LSCS1, LSCS2, ..., LSCS9). The various sibling relationships between the 20 test bulls are also indicated in **Table 9** and were determined from records of the bulls' sires and maternal grandsires resident at the National Association of Animal Breeders

Table 9. Rankings of Jersey Bulls by Somatic Cell Score (SCS) Predicted Transmitting Ability (PTA).

Bull Identification	Milk SCS PTA	Milk Protein PTA	Milk Yield PTA
HSCS1‡	3.69	39	1061
HSCS2† §	3.66	9	996
HSCS3† §	3.66	-2	-55
HSCS4	3.62	24	1387
HSCS5† §	3.61	28	1009
HSCS6‡	3.58	39	748
HSCS7† §	3.58	19	1050
HSCS8†	3.57	27	1490
HSCS9	3.55	22	1289
HSCS10† §	3.51	20	900
HSCS11	3.50	13	374
Contemporary Mean SCS PTA = 3.27			
LSCS9◊	3.28	26	807
LSCS8	3.26	23	734
LSCS7	3.25	14	303
LSCS6	3.24	30	1122
LSCS5† Δ	3.18	27	234
LSCS4	3.16	21	328
LSCS3† Δ	3.16	5	840
LSCS2	3.16	21	1145
LSCS1◊	3.04	24	731

†Denotes animals which are in half-sibling group 1;
‡Denotes animals which are in 3/4 sib group 1;
◊Denotes animals which are in 3/4 sib group 2;
ΔDenotes animals in 3/4 sib group 3;
§Denotes animals in 3/4 sib group 4.

Table 9. Rankings of Jersey Bulls by Somatic Cell Score (SCS) Predicted Transmitting Ability (PTA). The left-hand column depicts the Jersey bulls ranked by SCS PTA and coded as high SCS (HSCS) or low SCS (LSCS), from highest to lowest scores. The traits studied are listed across the top and each bull's PTA for that trait listed in the table. Bulls related to each other as half-sib or three-quarter sib groups are indicated by symbols.

web site (NAAB, http://www.naab-css.org; see **Appendix F** for sire and maternal grandsire information) and provided by the American Jersey Cattlemen's Club (AJCC, Reynoldburg, OH). Straws of semen for each bull were located in the field and purchased by AJCC for this study, and the straws shipped in liquid nitrogen to our laboratory at Michigan State University for isolation of genomic DNA.

ii. Isolation of Genomic DNA from Semen, PCR Amplification of GR2, and Molecular Cloning and DNA Sequencing of GR2

Genomic DNA was isolated from the donated semen samples as described in **Chapter 4,Section B*ia***. GR2 fragments were amplified from genomic DNA using PCR as described in **Chapter 4, Section B*ii***, and cloned into pGEM-T Easy vectors for subsequent DNA sequencing, as described in **Chapter 4, Section B*iv*** and ***v***.

iii. GR2 Polymorphism Identification at the DNA and Predicted Amino Acid Sequence Levels

GR2 allelic sequences were analyzed for mutations using GCG (Madison, WI) software as described in **Chapter 4, Section B*vi***. Briefly, 'chopup', 'reformat', and 'assemble' commands of GCG were used to align and join the forward and reverse sequences of GR2 alleles. The 'map' and 'translate' commands of GCG were then used to determine the open reading frame and obtain predicted amino acid sequences from each GR2 DNA sequence. SNPs were compared across half-sibling groups to ensure Mendelian inheritance was not violated. Consensus DNA and amino acid sequences and multiple sequence files were generated using the 'pileup' and 'pretty' commands of GCG, and the sequence files for all GR2 alleles imported into an Excel spreadsheet (Microsoft Office v. x, Redmond, WA) for generation of plots and figures used for data presentation. This approach allowed ready visualization and

summary of SNPs and resulting amino acid sequence changes in the bovine GR2 gene.

iv. Estimation of Association Between GR2 SNPs and Milk Production PTAs

Original PTA for bulls' milk SCS (Sept. 1999), and recent (Sept. 2002) PTAs for milk yield and milk protein yield were obtained from the Animal Improvement Programs Laboratory of the United States Department of Agriculture (AIPL-USDA; www.aipl.arsusda.gov). Associations of milk yield PTA, milk protein yield PTA, and milk SCS PTA with individual GR2 SNPs were determined by using the MIXED procedure of SAS to conduct one-way ANOVAs. Results were graphed as 'SNP position' against the '*P*-value' of the F-statistic with a line of significance added at $P = 0.05$. SNP positions falling on or below this line were considered significantly associated with the trait.

C. Results

Images in this dissertation are presented in color

i. The GR2 Locus is Polymorphic in American Jersey Bulls

DNA sequence analysis of GR2 from 20 American Jersey bulls revealed mutations (SNPs) at a total of 38 nucleotide positions within the 915 bp stretch of amplified GR2 (**Figure 20a**). When compared across half-sibling groups, SNPs did not violate Mendelian inheritance. The frequency of most SNPs was very low (one or two occurrences in the population of 20 bulls), but the SNPs at nucleotide positions 319, 429, 562, 660, and 703 occurred more often with allelic frequencies ranging from 10.5% to 18% (**Figure 20b**). The SNP at position 561 was very polymorphic (52% frequency) in this population of Jersey bulls. It should be noted that two alleles from two separate bulls produced poor quality DNA sequences, so these sequences were discarded. Thus, the

SNP frequencies reported here are based on 38 instead of 40 alleles for the population of 20 bulls.

Figure 20b also shows that there were SNPs at nucleotide positions 561 and 562, with multiple combinations of possible nucleic acid changes apparent in this area of GR2. While most of the 38 SNP locations occurred in DNA sequence encoding regions of GR that flank the actual τ1 core, 4 SNPs at nucleotides 319, 378, 422, and 429 lying within the τ1 core-encoding DNA also harbored SNPs (underlined in the consensus sequence in **Figure 20a** and highlighted by hatched bars in **Figure 20b**). Therefore, the GR2 locus that encodes the τ1 domain of bovine GR are polymorphic in Jersey bulls with high versus low milk SCS PTAs.

II. GR2 Mutations Translate into Amino Acid Substitutions in Predicted Protein Sequence

Twenty-three of the 38 GR2 nucleotide positions harboring SNPs were shown to change amino acids in predicted protein sequences (**Figures 21a** and **b**). Amino acid changes were both conservative (stayed within an amino acid class) and non-conservative (changed the class of amino acid from hydrophobic to hydrophilic, charged to uncharged, etc. See **Appendix B** for residue classification). Of the 23 predicted amino acid changes, 10 were conservative and 14 non-conservative, with residue 187 containing both conservative and non-conservative amino acid changes (**Figure 21a**). Due to the high degree of DNA sequence polymorphism at multiple SNP locations (**Figure 20**), many amino acid residue changes indicated more than 2 possible residue substitutions (**Figure 21b**). In particular, amino acid residue 187, which is translated from the codon at nucleotides 561, 562, and 563, was highly polymorphic across the 20 Jersey bulls. Within the τ1 core itself, four residue changes occurred (D106Q, T126R, C141G, L143R; **Figure 21**; see **Appendix B** for

one-letter residue coding), corresponding to the nucleotide substitutions at residues 319, 378, 422, and 429 (**Figure 20**). Four amino acid substitutions occurred in the amino-terminal region flanking the τ1 core but the majority of protein sequence polymorphisms occurred in the carboxy-terminal region of the τ1 domain (**Figure 21**). The C141G (in τ1), C220S, and Q262P (carboxy-terminal flanking region) non-conservative amino acid substitutions are noteworthy because they might be expected to have effects on steric hindrance and (or) protein folding properties of GR's τ1 domain. Additionally, changes to amino acids at positions 195 and 211 fall within a synergy control motif, which has been found to enhance transcription by human GR by 9 to 12 fold when mutated *in vitro* (Iniguez-Lluhi and Pearce, 2000).

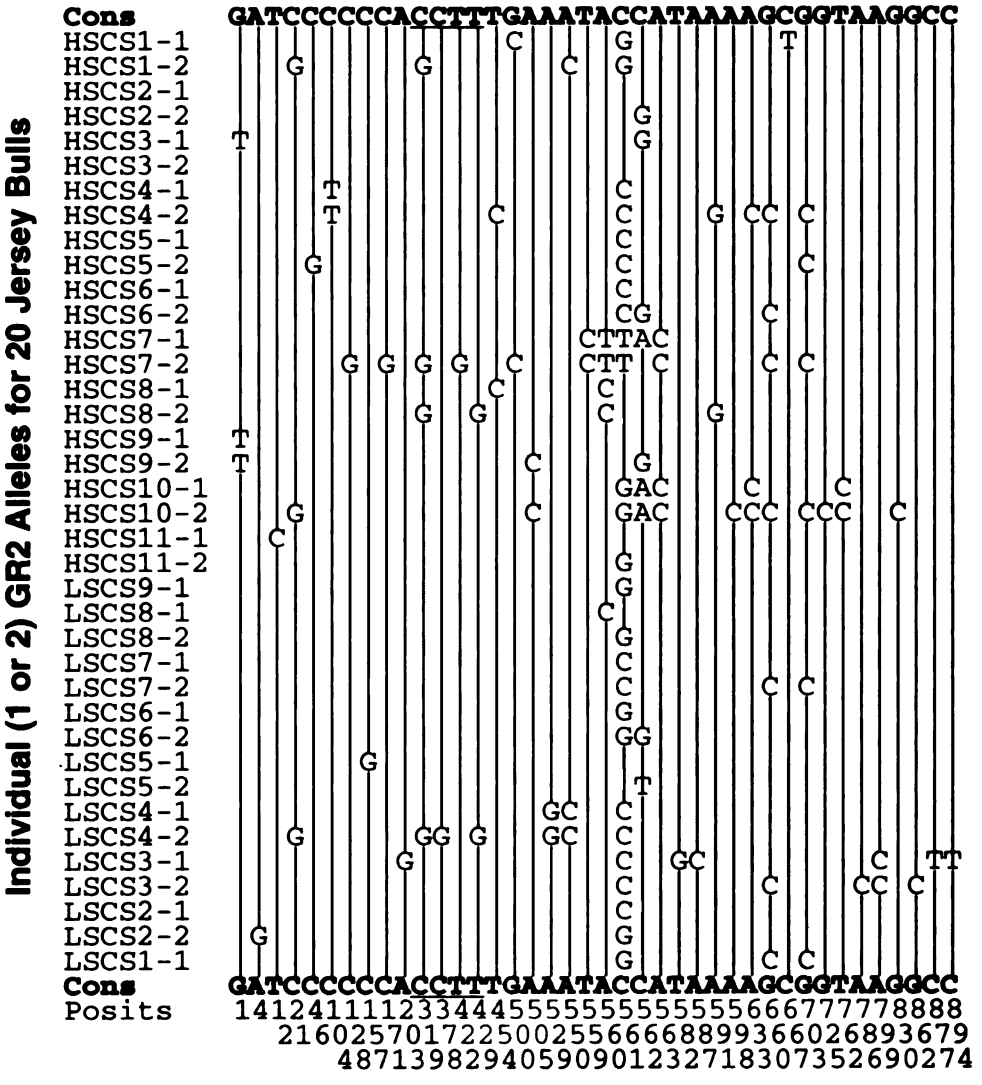
iii. GR2 SNPs Associate with Milk Production PTAs But Not Milk SCS PTA

Results of the one-way ANOVAs associating GR2 SNPs with mammary health and milk production PTAs are shown in **Figure 22**, which presents a series of plots where the X-axes indicate the nucleotide positions within GR2 where SNPs occurred and the Y-axes indicate the level of significance of associations between individual SNPs and the corresponding PTAs. One SNP, at position 598, was identified in this study as being significantly associated with milk production PTAs at $P \leq 0.05$. SNPs at nucleotide position 598 were also significantly associated with milk protein yield PTA (**Figure 22b**). While there were no SNP-milk SCS PTA associations detected that were significant at $P \leq 0.05$, several SNPs at positions 378, 454, 529, 582, 587, 786, 799, 862, 877, and 894 tended toward an association with milk SCS PTA at $P \leq 0.15$ (**Figure 22c**). None of these SNPs were tending towards significance with milk protein or milk yield PTAs. These results suggest that SNPs at position 598 may

Figure 20a. Summary of GR2 Polymorphism Observed in 20 Jersey Bulls.

Thirty-eight SNP locations were identified in the 915-bp region of GR2 amplified by PCR and then cloned for sequencing of individual alleles. In this figure, the vertical axis lists bull identification numbers (ie. HSCS11, HSCS10, HSCS9, etc.) along with the allele (1 or 2) shown for a particular row of DNA sequence. The consensus sequence identified by GCG (**Cons**) is shown at the top and bottom of the figure. The horizontal axis at the bottom of the figure shows the nucleotide positions within the original 915 bp fragments where SNPs were identified. Letters interrupting vertical lines are the actual changed nucleotides relative to consensus. Four SNP positions (319, 378, 422, 429) are located within the $\tau 1$ core-encoding DNA of GR2 and are underlined in the consensus sequences. Highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 20a. Summary of GR2 Polymorphism Observed in 20 Jersey Bulls.



Key:
HSCS = high somatic cell score bulls
LSCS = low somatic cell score bulls
Cons = consensus sequence

Figure 20b. Summary of GR2 SNP Frequencies in a Population of 20 Jersey Bulls.

This figure shows frequencies of SNPs observed in the 915 bp GR2 loci in the Jersey bull population studied expressed as percentages of all alleles studied. The Y-axis shows the percentage of allele differences observed and the X-axis shows the nucleotide position of these alleles. The hatched bars at positions 319, 378, 422, and 429 show SNPs found within the τ1 core-encoding DNA. Solid bars to the left and right of the hatched bars show SNPs found 5' and 3' (respectively) of the τ1 core-encoding DNA. While most SNPs occurred at relatively low frequencies (2.6%), several at positions 319, 429, 562, 660, and 703 occurred at higher frequencies (10.5% to 18%). One nucleotide at position 561 was particularly variable in this population of bulls. Highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 20b. Summary of GR2 SNP Frequencies in a Population of 20 Jersey Bulls.

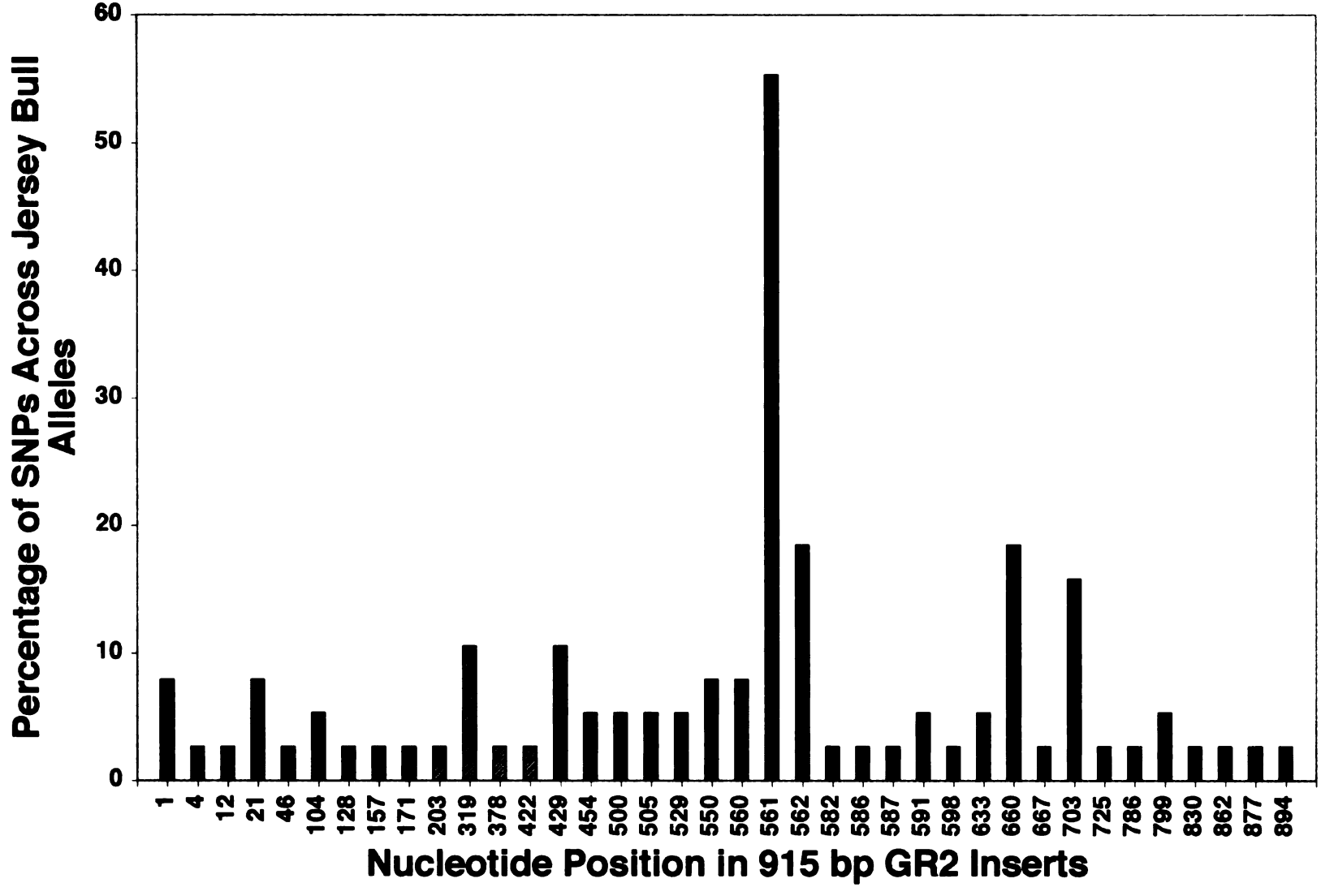


Figure 21a. Summary of Polymorphism Observed in Predicted Amino Acid Sequence Encoded by the GR2 Locus.

Twenty-three amino acid residue locations were predicted to contain substitutions encoded by the GR2 SNPs shown in **Figure 20**. In this figure, the vertical axis lists the bull's identification (ie. HSCS11, HSCS10, HSCS9, etc.) along with the allele (1 or 2) shown for the particular row of amino acid sequence. Consensus sequences (**Cons**) are shown at the top and bottom of the figure. The horizontal axis at the bottom of the figure shows the amino acid positions within the full $\tau 1$ region predicted protein sequence where amino acid substitutions occurred. Letters interrupting vertical lines are the actual residue changes relative to consensus. Four amino acid positions within the $\tau 1$ core were predicted to be changed and are underlined in the consensus sequences. Highlighted in bold red letters are those SNPs which fell within double-pass sequence, while the remaining SNPs on either side were generated from single-pass sequence.

Figure 21a. Summary of Polymorphism Observed in Predicted Amino Acid Sequence Encoded by the GR2 Locus.

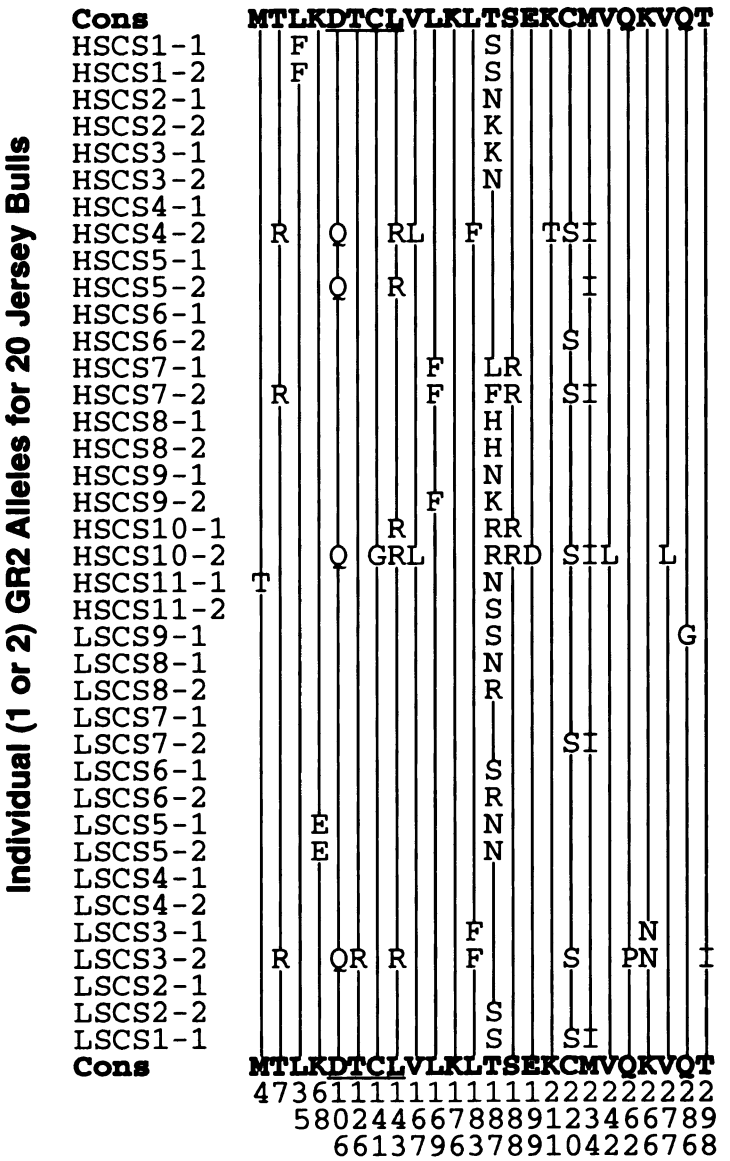


Figure 21b. Summary of Polymorphism Observed in the Predicted Amino Acid Sequences for GR2 Loci in a Population of 20 Jersey Bulls.

This figure shows frequencies of allelic amino acid residue substitutions predicted from GR2 SNPs in **Figure 20** and expressed as percentages of all alleles studied. The Y-axis shows the percentage of allele differences detected and the X-axis shows the residue position of these allele changes. Actual amino acid substitutions at each residue location are given above each bar. Amino acid residues predicted to be changed within the $\tau 1$ core, at amino acid positions 106, 126, 141, 143, are identified by hatched bars. The majority of the amino acid changes had frequencies of 2.6% to 5.3%, but several polymorphisms had frequencies ranging from 8% to 16% within this Jersey bull population. The amino acid at position 187 was highly variable in this population. Changes at amino acid positions 195 and 211 (indicated by an asterix) are located in a GR synergy control motif.

Figure 21b. Summary of Polymorphism Observed in the Predicted Amino Acid Sequences for GR2 Loci.

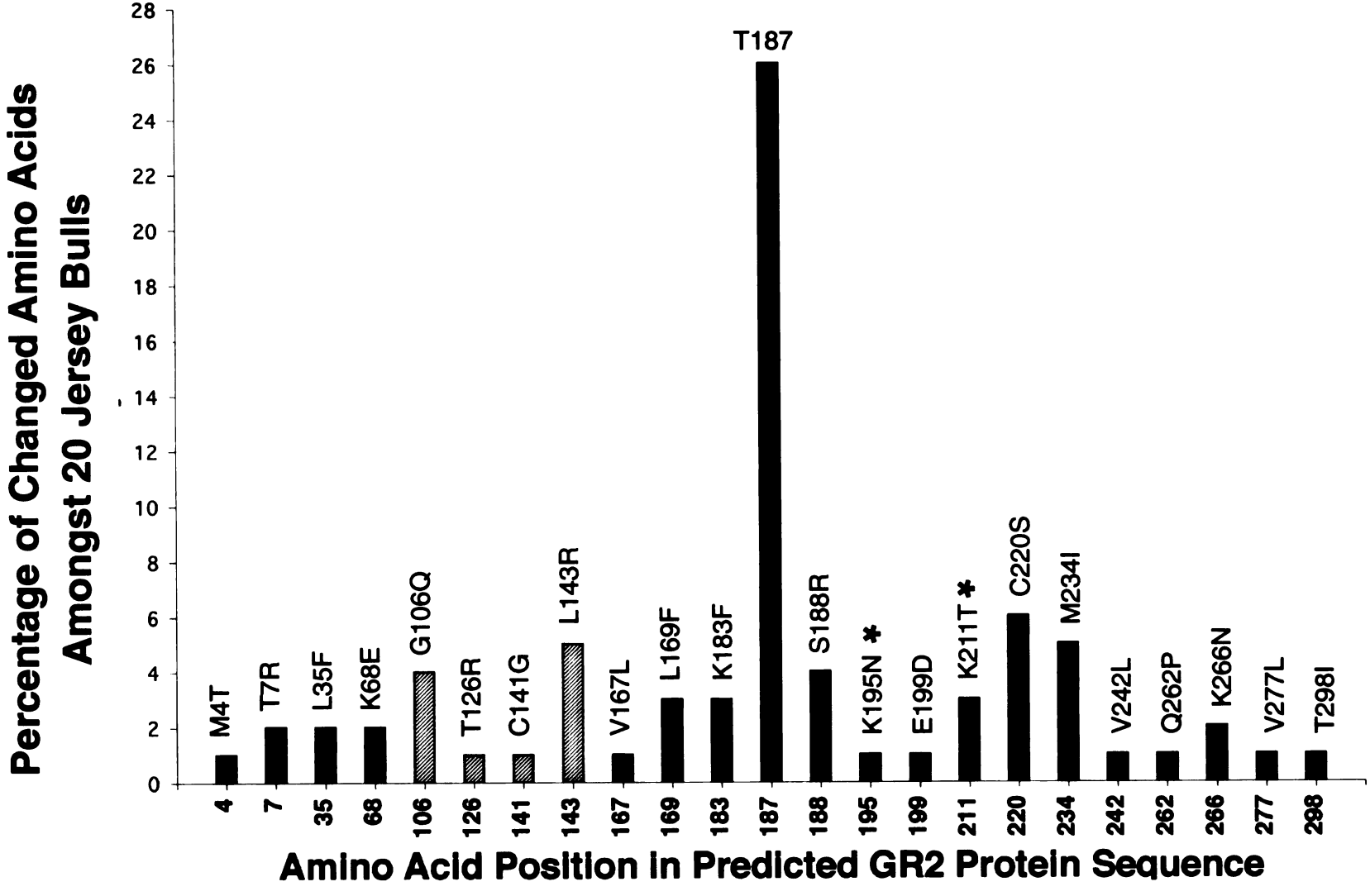


Figure 22. Associations between individual GR2 SNPs and milk production PTAs for Jersey bulls.
In each panel, *P*-values from one-way analysis of variance of associations between SNPs and PTAs are given on the Y-axis ($\log_{10}$ scale), with a horizontal black line representing significance at $P \leq 0.05$. The X-axes in each panel show nucleotide positions within GR2 where SNPs were identified. SNPs that associated with more than one trait in panels **(a)** through **(c)** are indicated with solid black circles (●), SNPs that associated with only the trait in the panel are indicated by hatched circles (⊘), and SNPs that were not significantly associated with any measured trait are indicated by open circles (◯). **(a)** milk yield PTA; **(b)** milk protein yield PTA; **(c)** milk SCS PTA. SNPs at position 598 were significantly ($P \leq 0.05$) associated with both milk yield **(a)** and protein yield PTAs **(b)**. GR2 SNPs at nucleotides 378, 454, 529, 582, 587, 786, 799, 862, 877, and 894 tended toward associations ($P \leq 0.15$) with milk SCS PTA **(c)**.

Figure 22. Associations between individual GR2 SNPs and milk production PTAs for Jersey bulls.

Figure 22a. Milk Yield PTA

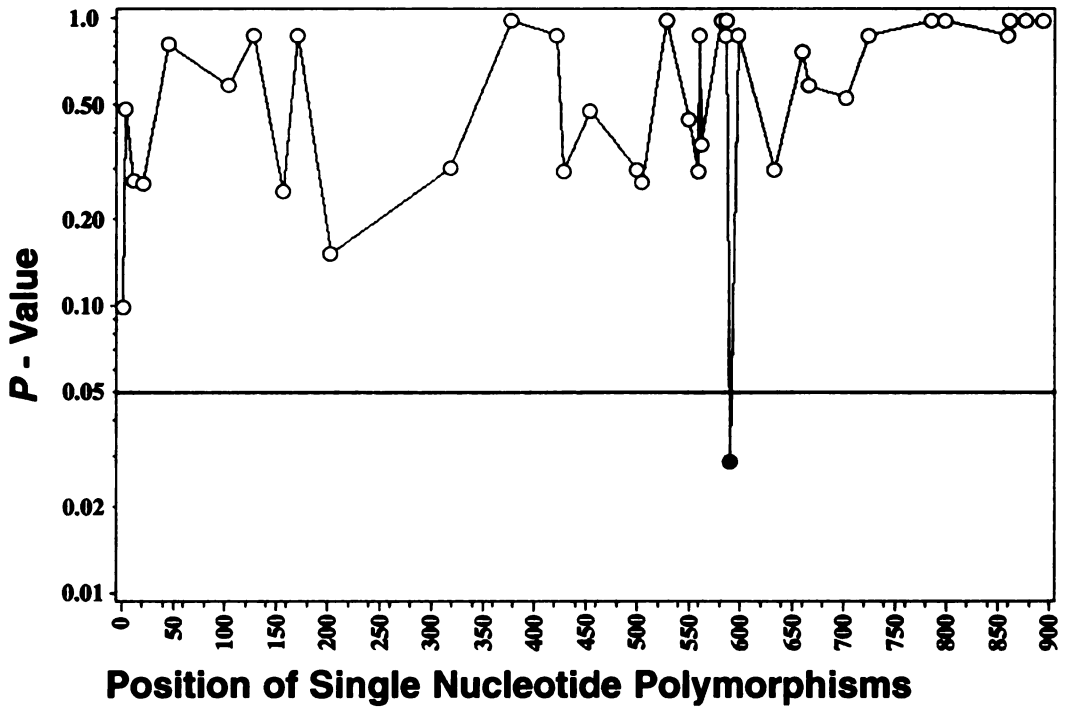
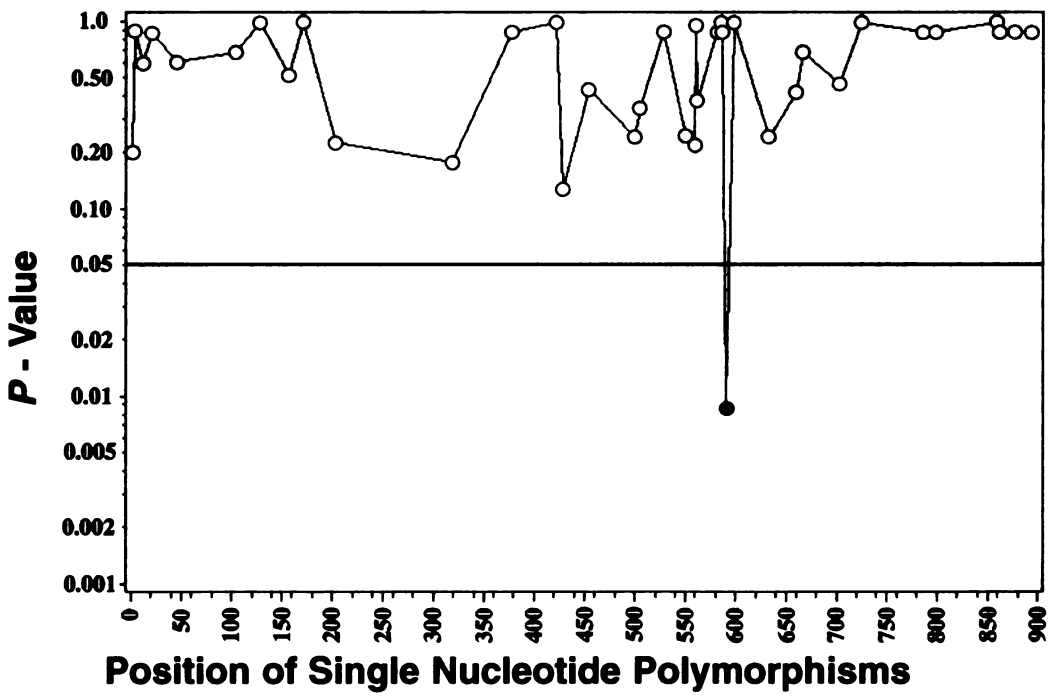


Figure 22b. Milk Protein Yield PTA



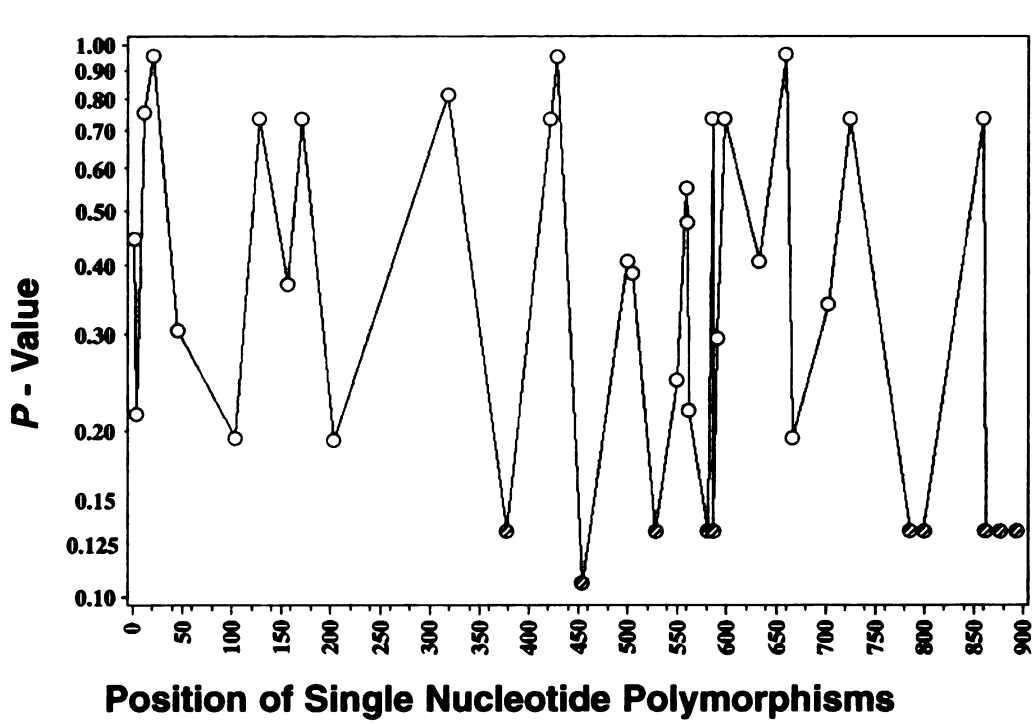
= Associates with only this trait

= No association

= Associates with 2+ traits

Figure 22. (cont'd)

Figure 22c. Milk SCS PTA



⊘ = Associates with only this trait

● = Associates with 2+ traits

○ = No association

account for some of the genetic variation in milk production traits, while different SNPs at positions 378, 454, 529, 582, 587, 786, 799, 862, and 894 may influence mammary health, but each must be studied in a larger population of animals before final conclusions can be drawn.

D. Discussion

The study reported here has identified numerous mutations in and surrounding the τ1 core-encoding locus of GR genes (GR2 locus) in a population of 20 active AI Jersey bulls. Several of the identified GR2 mutations were predicted to change amino acid sequence, including 2 changes within GR's synergy control motif; a mutation at SNP position 598 associated significantly with both milk yield and milk protein yield PTAs; and several SNPs at various 3' positions tended toward associations with milk SCS PTA. These results were not surprising given that expression of at least three milk protein genes β-casein, α-lactalbumin, and whey associated protein are controlled by glucocorticoids, most likely through interactions between GR and certain transcriptional coactivators (Houdebine et al., 1985; Reichardt et al., 2001). Indeed, increased blood glucocorticoid concentrations have been shown to reduce milk production in lactating ruminants (Bruckmaier et al., 1993; Sevi et al., 2001). While results of the current study must be substantiated in a larger population of bulls and their daughters, combined with available literature on glucocorticoid regulation of milk protein production, they suggest that variation in the DNA sequence of GR2 may be partially responsible for genetic regulation of milk production during stress.

Several studies other than this one have looked for associations between milk production traits and polymorphism in bovine candidate genes. In particular, highly polymorphic alleles of the bovine DRB3 locus (encoding the antigen binding groove of MHC II) have been associated with milk SCS, milk yield, milk

protein yields, the incidences of milk fever, retained placenta, and mastitis (Sharif et al., 1998a, b; Dietz et al., 1997a), and the competency of the immune system in dairy cattle (Dietz et al., 1997b). Yao et al., (1996) also identified associations between growth hormone gene polymorphism and milk yield, milk fat yield, and milk protein yield in Holstein cattle. Additionally, recent work from our group has shown that several GR2 mutations associate with glucocorticoid-sensitive traits in bovine leukocytes, including neutrophil expression of CD18 and the percentages of circulating T cells expressing CD4 and CD8 accessory molecules (**Chapter 5** and Jacob et al., in preparation). Three GR2 mutations also associated ($P \leq 0.05$) with milk yield PTA in the population of 40 Holsteins bulls used for that study, but those were at different GR2 nucleotide positions (**Figure 19f**) than the one position identified in the current study (**Figure 22a, b**).

Interestingly, when SNPs identified in the current study are compared to those present in a population of Holstein bulls (**Chapter 5** and Jacob et al., in preparation) several SNPs tending toward an association with milk SCS PTA ($P \leq 0.20$) were found to be held in common (or in close proximity within the GR2 gene) between the two populations of bulls (**Table 10**). Within the current Jersey population these occurred at nucleotide positions 203, 550, 582, 587, 598, 703, and 894, and within the Holstein population (**Chapter 5**) the SNPs occurred at positions 200, 545, 586, 597, 702, and 895 (**Table 10**). Three SNP positions located within 1 bp of each other (586/587, 597/598, and 702/703) translated into the same amino acid substitutions (at residues K195N, D199D, and M234I/T) in both populations, while two additional SNPs translated into neighboring amino acids (L182F in Jersey and I183V in Holstein). SNPs that translated into amino acid substitutions at residues 182 and 183 (I182V and L183F) tended to associate with milk protein yield PTA in both pop-

ulations of bulls, while SNPs that translated into a K195N substitution tended to associate with milk SCS PTA in both populations, and SNPs that translated into E199D substitutions tended to associate with milk yield PTA in the Holstein population ($P \leq 0.20$) and associated significantly ($P \leq 0.05$) with milk yield PTA in the Jersey population (**Table 10**). These results raise the suggestion that common GR2 SNPs may be identified that are conserved across dairy breeds which may have an impact on milk production traits following a stress challenge. Given the known effects of glucocorticoids on lactation, additional studies using larger populations of animals are warranted to substantiate this possibility.

In conclusion, this study reports the identification of numerous naturally occurring mutations within the GR2 locus of a small population of active Jersey bulls, chosen for study based upon their divergence in milk SCS PTA. Because SNPs at one of these GR2 nucleotide positions associated significantly with genetic merit for milk production traits of key economic importance to the dairy industry in this small population of Jersey bulls, GR2 polymorphism may prove useful for future studies on glucocorticoid regulation of mammary gland health and productivity. Ultimately, certain GR2 polymorphisms may be proven to be useful molecular genetic markers for genetic improvement of mastitis resistance and milk production in high producing, intensively reared dairy cattle.

Table 10. Summary of GR2 SNP Positions Commonly Held Between Holstein and Jersey Bull Populations.

Comparison of SNPs which tended ($P \leq 0.20$) toward associations with milk production traits in both Holstein and Jersey bulls identified seven nucleotide positions in GR2 that were either held in common or occurred in close proximity to each other (within 5 bp). These are listed in the left-hand column, followed by the population the SNPs occurred in. The right-hand column lists the amino acid changes resulting from the SNPs at each position. Three amino acid changes were common between the populations, at residues 195, 199, and 234. SNPs occurring at positions 545 (Holstein) and 550 (Jersey) translated into neighboring amino acids (182 and 183) and were associated with milk protein yield PTA in both populations, while SNPs at position 586 (Holstein) and 587 (Jersey) changed the same amino acid (K195N) and were associated with milk SCS PTA in both populations. Additionally, SNPs at positions 597 (Holstein) and 598 (Jersey) which changed the same amino acid (E199D) were associated with milk yield PTA in both populations.

Table 10. Summary of GR2 SNP Positions Commonly Held Between Holstein and Jersey Bull Populations.

SNP Position	Population	Trait Association	Amino Acid Change
200	Holstein	Protein Yield PTA, CD18 Expression	Silent
203	Jersey	Milk Yield PTA	K68E
545	Holstein	Milk Protein Yield PTA	I183V
550	Jersey	Milk Protein Yield PTA, Milk SCS PTA	L182F
560	Jersey	Milk SCS PTA	Silent
562	Holstein	Milk Yield PTA, CD18 Expression	Silent
586	*Jersey*	*No significant associations*	*K195N*
587	Jersey	Milk SCS PTA	Silent
586	Holstein	Milk SCS PTA, CD18 Expression	K195N
597	Holstein	Milk Yield PTA	E199D
598	Jersey	Milk Yield PTA, Milk Protein Yield PTA	E199D
702	Holstein	Milk Yield PTA, CD18 Expression	M234T
703	Jersey	Milk SCS PTA	M234V M234I
799	Jersey	Milk SCS PTA	K266N
801	Holstein	Milk Yield PTA, Milk Protein Yield PTA CD18 Expression	P267H
894	Jersey	Milk SCS PTA	T298I
895	Holstein	CD18 Expression	Silent

CHAPTER 7. GENERAL DISCUSSION AND CONCLUSION

The response to glucocorticoid challenge is varied among species, individuals within a species, and different tissues, cell types, and stages of the cell cycle within individuals (Yudt and Cidlowski, 2002, and references therein). Responsiveness is mediated at the molecular level through GR and the transcriptional co-factors with which the receptor associates. Hormone-bound GR interacts either with specific DNA motifs in promoters of target genes and (or) a variety of transcriptional coactivators/suppressors to regulate gene expression and change the phenotype of cells during stress. Following the identification of variation in sensitivity to glucocorticoids between human, marmoset, and guinea pig GRs (Brandon et al., 1991; Keightley and Fuller, 1994; Keightley and Fuller, 1995), numerous naturally occurring mutations were identified in key domains of hGR which affect their transcriptional functions (**Tables 1** and **2, Chapter 3**). Many of these hGR gene mutations affected the phenotype of individuals harboring them. Recently, studies have also shown that there is significant additive genetic variation in the sensitivity of the bovine immune system to glucocorticoid challenge (Tempelman et al., 2002; Abdel-Azim et al., in review; Burton et al., in review; Kelm et al., in review; **Table 4, Chapter 3**). Therefore, the hypothesis of the current series of studies was that bovine GR genes are polymorphic and that corresponding SNPs associate with traits of immunological and economic relevance to the cattle industry. The goals were to determine whether the bovine GR2 locus harbors polymorphism, to characterize this polymorphism at the DNA and amino acid sequence levels, and to elucidate possible associations between polymorphisms and a variety of immunological and production traits measured in populations of pedigreed cattle.

This dissertation describes the presence of complex GR2 SNPs in three populations; 6 cattle breeds selected for either milk production (Brown Swiss, Holstein, and Jersey), beef production (Angus and Polled Hereford), or dual milk and beef production (Brahman; **Chapter 4**); a population of 40 active AI Holstein bulls with known leukocyte sensitivities to glucocorticoid challenge (**Chapter 5**); and a population of active AI Jersey bulls divergent (≥ 2 standard deviations from their contemporaries) in milk SCS PTA (**Chapter 6**). Many of the identified SNPs translated into changes in predicted amino acid sequences. Three-dimensional homology modeling of divergent Holstein alleles predicted only modest changes in tertiary structure of $\tau 1$ regions encoded by mutant GR2s (**Chapter 4**), however this approach does not reveal changes in hydrophobicity or hydrophilicity, charge, or bonding. Holstein bulls with documented genetic variation in leukocyte sensitivity to glucocorticoids harbored numerous SNPs within their GR2 loci (**Chapter 5**). In these glucocorticoid challenged bulls, several SNPs associated significantly ($P \leq 0.05$) with changes in neutrophil CD18 expression, MHC II expression on mononuclear cells, and percentages of circulating CD4 and CD8 T cells. Additionally, several SNPs associated significantly with the bulls' PTAs for milk yield (**Chapter 5**). Associations between GR2 SNPs, neutrophil CD18 expression, and milk yield PTA in the Holstein bulls suggested that GR2 polymorphism may be linked to mammary health and productivity in this breed of cattle. In the dairy industry, mammary health is monitored using milk SCS as an indicator trait, which is a reflection of the ability of neutrophils to migrate (via CD18 adhesions) into infected quarters. Following identification of complex polymorphism in a population of 20 Jersey bulls of divergent milk SCS PTAs, some GR2 SNPs associated significantly with milk yield and protein yield PTA ($P \leq 0.05$), but no SNPs associated significantly at $P \leq 0.05$ with milk SCS PTA (**Chapter 6**). However,

this may have been due to the small number of animals studied and the tendency ($P \leq 0.20$) for one key SNP to associate with milk SCS PTA suggests that this work should be repeated in a larger number of animals.

In order to elucidate the extent of GR2 polymorphism, all sequenced GR2s across populations were analyzed for SNP location and frequency. GR2 loci from a total of 89 animals have been sequenced, resulting in 167 usable alleles. All totaled, these alleles harbored 103 unique SNP positions (**Figure 23** and **Table 11**), 43 of which are found in more than one population [Divergent Breeds, Jersey, and (or) Holstein; **Table 12**]. In addition, several SNPs were found exclusively in each breed (**Table 5**), and may be markers for breed-specific variation in glucocorticoid-responsiveness. Additionally, several SNP positions occurred in relatively high frequency (≥ 3%) across alleles, with nucleotide position 561 being particularly variable (30%; **Figure 23**). The majority of SNPs were located in 5' and 3' regions flanking the τ1 core-encoding DNA, with 8 SNPs falling within the τ1 core itself (highlighted by white bars in **Figure 23**). These changes may result in alteration of secondary and (or) tertiary protein structure, bonding, or charge, and therefore be partially responsible for variation in a cell's responsive to glucocorticoids.

Ultimately, alterations in transcriptional regulation by GR's τ1 domain are a result of changes to the protein structure or charge, therefore predicted amino acid changes resulting from GR2 SNPs were analyzed across populations. Across all animals studied, a total of 62 unique amino acid changes were predicted to occur within the τ1 region of GR (**Figure 24** and **Table 13**), 22 of which were found in more than one cattle population (**Table 14**). Twelve residue changes had relatively high frequencies (≥ 3%) across animals (**Figure 24**). Most predicted protein changes were located in the amino- and carboxy-terminal regions surrounding the τ1 core, however, 5 residue changes

occurred within core τ1 (highlighted by hatched bars in **Figure 24**). Such changes in predicted amino acid sequences would most likely result in alteration of tertiary protein structure or charge, and lead to changes in τ1 transactivation activity. Examples of residue substitutions which could possibly alter protein structure or charge are L35P found in Holsteins, which may result in steric hindrance due to a bulky proline residue; S46L also in Holsteins, which could be expected to change the polarity of the residue and perhaps orientation (**Table 14**; see **Appendix B** for classification of amino acid residues); N52K found in Brown Swiss and Angus cattle (**Figure 12a**) or K195N which is found in Jersey and Holstein (**Table 14**), both of which would be expected to alter the charge of the residue; S188R found in Jersey, which could change both charge and polarity (**Table 14** and **Figure 12a**); and C220S in all of the six cattle breeds, which has the possibility of disrupting disulfide bridges, and S225F in Holsteins which would change the polarity of the amino acid (**Table 14**). Indeed, changes to the bovine τ1 region at amino acid positions 143 (L143R) and 158 (S158N) in hGR have been found to influence GR's ability to transcribe a reporter gene from 28-48% and 24-39% relative to wild type GR, respectively (**Table 15**; Almlof et al., 1997). Additionally, changes at amino acid position 158 (F153S in bovine) in humans reduces the ability of hGR to bind the TATA binding protein (84% reduction) as well as the CREB binding protein (28% reduction), thus reducing transcription by mutant GR relative to wild type GR (Almlof et al., 1999). In 2000, Iniguez-Lluhi and Pearce identified a region in the human GR τ1 which appears to suppress transcription, termed the synergy control motif. This motif is highly conserved across the members of the hormone receptor superfamily as (I/V)KXE where I/V indicates the presence of either an isoleucine or valine at that position and X is any amino acid. Disruption of this motif by mutation of these conserved amino acid residues

results in a 3 to 12 fold increase in GR transcriptional activity relative to wild type GR (Iniguez-Lluhi and Pearce, 2000). Interestingly, bovine τ1 regions contain mutations at 2 amino acid residues within the synergy control motif (**Table 15**). Mutation of residue 298 (195 in bovine GR) results in a 6 fold increase in hGR transcriptional activity *in vitro*, while mutation of residue 315 (211 in bovine GR) resulted in a 12 fold increase in hGR activity relative to wild type (Iniguez-Lluhi and Pearce, 2000). Therefore, not only is the bovine GR2 locus polymorphic, but many of the identified SNPs change predicted amino acid charge and polarity and presumably the ability of GR to regulate target gene transcription.

It has been suggested that GR τ1 regions regulate transcription of glucocorticoid-responsive genes by using active surfaces to interact with other transcriptional co-regulators (Iniguez-Lluhi et al., 1997). Therefore, mutations harbored by bovine GR2 loci that changed predicted amino acids may alter the folding of GR's τ1 region, or its overall charge, and ultimately the phenotype of glucocorticoid-responsive cells.

Why would variation in τ1 region-encoding GR2 be present in animal populations? Changes to GR τ1 regions resulting from polymorphism in GR2 could result in changes to the folding and (or) charge of the τ1 region. Such changes in the τ1 domain of GR could affect how it interacts with other transcriptional co-regulators (τ1-protein interactions), leading to alterations in GR's regulation of glucocorticoid-responsive gene expression. Formation of unstable τ1-protein interactions as a result of identified mutations could lead to reductions in the transactivation or transrepression of gene expression by GR. Alternatively, more stable τ1-protein interactions could increase GR's transactivation or transrepression of target gene expression. Such changes in GR's ability to regulate transcription could cause changes in the mRNA levels of glu-

cocorticoid-responsive genes, and might alter the amount of protein expressed by the cell. Protein changes could result in changes in the cell's phenotype, which in turn, can change the phenotype of an organ or tissue, and ultimately the entire animal. Changes in an animal's responsiveness to glucocorticoids, and therefore to stressful stimuli, could influence the evolutionary selection of animals with superior genetics. Prior to the domestication of cattle, increases in blood glucocorticoid concentrations in response to perceived stress (**Appendix A**) enabled them to react to predatory danger through increased blood glucose concentrations and heart and respiration rates, thus allowing for an enhanced fight or flight response. Animals who were able to evade predation and remain healthy would be more likely to pass on their genetics. Therefore, any genetic changes to GR, including its τ1 domain, which resulted in appropriate adaptation of gene expression required for health and survival would be preserved. Today, presence of polymorphism in the τ1-encoding GR2 locus could reflect these evolutionary struggles for survival and still underlie subtle variations in glucocorticoid (stress)-responsive phenotypes of the immune, mammary, reproductive, and circulatory systems that partially determine an animal's genetic merit. Clearly, more studies using larger animal numbers and precise measurements of glucocorticoid-sensitive traits of economic importance need to be performed to substantiate the findings of the current study and determine if the GR2 locus is a critical factor in predicting animal performance in today's husbandry environments. In addition, studies are required to define precisely which GR2 SNPs or SNP clusters are critical in regulating expression of glucocorticoid-responsive genes with known involvement in animal health, reproduction, and production.

Table 11. Summary of SNPs Identified Across All Sequenced GR2 Loci.

SNP Position & Population	SNP Position & Population	SNP Position & Population	SNP Position & Population
1 Jer	203 Jer	531 Breeds, Hol	701 Hol
4 Jer, Breeds	247 Hol	545 Breeds, Hol	702 Hol
5 Hol	289 Hol	550 Jer	703 Breeds, Jer
12 Breeds, Jer	***319 Jer***	560 Breeds, Jer	725 Breeds, Jer
21 Breeds, Jer	***346 Breeds, Hol***	561 Breeds, Jer	733 Hol
24 Hol	***377 Breeds***	562 Hol, Jer	754 Hol
28 Hol	***378 Jer***	563 Breeds	759 Hol
46 Jer	***415 Hol***	565 Breeds	780 Hol
62 Hol	***417 Breeds***	571 Hol	783 Breeds
77 Breeds, Hol	***422 Jer***	578 Breeds, Hol	786 Breeds, Jer
95 Hol	***429 Breeds, Jer***	579 Hol	799 Hol, Jer
104 Jer	441 Breeds	582 Jer	801 Hol
105 Hol	442 Hol	586 All	811 Hol
123 Breeds, Hol	454 Breeds, Jer	587 Jer	813 Breeds, Hol
128 Breeds, Jer	459 Breeds	591 Jer	820 Breeds, Hol
138 Hol	472 Breeds	592 Breeds	828 Hol
139 Hol	474 Breeds	597 Hol	830 Breeds, Jer
140 Hol	480 Breeds	598 Breeds, Jer	841 Hol
145 Hol	492 Breeds, Hol	613 Hol	848 Hol
147 Breeds, Hol	496 Hol	617 Breeds, Hol	851 Hol
154 Hol	500 Jer	621 Hol	862 Breeds, Jer
156 Hol	505 Jer	633 Breeds, Jer	877 Jer
157 Breeds, Jer	510 Breeds	645 Hol	881 Hol
171 Breeds, Jer	520 Breeds	660 Breeds, Jer	894 Jer
193 Breeds	528 Breeds	667 Jer	895 Hol
200 Hol	529 Breeds, Jer	675 Hol	

Key: Breeds = All six cattle breeds, Jer = Jersey bull population, Hol = Holstein bull population.

Table 11. Summary of SNPs Identified Across All Sequenced GR2 Loci.

Following analysis of the presence of SNPs across sequenced GR2 loci, 103 unique nucleotide SNP locations were identified in the 915 bp sequences of GR2. SNPs are listed in the columns followed by the population in which they are identified. SNP locations that fall within the $\tau 1$ core-encoding DNA are italicized and boldfaced.

Table 12. Summary of SNPs Found in GR2 Loci from More Than One Cattle Population.

Twenty-nine SNP positions were identified in GR2 loci of more than one cattle population. The first column lists the position of the identified SNP, with the exact change found across the breeds (**Breeds**; **Chapter 4**) following it. SImilarly, the change at each SNP position found within the Jersey bull (**Jersey Bulls**; **Chapter 6**) and Holstein Bull (**Holstein Bulls**; **Chapter 5**) populations indicated in the next columns. The right column lists the amino acid position encoded by the SNP(s) found within the cattle populations studied. SNPs that fell in the $\tau 1$ core-encoding DNA are highlighted by a gray box.

Table 12. Summary of SNPs Found in GR2 Loci from More Than One Cattle Population.

Position of SNP	Breeds	Jersey Bulls	Holstein Bulls	Amino Acid Position
12	T12A (K)	T12C (I)		M4K, I
21	C21G	C21G		T7R
46	C46G	C46G		G15E
77	C77G (V)		C77T (F)	L26V, F
104		C104T		L35F
105			T105C	L35P
123	C123G (G)		C123T (V)	A41G, V
128	C128G	C128G		L43V
147	T147C		T147C	V49A
156			A156G	N52S
157	C157G	C157G		N52K
171	C171G	C171G		A57G
346	*G346A*		*G346A*	*Silent*
377	*A377G*			*T126A*
378		*C378G*		*T126R*
429	*T429G*	*T429G*		*L143R*
454	T454C	T454C		Silent
492	A492T (M)		A492G (R)	K164M, K164R
528	A528G			K176R
529	A529C (N)	A529G (K)		K176N
531	A531G		A531G	D177G
545	A545G		A545G	I182V
560	A560T	A560C, T		(187)
561	G561A, C	C561G, T		S187T,Y,N
562		C562T, G, A	C562G (T187I)	T187S,I,R,L,F,P
578	C578A		C578A	Q193K
586	A586C	A586C	A586C	K195N
591		A591G		E197G
592	A592G(E), T(D)			E197D, Silent
597			A597C	E199D
598	A598C	A598C		E199D
617	A617T		A617G	T206S
633	A633C	A633C		K211T
660	G660C	G660C		C220S
702			T702C	M234T
703	G703C	G703C		M234I
725	G725C	G725C		V242L
786	A786C	A786C		Q262P
799		A799C (N)	A799G (K)	K266N, Silent
813	T813C		T813C	V271A
820	C820T		A820G	Silent
830	G830C	G830C		V277L
862	G862C	G862C		Silent

Figure 23. Summary of the Occurrence of SNPs Across all GR2 Loci Sequenced.

This figure shows frequencies of SNPs observed across all sequenced GR2 alleles (shown in **Table 10**) expressed as percentages of all alleles studied. The X-axis shows the nucleotide position of SNPs and the Y-axis shows the percentage of allele differences at each SNP position. The white bars at nucleotide positions 319, 346, 377, 378, 415, 417, 422, and 429 indicate SNPs found within the τ1 core-encoding DNA. Solid bars to the left and right of the white bars shows SNPs found in the 5' and 3' DNA flanking regions. The majority of SNPs occurred with a frequency ≤ 3%. Several other SNPs had frequencies ranging from 3.6% to 50% (nucleotide position 561).

Figure 23. Summary of the Occurrence of SNPs Across all GR2 Loci Sequenced.

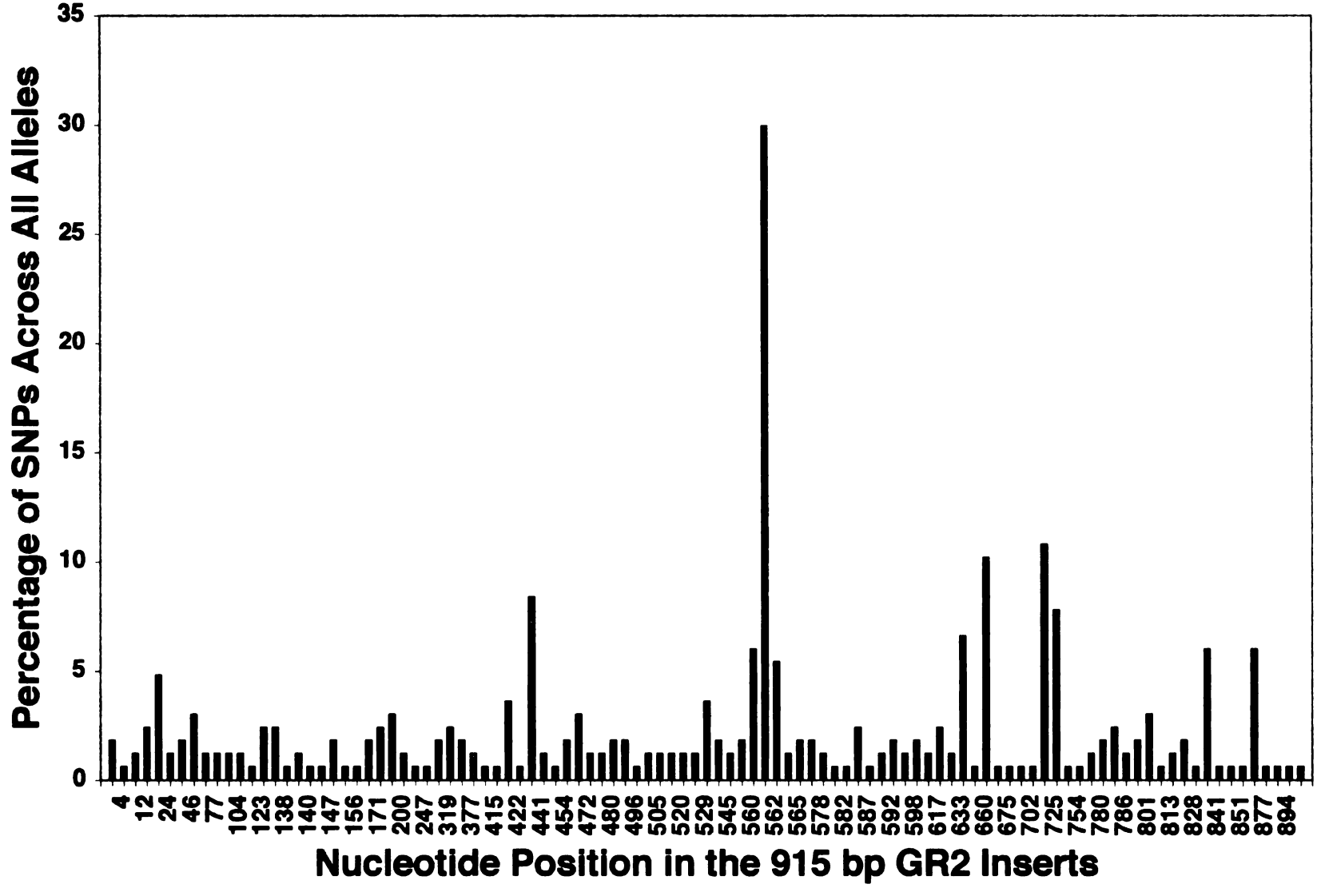


Table 13. Summary of Amino Acid Changes Identified in the GR2-Encoding τ1 Region.

Amino Acid Position & Population	Amino Acid Position & Population	Amino Acid Position & Population
L2V Breeds	***E139G Breeds***	E199D Breeds, Jer
M4K Breeds	***C141G Jer***	E199G Hol
M4T Jer	***L143R Breeds, Jer***	T206S Breeds
T7R Breeds, Jer	A147R Breeds	T206A Hol
E8G Hol	F153S Breeds	P207I Hol
D15E Breeds	S158N Breeds	K211T Breeds, Jer
L26V Breeds	N160I Breeds	C220S Breeds, Jer
L35F Jer	K164M Breeds	S225F Hol
L35P Hol	K164R Hol	M234I Breeds, Jer
A41G Breeds	V167L Jer	M234T,V Hol
A41V Hol	L169F Jer	V242L Breeds, Jer
L43V Breeds	D170G Breeds	H251Q Hol
S46L Hol	K176R,N Breeds	D253G Hol
T47S Hol	D177G Breeds, Hol	S260Y Hol
V49A Breeds, Hol	I182V Breeds, Hol	Q261L Breeds
E51D Hol	L183F Jer	Q262P Breeds, Jer
N52K Breeds	S187T,Y,N Breeds	K266N Jer
N52S Hol	T187S,N,K,L,F,H,R Jer	P267H Hol
A57G Breeds	T187I Hol	V271A Breeds
K68E Jer	S188R,G Breeds	V277L Breeds, Jer
Q82H Hol	S188R Jer	E280D Hol
K96N Hol	Q193K Breeds	N283D Hol
D106Q Jer	Q193K,P Hol	R284G Hol
T126A Breeds	K195N All	T294A Hol
T126R Jer	E197D Breeds	T298I Jer

Key: Breeds = All six cattle breeds, Jer = Jersey bull population, Hol = Holstein bull population.

Table 13. Summary of Amino Acid Changes Identified in the GR2-Encoding τ1 Region

Seventy-five unique amino acid residue changes were identified across the 304 residue bovine τ1 region of the 89 animals studied. Residue changes that fell within the τ1 core are italicized and boldfaced.

Table 14. Summary of Amino Acid Changes Found in GR τ1 Regions of More Than One Cattle Population.

Position of AA	Breeds	Jersey Bulls	Holstein Bulls	SNP Position(s)
4	M4K	M4T		12
7	T7R	T7R		21
35		L35F	L35P	104, 105
41	A41G		A41V	123
49	V49A		V49A	147
52	N52K		N52S	156, 157
126	*T126A*	*T126R*		*377, 378*
143	*L143R, P*	*L143R*		*429*
164	K164M		K164R	492
177	D177G		D177G	531
182	I182V		I182V	545
187	S187Y,T,N	T187S,N,R, K,L,F	T187I	560, 561, 562
193	Q193K		Q193K	578
195	K195N	K195N	K195N	586
199	E199D	E199D	E199G	597, 598
206	T206S		T206A	617
211	K211T	K211T		633
220	C220S	C220S		660
234	M234I	M234I	M234T	702, 703
242	V242L	V242L		725
262	Q262P	Q262P		786
277	V277L	V277L		830

Table 14. Summary of Amino Acid Changes Found in GR τ1 Regions of More Than One Cattle Population.

Twenty-two unique amino acid residue positions were found in GR2-encoded τ1 regions of more than one cattle population. The first column lists the position of amino acid changes encoded by identified SNPs, with the exact change found across the breeds (**Breeds**; **Chapter 4**) following it. SImilarly, the change at each amino acid position found within the Jersey bull (**Jersey Bulls**; **Chapter 6**) and Holstein Bull (**Holstein Bulls**; **Chapter 5**) populations indicated in the next columns. The right column lists the SNP position(s) found within the cattle populations studied. Residue changes within the τ1 core are highlighted by a gray box.

Figure 24. Summary of the Frequencies of Amino Acid Changes Across All GR2 Loci Sequenced.

Frequencies of allelic amino acid residue substitutions predicted from GR2 SNPs in **Table 10** expressed as percentages of all alleles studied. The Y-axis shows the percentage of allele differences detected and the X-axis shows the residue position of these allele changes. Hatched bars at residues 106, 126, 139, 141, and 143 indicates predicted changes in the $\tau 1$ core, while solid bars to the left and right indicate changes in the amino- and carboxy-terminals (respectively) protein sequences flanking $\tau 1$. The majority of changes occurred with a frequency $\leq 3\%$. However, several residue positions had frequencies ranging from 10 (residues 143, 220, and 234) to 30% (residue 187).

Figure 24. Summary of the Frequencies of Amino Acid Changes Across All GR2 Loci Sequenced.

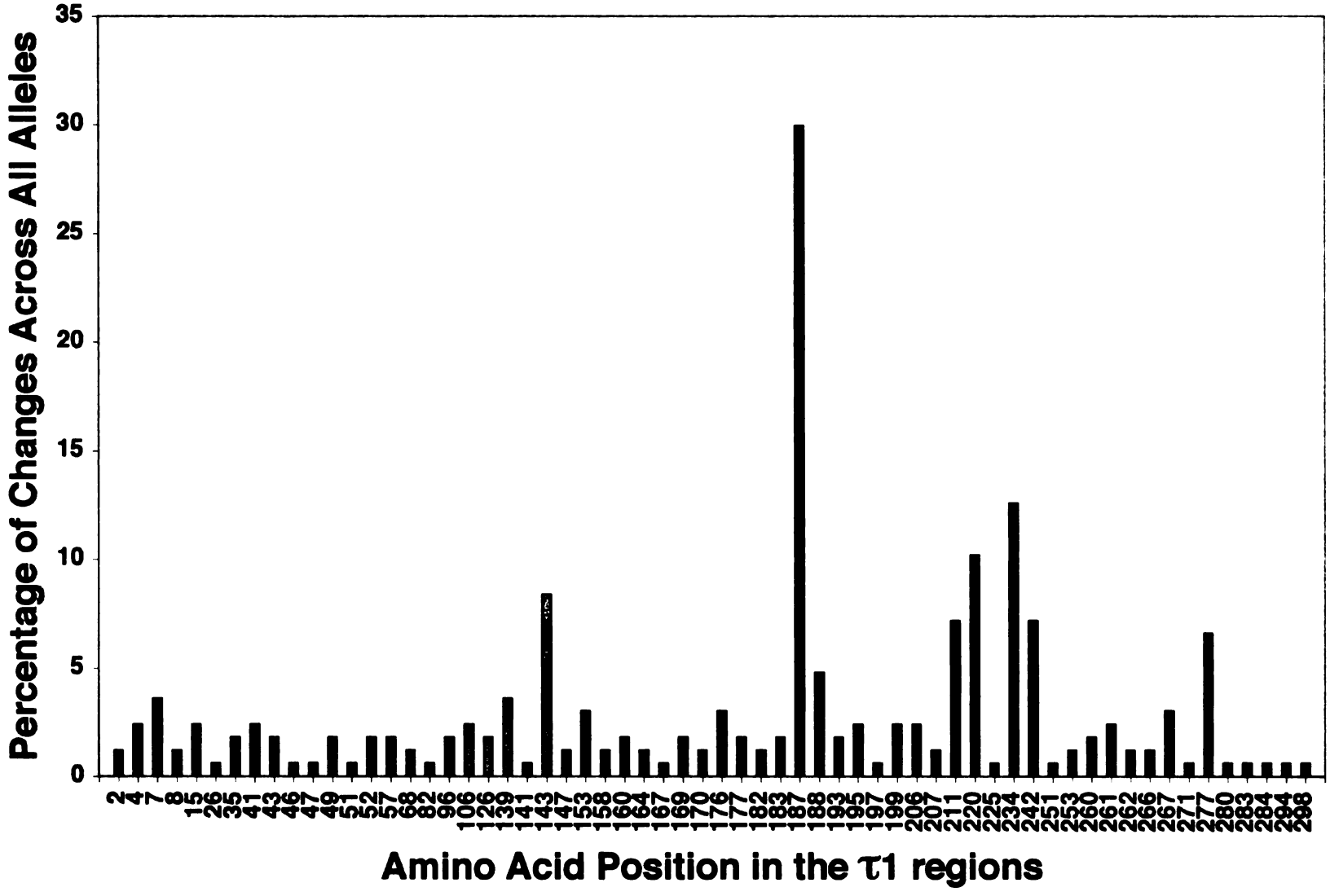


Table 15. Summary of Significant Amino Acid Changes in Human and Bovine τ1 Regions and the Resulting Change in hGR Activity.

Position of hGR Amino Acid Change	Corresponding Amino Acid Change in Bovine GR2	Transactivation of a Reporter Gene as Compared to WT hGR	Reference for hGR
L225V	L143R	28-48%	Almlof et al., 1997
F235L or V	F153NS	84% decrease in binding affinity for TBP and 28% decrease in CBP binding	Almlof et al., 1998
N240D	S158N	24-39%	Almlof et al., 1997
K298E	*K195N*	*6-fold increase*	*Iniguez-Lluhi and Pearce, 2000*
K315E	*K211T*	*12-fold increase*	*Iniguez-Lluhi and Pearce, 2000*

Table 15. Summary of Significant Amino Acid Changes in Human and Bovine τ1 Regions and Resulting Changes in hGR Activity.

Amino acid residue changes identified within hGR τ1 regions or the synergy control motif are listed in the left-hand column with their bovine counterparts listed in the next column (τ1 core are indicated by **bold blue**). The functional change observed in an *in vitro* reporter gene system or protein binding affinity is reported in the third column and the reference is listed in the right-hand column. Changes to hGR residues 225 and 240 result in decreased GR transcription while changes to residue 235 reduce the ability of GR to bind the TATA binding protein (TBP) as well as the CREB binding protein (**CBP**). Residues 298 and 315 (italicized) are located within the synergy control motif and enhance hGR transcription when mutated.

CHAPTER 8. RECOMMENDATIONS FOR FUTURE WORK

In order to determine true biological relevance of the GR2 mutations identified through the current series of studies, several additional studies would need to be conducted both *in vivo* and *in vitro*. First of all, associations of GR2 SNPs with other heritable glucocorticoid-responsive leukocyte traits, such as lymphocyte blastogenesis, random migration of neutrophils, and neutrophil oxidative metabolism and generation of superoxide anions, etc. (described in Kelm et al., in review), should be conducted. Additionally, modification of the current one-way ANOVA protocol to test multiple SNPs (instead of individual SNPs) for associations with the glucocorticoid-responsive traits studied may reveal significant clusters of SNPs that form response surfaces that may be worthy of further study. In addition, association of individual and multiple amino acid changes with glucocorticoid-responsive immune traits could highlight important protein residues in $\tau 1$ regions. Furthermore, development of an efficient PCR-based genotyping method to facilitate future studies in larger populations is essential. One such study could be the genotyping of significantly larger populations of bulls and their daughters, from the Jersey or Holstein breeds, for analysis of milk production and peripartum immune traits and determination of associations between key GR2 SNPs and these traits. On a larger population of animals, truly significant ($P \leq 0.001$) GR2 SNPs may be revealed and allow for effective GR2 genotyping and (or) haplotyping for genetic selection purposes.

To further identify biological relevance of GR2 SNPs and also to continue linking glucocorticoid-sensitive immune traits to mammary health, animals which have been genotyped or haplotyped for GR2 SNPs could be challenged with glucocorticoids (dexamethasone) and immune traits collected and ana-

lyzed, particularly those which have been shown to possess genetic variation (Tempelman et al., 2002; Burton et al., in review; Kelm et al., in review) and also to be significantly associated with GR2 mutations in the current study (ie. % CD4 and CD8 T cells, CD18 expression by neutrophils, MHC II expression by mononuclear cells, etc.). Not only would this type of study allow further analysis of glucocorticoid-responsive immune functions, but would allow for SNP-trait associations better poised to reveal truly significant SNPs. Additionally, microarrays could be run on GR2 "glucocorticoid-sensitive" and "glucocorticoid-resistant" genotyped animals for the identification of target genes which might be differentially effected in expression by GR2 mutations.

The studies outlined above are mainly designed to screen for GR2 mutations and to determine whether they are truly biologically relevant to immune function, mammary health, and milk production of the whole animal. A concurrent study could be conducted to determine the effect and importance of individual amino acid changing SNPs on GR function *in vitro*. This study would make use of a highly defined gene expression system to study GR2 haplotypes that associate with transactivation of a specific reporter gene, thus identifying key GR2 alleles that may be important to stress susceptibility. Making use of the yeast transcription factor Gal4, which is not found in other higher mammalian cells, various GR2 mutants can be fused to the Gal4 DNA-binding domains and used to transactivate expression of a reporter gene such as the green fluorescent protein (GFP) or luciferase gene. Following identification of 'functionally relevant' GR2 haplotypes *in vitro*, relevant SNPs could be identified that were responsible for changes to $\tau 1$ transactivation/transrepression function. These may make the best candidates for future studies of GR2 polymorphism i*n vivo*. Finally, screening and investigation of the remaining exons

of bovine GR gene may reveal significant polymorphisms which are associated with sensitivity or resistance to glucocorticoids.

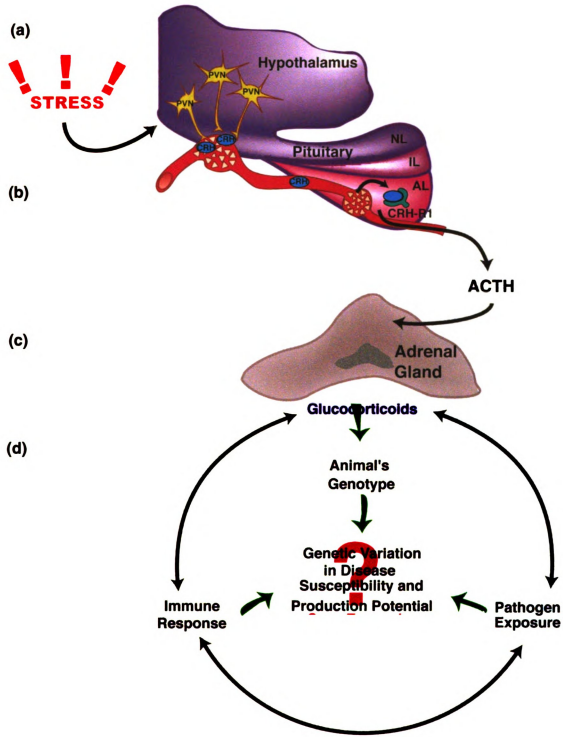
Ultimately, these suggested studies would identify functionally relevant GR2 SNPs, both *in vitro* and *in vivo*, which could be used in the future for management decisions or genetic selection of cattle whose immune systems will be less sensitive to stressful husbandry practices, leading to healthier and more productive progeny.

APPENDICES

Appendix A. Activation of the Hypothalamic-Pituitary-Adrenal, or Stress, Axis.

Following exposure of an animal to stimuli perceived as stressful (ie. extremes in heat and cold, pain and fear, infection, common husbandry practices), paraventricular nuclei (PVN) within the hypothalamus release corticotrophin releasing hormone (CRH) into the portal blood **(a)**. CRH binds to CRH receptors (CRH-R1) in the anterior lobe (AL) of the pituitary **(b)**, resulting in the release of adrenocorticotropin releasing hormone (ACTH) into the circulation. ACTH, in turn, targets the adrenal glands to synthesize and release glucocorticoids **(c)**. Genetic variation in disease susceptibility and production potential is observed following glucocorticoid challenge, pathogen exposure and the subsequent immune response **(d)**, and an animals' genotype will determine gene expression following a stress challenge. As the receptor for glucocorticoids, molecular genetic variation in GR and resulting glucocorticoid-responsive gene expression is an intriguing possibility to explain genetic variation in the health and productivity of husbandry-stressed cattle.

Appendix A. Activation of the Hypothalamic-Pituitary-Adrenal, or Stress, Axis.



APPENDIX B. REFERENCE OF AMINO ACIDS.

Listed are amino acid residue names and their three letter and one letter codes. One letter codes are used throughout this dissertation. In the right-hand column is listed the classification (or class) of the amino acid residue.

Amino Acid Full Name	Three-Letter Code	Single-Letter Code	Residue Classification
Alanine	Ala	A	Neutral, Nonpolar
Cysteine	Cys	C	Basic, Polar
Aspartic Acid	Asp	D	Acidic
Glutamic Acid	Glu	E	Acidic
Phenylalanine	Phe	F	Neutral, Nonpolar
Glycine	Gly	G	Neutral, Nonpolar
Histidine	His	H	Acidic
Isoleucine	Ile	I	Neutral, Nonpolar
Lysine	Lys	K	Basic
Leucine	Leu	L	Neutral, Nonpolar
Methionine	Met	M	Neutral, Nonpolar
Asparagine	Asn	N	Neutral
Proline	Pro	P	Neutral, Nonpolar
Glutamine	Gln	Q	Neutral, Polar
Arginine	Arg	R	Basic, Polar
Serine	Ser	S	Neutral, Polar
Threonine	Thr	T	Neutral, Polar
Valine	Val	V	Neutral, Nonpolar
Tryptophan	Trp	W	Neutral, Nonpolar
Tyrosine	Tyr	Y	Basic, Polar

Appendix C. Coding for Animals Used in the Multi-Breed Study of Chapter 4.

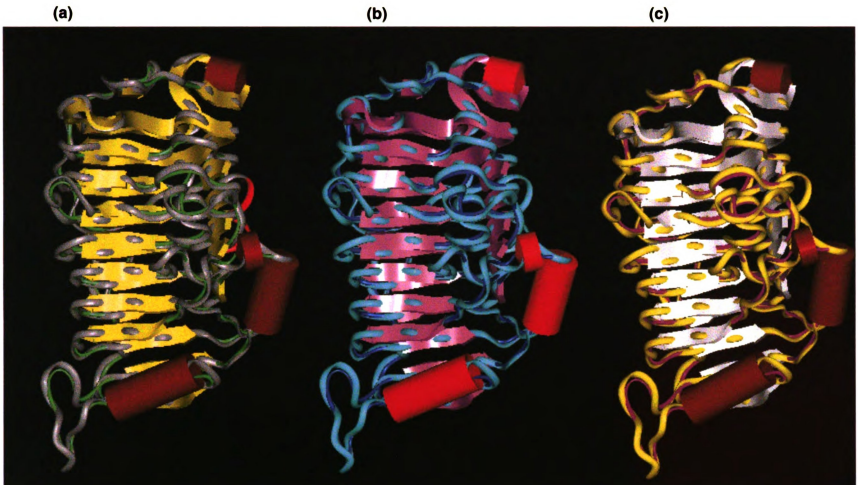
Codes for animals making up the Breeds Populations are listed on the left-hand side, along with their breed. Barn identifiers and the source of the genomic DNA are listed on the right.

Code	Breed	Barn ID	Source of DNA
A1	Angus	a3d28	MSU Beef Teaching and Research
A2	Angus	a9603	MSU Beef Teaching and Research
A3	Angus	a9609	MSU Beef Teaching and Research
A4	Angus	a9707	MSU Beef Teaching and Research
Br1	Brahman	br21	Donated By Dr. Pagan, Puerto Rico
Br2	Brahman	br22	Donated By Dr. Pagan, Puerto Rico
Br3	Brahman	br23	Donated By Dr. Pagan, Puerto Rico
Br4	Brahman	br26	Donated By Dr. Pagan, Puerto Rico
Br5	Brahman	br27	Donated By Dr. Pagan, Puerto Rico
Bs1	Brown Swiss	bs31	Donated by Dr. Mashek, Iowa
Bs2	Brown Swiss	bs38	Donated by Dr. Mashek, Iowa
Bs3	Brown Swiss	bs51	Donated by Dr. Mashek, Iowa
Bs4	Brown Swiss	bs60	Donated by Dr. Mashek, Iowa
H1	Holstein	h1175	MSU Dairy Teaching and Research
H2	Holstein	h2941	MSU Dairy Teaching and Research
H3	Holstein	h3159	MSU Dairy Teaching and Research
H4	Holstein	h3223	MSU Dairy Teaching and Research
H5	Holstein	h3229	MSU Dairy Teaching and Research
He1	Polled Hereford	he608	MSU Beef Teaching and Research
He2	Polled Hereford	he40e	MSU Beef Teaching and Research
He3	Polled Hereford	he40g	MSU Beef Teaching and Research
He4	Polled Hereford	he58c	MSU Beef Teaching and Research
He5	Polled Hereford	hes35	MSU Beef Teaching and Research
J1	Jersey	j211	NorthStar Select Sire, Michigan
J2	Jersey	j311	NorthStar Select Sire, Michigan
J3	Jersey	j344	NorthStar Select Sire, Michigan
J4	Jersey	j355	NorthStar Select Sire, Michigan
J5	Jersey	j357	NorthStar Select Sire, Michigan

APPENDIX D. TERTIARY STRUCTURE PREDICTIONS FOR THE REMAINING THREE HOLSTEINS COWS STUDIED IN CHAPTER 4.

GR2 alleles for Holstein H2 **(a)**, H4 **(b)**, and H5 **(c)** were homology modeled using the same techniques described in **Chapter 4, Section B*v***. Unlike the structural changes predicted for Holsteins H3 (In **Figure 15a** of **Chapter 4**), these GR2-encoding τ1 regions were not predicted to be significantly different from the Holstein H1-1 allele **(Figure 15a)**.

Appendix D. Tertiary Structure Predictions for the Remaining Three Holsteins Cows Studied in Chapter 4.



Appendix E. Pedigree Information for Holstein Bulls Studied in Chapter 5.

Holstein bull identifications are listed in the left-hand column, followed by their registration numbers, sire registration numbers, and maternal grandsire registration numbers. Several half-sibling and three-quarter sibling groups were included in this population.

Appendix E. Pedigree Information for Holstein Bulls Studied in Chapter 5.

Bull ID	Registration	Sire	Maternal Grandsire
4536	2194316	2030882	1806201
4591	2197294	2012343	1929410
4628	2202276	2035598	1856904
4658	2212508	2030882	1929410
4661	2212717	2022844	1879085
4665	2218401	2030882	1806201
4677	2226286	2012343	1856904
4688	2218538	1874634	1829881
4721	2215908	1874634	1927133
4732	2215898	2049679	1929410
4736	2215547	1874634	1983348
4756	2217590	2027062	1879085
4757	2218460	1841366	1879085
4765	2237843	2035598	1875896
4771	2229984	2027062	1879085
4777	2228888	2030882	1875896
4778	2223695	2035598	1879085
4793	2230301	1875356	1929410
4796	2237962	2018469	1879085
4797	2235883	2027062	1875896
4821	2247859	2027062	1883228
4832	2235504	392457	1881163
4836	2238426	2035598	1929410
4837	2239203	2032124	1875896
4843	2233231	2035598	1856904
4857	2239432	2022844	1841366
4861	2228367	2022129	1806201
4865	2250145	2069266	1841366
4874	2256961	2049679	1856904
4879	2257325	392457	1879085
4882	2259353	2037045	1879085
4883	2242921	392457	1929410
4888	2250354	2035598	1841366
4896	2242679	2027062	1875356
4898	2239110	2070579	1856904
4937	2251614	2049679	1879149
4941	2253774	1874634	1841366
4946	2247616	2049679	1929410
4947	2263282	2019612	1856904
4951	2249886	2065871	1887096

Appendix F. Identification Coding and Pedigree Information for Jersey Bulls Studied in Chapter 6.

Coding for Jersey bulls, referred to as the Jersey Population, are listed in the left-hand column, followed by their National Association of Animal Breeders (NAAB) identifiers, and their registration numbers. Sire and maternal grandsire NAAB identifiers are also shown. Several half-sibling and three-quarter sib groups are included in this population.

Code	NAAB Code	Registration	Sire	Maternal Grandsire
HSCS1	j148	660344	J4093	J159
HSCS2	j3028	657298	J2875	J159
HSCS3	j545	660315	J2875	J159
HSCS4	j3100	654035	J159	J177
HSCS5	j3053	657913	J2875	J159
HSCS6	j376	660324	J4093	J159
HSCS7	j430	658103	J2875	J159
HSCS8	j129	657279	J2875	J240
HSCS9	j7081	660317	J345	J159
HSCS10	j347	658479	J2875	J159
HSCS11	j212	647162	J177	J194
LSCS9	j596	657423	J2877	J177
LSCS8	j284	654780	J2850	J337
LSCS7	j629	661446	J254	J337
LSCS6	j239	654512	J221	J354
LSCS5	j134	658575	J2875	J337
LSCS4	j290	654500	J177	J337
LSCS3	j599	658279	J2875	J337
LSCS2	j274	657768	J2890	J337
LSCS1	j126	656632	J2877	J177

BIBLIOGRAPHY

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Abdel-Azim, G.A., A.E. Freeman, M.E. Kehrli, Jr., S.C. Kelm, J.L. Burton, A.L. Kuck, and S. Schnell. Analysis of disease incidence data and relationship of probability of disease incidence with immunity. In review.

Agelaki, S., C. Tsatsanis, A. Gravanis, A.N. Margioris. 2002. Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages *in vitro* and in lipopolysaccharide-induced endotoxin shock in mice. Infect Immun 70:6068-6074.

Agura, E.D., M. Howard, S.J. Collins. 1992. Identification and sequence analysis of the promoter for the leukocyte integrin β-subunit (CD18): a retinoic acid-inducible gene. Blood 79:602-609.

Almawi, W.Y., H.N. Beyhum, A.A. Rahme, and M.J. Rieder. 1996. Regulation of cytokine and cytokine receptor expression by glucocorticoids. J Leuk Biol 60:563-572.

Almawi, W.Y., and N. Irani-Hakime. 1998. The antiproliferative effect of glucocorticoids: is it related to induction of TGF-β? Nephrol Dial Transplant 13:2450.

Almawi, W.Y., and O.K. Melemedjian. 2002. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. J Leuk Biol 71:9-15.

Almlof, T., J-A. Gustafsson, A.P.H. Wright. 1997. Role of hydrophobic amino acid clusters in the transactivation activity of the human glucocorticoid receptor. Mol Cell Bio 17:934-945.

Almlof, T., Wallberg, A.E., Gustafsson, J.A., Wright, A.P. 1998. Role of important hydrophobic amino acids in the interaction between the glucocorticoid receptor tau 1-core activation domain and target factors. Biochemistry 37:9586-9594.

Amano, Y., S.W. Lee, A.C. Allison. 1993. Inhibition by glucocorticoids of the formation of interleukin-1α, interleukin-1β, and interleukin-6: mediation by decreased mRNA stability. Mol Pharmacol 43:176-182.

Amsterdam, A., K. Tajima, R. Sasson. 2002. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem Pharmacol 64:843-850.

Anderson, B.H., D.L. Watson, I.G. Colditz. 1999. The effect of dexamethasone on some immunological parameters in cattle. Vet Res Commun 23:399-413.

Arriza, J.L., C. Weinberger, G. Cerelli, T.M. Glaser, B.L. Handelin, D.E. Housman, R.M. Evans. 1987. Cloning of human mineralcorticoid receptor complimentary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268-275.

Ashraf, J., and E.B. Thompson. 1993. Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes. Mol Endocrinol 7:631-642.

Ashwell J.D., F.W.M. Lu, and M.S. Vacchio. 2000. Glucocorticoids in T cell development and function. Annu Rev Immunol 18:309-345.

AyanlarBatuman, O., A.P. Ferrero, A. Diaz, S.A. Jimenez. 1991. Regulation of transforming growth factor-β1 gene expression by glucocorticoids in normal T lymphocytes. J Clin Invest 88:157401580.

Azizkhan, J.C., D.E. Jensen, A.J. Pierce, M. Wade. 1993. Transcription from TATA-less promoters: dihydrofolate reductase as a model. Crit Rev Eukaryot Gene Expr 3:229-254.

Baarends, W.M., A.P. Themmen, L.J. Blok, P. Mackenbach, A.D. Brinkmann, D. Meijer, P.W. Faber, J. Trapman, J.A. Grootegoed. 1990. The rat androgen receptor promoter. Mol Cell Endocrinol 74:75-84.

Baeuerle, P.A., and D. Baltimore. 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Gene Dev 3:1689-1698.

Baeuerle, P.A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. Annu Rev Immunol 12:141-179.

Baldwin, C.L., T. Sathiyaseelan, B. Naiman, A.M. White, R. Brown, S. Blumerman, A. Rogers, S.J. Black. 2002. Activation of bovine peripheral blood γδ T cells for cell division and IFN-γ production. Vet Immunol Immunopath 87:251-259.

Ballard, P.L., J.D. Baxter, S.J. Higgins, G.G. Rousseau, G.M. Tomkins. 1974. General presence of glucocorticoid receptors in mammalian tissues. Endocrinol 94:998-1002.

Bamberger et al., 1995. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest 95:24-35.

Bamberger, C.M., H.M. Schulte, G.P. Chrousos. 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev 17:245-61.

Bamberger, C.M., T. Else, A.M. Bamberger, F.U. Beil, H.M. Schulte. 1997. Regulation of the human interleukin-2 gene by the alpha and beta isoforms of the glucocorticoid receptor. Mol Cell Endocrinol 136:23-28.

Barnes, P.J., and I. Adcock. 1993. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 14:436-441.

Barnes, P.J. 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci 94:557-572.

Barnes, P.J. 2001. Corticosteroids, IgE, and atopy.. J Clin Invest 107:265-266.

Beato, M. 1989. Gene regulation by steroid hormones. Cell 56:335-344.

Beato, M. 1991. Transcriptional control by nuclear receptors. FASEB J 5:2044-2051.

Benjamini, E., R. Coico, G. Sunshine. 2000. *Immunology; A Short Course, 4th Ed.* Wiley & Sons, Inc. New York, NY.

Bohmann, D., T.J. Bos, A. Admon, T. Nishimura, P.K. Vogt, R. Tjian. 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238:1386-1392.

Brack, A., H.L. Rittner, B.R. Younge, C.Kaltschmidt, C.M. Weyand, and J.J. Goronzy. 1997. Glucocorticoid-mediated repression of cytokine gene transcription in human artheritis-SCID chimeras. J Clin Invest 99:2842-2850.

Brandon, D.D., A.J. Markwick, M. Flores, K. Dixon, B.D. Albertson, D.L. Loriaux. 1991. Genetic variation of the glucocorticoid receptor from a steroid-resistant primate. J Mol Endocrinol 7:89-96.

Breslin, M.B., and W.V. Vedeckis. 1998. The human glucocorticoid receptor promoter upstream sequences contain binding sites for the ubiquitous sequences contain binding sites for the ubiquitous transcription factor, Yin Yang 1. J Steroid Biochem Mol Biol 67:369-381.

Breslin, M.B., C.D. Geng, W.Y Vedeckis. 2001. Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol 15:1381-1395.

Brewer, J.A., B.P. Sleckman, W. Swat, L.J. Muglia. 2002. Green fluorescent protein-glucocorticoid receptor knockin mice reveal dynamic modulation during thymocyte development. J Immunol 169:1309-1318.

Brogan, I.J., I.A. Murray, G. Cerillo, M. Needham, A. White, J.R. Davis. 1999. Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-κB lack of effect of glucocorticoid receptor β.

Bronnegard, M., S. Werner, J.A. Gustafsson. 1986. Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. J Clin Invest 78:1270-1278.

Brown, R.S., C. Sander, P. Argos. 1985. The primary structure of transcription factor TFIIIA has 12 consecutive repeats. FEBS Letters 186:271-274.

Bruckmaier, R.M., D. Schams, J.W. Blum. 1993. Milk removal in familiar and unfamiliar surroundings: concentrations of oxytocin, prolactin, cortisol, and β-endorphins. J Dairy Res 60:449-456.

Buemann, B., M-C. Vohl, M. Chagnon, Y.C. Chagnon, J. Gagnon, L. Perusse, F. Dionne, J-P. Depres, A. Tremblay, A. Nadeau, C. Bouchard. 1997. Abdominal visceral fat is associated with a *BclI* restriction fragment length polymorphism at the glucocorticoid receptor gene locus. Obes Res 5:186-192.

Burnstein, K.L., C.M. Jewell, J.A. Cidlowski. 1990. Human glucocorticoid receptor cDNA contains sequences sufficient for receptor down-regulation. J Biol Chem 265:7284-7291.

Burnstein, K.L., D.L. Bellingham, C.M. Jewell, F.E. Powell-Oliver, J.A. Cidlowski. 1991. Autoregulation of glucocorticoid receptor gene expression. Steroids 56:52-58.

Burnstein, K.L., C.M. Jewell, J.A. Cidlowski. 1994. Intragenic sequences of the human glucocorticoid receptor complimentary DNA mediate hormone-inducible receptor messenger RNA down-regulation through multiple mechanisms. Mol Endocrinol 8:1764-1773.

Burton, J.L., M.E. Kehrli, S. Kapil, R.L. Horst. 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J Leuk Biol 57:317-25.

Burton, J.L. and M.E. Kehrli. 1995. Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol- and dexamethasone-treated cows. Am J Vet Res 56:997-106.

Burton, J.L. and M.E. Kehrli. 1996. Effects of dexamethasone on bovine circulating T lymphocyte populations. J Leuk Biol 59:90-9.

Burton, J.L., S.A. Madsen, J. Yao, S. Sipkovsky, P.M. Coussens. 2001. An Immunogenetics approach to understanding periparturient immunosuppression and mastitis susceptibility in dairy cows. Acta Vet Scand 42:407-424.

Burton, J.L. and R.J. Erskine. 2003. Mastitis and immunity: some new ideas for an old disease. Vet Clin North Am Food Anim Pract 19:1-45.

Burton, J.L., J.B. Jacob, P.M. Saama, M.E. Kehrli, Jr., A.E. Freeman, S.C. Kelm, A.L. Kuck, R.J. Tempelman. 2003. Genetic variation in bovine leukocyte responses to dexamethasone challenge. (In preparation for submission to J Dairy Sci).

Buttgereit, F., and A. Scheffold. 2002. Rapid glucocorticoid effects on immune cells. Steroids 67:529-534.

Caamano, C.A., M.I. Morano, H. Akil. 2001. Corticosteroid receptors: a dynamic interplay between protein folding and homeostatic control. Psychopharm Bull 35:6-23.

Caldenhoven, E., J. Liden, S. Wissink, A. Van de Stolpe, J. Raaijmakers, L. Koenderman, S. Okret, J-A. Gustafsson, P.T. Van der Saag. 1995. Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the anti-inflammatory action of glucocorticoids. Mol Endocrinol 9:401-412.

Carding, S.R., and P.J. Egan. 2002. γδ T cells: functional plasticity and heterogeneity. Nat Rev Immunol 2:336-345.

Carlstedt-Duke, J., P.E. Stromstedt, B. Persson, E. Cederlund, J.A. Gustafsson, H. Jornvall. 1988. Identification of hormone-interacting amino acid residues within the steroid-binding domain of the glucocorticoid receptor in relation to other steroid hormone receptors. J Biol Chem 263:6842-6846.

Cato, A.C.B., S. Geisse, M. Wenz, H.M. Westphal, M. Beato. 1984. The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. EMBO J 3:2771-2778.

Chandran, U.R., B. Attardi, R. Friedman, Z-W. Zheng, J.L. Roberts, D.B. DeFranco. 1996. Glucocorticoid repression of the mouse gonadotropin-releasing hormone gene is mediated by promoter elements that are recognized by heteromeric complexes containing glucocorticoid receptor. J Biol Chem 271:20412-20420.

Chen, F., C.S. Watson, B. Gametchu. 1999a. Multiple glucocorticoid receptor transcripts in membrane glucocorticoid receptor-enriched S-49 mouse lymphoma cells. J Cell Biochem 74:418-429.

Chen, F., C.S. Watson, B. Gametchu. 1999b. Association of the glucocorticoid receptor alternatively-spliced transcript 1A with the presence of the high molecular weight membrane glucocorticoid receptor in mouse lymphoma cells. J Cell Biochem 74:430-446.

Chen, Y., S.L. Carpenter, S. J. Lamont. 2000. Detection of protein binding to a glucocorticoid response element-like sequence in a chicken major histocompatibility complex class II promoter. Vet Immunol Immunopath 75:1-7.

Christodoulopoulos, P., D.V. Leung, M.W. Elliott, J.C. Hogg, S. Muro, M. Toda, S. Laberge, Q.A. Hamid. 2000. Increased number of glucocorticoid receptor-β-expressing cells in the airways in fetal asthma. J Allergy Clin Immunol 106:479-484.

Chrousos, G.P., A. Vingerhoeds, D. Brandon, C. Eil, M. Pugeat, M. DeVroede, D.L. Loriaux, M.B. Lipsett. 1982. Primary cortisol resistance in man. A glucocorticoid receptor-mediated disease. J Clin Invest 69:1261-1269.

Cohen, J.J. 1992. Glucocorticoid-induced apoptosis in the thymus. Semin Immunol 4:363-369.

Cole, T.J., J.A. Blendy, W. Schmid, U. Strahle, G. Schutz. 1993. Expression of mouse glucocorticoid receptor and its role during development. J Steroid Biochem Mol Biol 47:49-53.

Cole, T.J., J.A. Blendy, A.P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, G. Schutz. 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Gene Dev 9:1608-1621.

Collins, R.A., D. Werling, S.E. Duggan, A.P. Bland, K.R. Parsons, C.J. Howard. 1998. γδ T cells present antigen to CD4+ αβ T cells. J Leuk Biol 63:707-714.

Corlett, N.J., R.R. Peters, M.J. Paape, W.D. Schultze. 1984. Effect of intramammary device on new infection rate, milk yield, and milk somatic cell counts in Maryland dairy herds. J Dairy Sci 67:2571-2579.

Crabtree, G.R., S. Gillis, K.A. Smith, A. Munck. 1980. Mechanisms of glucocorticoid-induced immunosuppression: inhibitory effects of expression of Fc Receptors and production of T-cell growth factor. J Steroid Biochem 12:445-449.

Cranford, J.L., and R.E. Pearson. 2001. Relationships of sire predicted transmitting ability for somatic cell score with measures of daughter performance. J Dairy Sci 84:1501-1507.

Cress, W.D., and S.J. Treizenberg. 1991. Critical structural elements of the VP16 transcriptional activation domain. Science 251:87-90.

Cronstein, B.N., S.C. Kimmel, R.I. Levin, F. Martiniuk, G. Weissmann. 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc Natl Acad Sci 89:9991-5.

Czar, M.J., J.K.O. Grillo, K.D. Dittmar, K.A. Hutchison, A.M. Zacharek, K.L. Leach, M.R. Deibel, Jr., W.B. Pratt. 1994. Characterization of the protein-protein interactions determining the heat shock protein (hsp90-hsp70-hsp56) heterocomplex. J Biol Chem 269:11155-11161.

Dahlman-Wright, K., A. Wright, J.A. Gustafsson, J. Carlstedt-Duke. 1991. Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimmer is mediated by a short segment of five amino acids. J Biol Chem 266:3107-3112.

Dahlman-Wright, K., T. Almlof, I.J. McEwan, J.A. Gustafsson, A.P. Wright. 1994. Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene expression. Proc Natl Acad Sci 91:1619-1623.

Dahlman-Wright, K., H. Baumann, I.J. McEwan, T. Almlof, A.P. Wright, J.A. Gustafsson, T. Hard. 1995. Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor. Proc Natl Acad Sci 92:1699-1703.

Dahlman-Wright, K., and I. J. McEwan. 1996. Structural studies of mutant glucocorticoid receptor transactivation domains establish a link between transactivation activity *in vivo* and α-helix-forming potential *in vitro*. Biochem 35:1323-1327.

Danielsen, M., J.P. Northrop, G.M. Ringold. 1986. The mouse glucocorticoid receptor mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins. EMBO J 5:2513-2522.

Danielsen, M., J.P. Northrop, J. Jonklaas, G.M. Ringold. 1987. Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. Mol Endocrinol 1:816-822.

de Bosscher, M.L. Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers, G. Haegeman. 1997. Glucocorticoid-mediated repression of nuclear factor-κB-dependent transcription involves direct interference with transactivation. Proc Natl Acad Sci 94:13504-13509.

de Castro, M., S. Elliot, T. Kino, C. Bamberger, M. Karl, E. Webster, G.P. Chrousos. 1996. The non-ligand binding β-isoform of the human glucocorticoid receptor (hGR β) tissue levels, mechanism of action, and potential physiologic role. Mol Med 2:597-607.

DeGraves, F.J., and J. Fetrow. 1993. Economics of mastitis and mastitis control. Vet Clin North Am Food Anim Pract 9:421-434.

De Groot, B.J., J.F. Keown, L.D. Van Vleck, and E.L. Marotz. 2002. Genetic parameters and responses of linear type, yield traits, and somatic cell scores to divergent selection for predicted transmitting ability for type in Holsteins. J Dairy Sci 85:1578-1585.

de Lange, P., J.W. Koper, N.A.T.M. Huizenga, A.O. Brinkmann, F.H. de Jong, M. Karl, G.P. Chrousos, and S.W.J. Lamberts. 1997. Differential hormone-dependent transcriptional activation and –repression by naturally occurring human glucocorticoid receptor variants. Mol Endocrinol 11:1156-1164.

de Lange, P., C.M. Segeren, J.W. Koper, E. Wiemer, P. Sonneveld, A.O. Brinkmann, A. White, I.J. Brogan, F.H. de-Jong, S.W. Lamberts. 2001. Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res 61:3937-3941.

De Libero, G. 2000. Tissue distribution, antigen specificity and effector functions of γδ T cells in human diseases. Springer Semin Immunopathol 22:219-238.

Dellweg, H.G., A. Hotz, K. Mugele, U. Gehring. 1982. Active domains in wild-type and mutant glucocorticoid receptors. EMBO J 1:285-289.

DeRijk, R., and F. Berkenbosch. 1994. Suppressive and permissive actions of glucocorticoids: a way to control innate immunity and to facilitate specificity of adaptive immunity? *Endocrinology and Metabolism Vol 4: Bilateral Communications Between the Endocrine and Immune Systems.* Pg. 73-89. Charles J Grossman (Ed). Springer-Verlag, New York, NY.

Desarnaud, F., S. Bidichandani, P.I. Patel, E-E. Baulieu, M. Schumacher. 2000. Glucocorticosteroids stimulate the activity of the promoters of peripheral myelin protein-22 and protein zero genes in Schwann cells. Brain Res 865:12-16.

Detilleux, J.C., M.E. Kehrli, Jr., A.E. Freeman, L.K. Fox, and D.H. Kelley. 1994. Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet Immunol Immunopath 44:251-267.

Detilleux, J.C., K.J. Koehler, A.E. Freeman, M.E. Kehrli ,Jr., D.H. Kelley. 1995a. Immunological parameters of periparturient Holstein cattle: genetic variation. J Dairy Sci 77:2640-2650.

Detilleux, J.C., M.E. Kehrli, Jr., A.E. Freeman, L.K Fox, and D.H. Kelley. 1995b. Mastitis of periparturient Holstein cattle: a phenotypic and genetic study. J Dairy Sci 78:2285-2293.

Detilleux, J.C. 2002. Genetic factors affecting susceptibility of dairy cows to udder pathogens. Vet Immunol Immunopath 88:103-110.

Diamond, M.I., J.N. Miner, S.K Yoshinaga, K.R. Yamamoto. 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science 249:1266-1272.

Dietz, A.B., N.D. Cohen, L. Timms, M.E. Kehrli, Jr. 1997a. Bovine lymphocyte antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. J Dairy Sci 80:406-412.

Dietz, A.B., J.C. Detilleux, A.E. Freeman, D.H. Kelley, J.R. Stabel, M.E. Kehrli, Jr. 1997b. Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle. J Dairy Sci 80:400-405.

Dobson, M.G., C.P. Redfern, N. Unwin, J.U. Weaver. 2001. The N363S polymorphism of the glucocorticoid receptor: potential contribution to central obesity in men and lack of association with other risk factors for coronary heart disease and diabetes mellitus. J Clin Endocrinol Metab 86:2270-4.

Dong, Y., L. Pellinger, J.A. Gustafsson, S. Okret. 1988. Regulation of glucocorticoid receptor expression evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol 2:1256-1264.

Drouin, J., M.A. Trifiro, R.K. Plante, M. Nemer, P. Eriksson, O. Wrange. 1989a. Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. Mol Cell Biol 9:5305-5314.

Drouin, Y.L. Sun, M. Nemer. 1989b. Glucocorticoid repression of pro-opiomelanocortin gene transcription. J Steroid Biochem 34:63-69.

Drouin, J., M. Nemer, J. Charron, J.P. Gagner, L. Jeannotte, Y.L. Sun, M. Therrien, Y. Tremblay. 1989c. Tissue-specific activity of the pro-opiomelanocortin (POMC) gene and repression by glucocorticoids. Genome 31:510-519.

Eggert, M., C.C. Mows, D. Tripier, R. Arnold, J. Michel, J. Nickel, S. Schmidt, M Beato, R. Renkawitz. 1995. A fraction enriched in a novel glucocorticoid receptor-interacting protein stimulates receptor-dependent transcription *in vitro*. J Biol Chem 270:30755-30759.

Eiler, S., M. Gangloff, S. Duclaud, D. Moras, M. Ruff. 2001. Overexpression, purification, and crystal structure of native ER alpha LBD. Protein Expr Purif 22:165-173.

Eisen, L.P., M.S. Elasser, J.M. Harmon. 1980. Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. J Biol Chem 263:12044-12048.

Encio, I.J., and S.D. Detera-Wadleigh. 1991. The genomic structure of the human glucocorticoid receptor. J Biol Chem 266:7182-7188.

Erskine, R.J., R.J. Eberhart, L.J. Hutchinson, S.B. Spencer, M.A. Campbell. 1988. Incidence and types of clinical mastitis in dairy herds with high and low somatic cell counts. J Am Vet Med Assoc 192:761-765.

Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895.

Feng, J., J. Zheng, W.P. Bennett, L.L. Heston, I.R. Jones, N. Craddock, S.S. Sommer. 2000. Five missense variants in the amino-terminal domain of the glucocorticoid receptor: no association with puerperal psychosis or schizophrenia. Am J Med Genet 96:412-417.

Filep, J.G., A. Delalandre, Y. Payette, E. Foldes-Filip. 1997. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. Circulation 96:295-301.

Filion, L.G., P.J. Willson, H. Bielefeldt-Ohmann, L.A. Babiuk, R.G. Thomson. 1984. The possible role of stress in the induction of pneumonic pasteurellosis [Bovine herpesvirus-1, *Pasteurella haemolytica*]. Can J Comp Revue Can Med Comp:Can Vet Med Assoc. Pg 268-274.

Ford, J., I.J. McEwan, A.P. Wright, J.A. Gustafsson. 1997. Involvement of the transcription factor IID protein complex in gene activation by the N-terminal transactivation domain of the glucocorticoid receptor *in vitro*. Mol Endocrinol 11:1467-75.

Freeman, A.E., and G.L. Lindberg. 1993. Challenges to dairy cattle management: genetic considerations. J Dairy Sci 76:3143-3159.

Gagliardo, R., P. Chanez, A.M. Vignola, J. Bousquet, I. Vachier, P. Godard, G. Bonsignore, P. Demoly, M. Mathieu. 2000. Glucocorticoid receptor α and β in glucocorticoid dependent asthma. Am J Respir Crit Care Med 162:7-13.

Galon, J., D. Franchimont, N. Hiroi, G. Frey, A. Boettner, M. Ehrhart-Bornstein, J.J. O'Shea, G.P. Chrousos, and S.R. Bornstein. 2002. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J 16:61-71.

Gametchu, B., and R.W. Harrison. 1984. Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. Endocrinol 114:274-279.

Gametchu, B. 1987. Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis. Science 236:456-461.

Gametchu, B., C.S. Watson, C. C-Y. Shih, B. Dashew. 1991. Studies on the arrangement of glucocorticoid receptors in the plasma membrane of S-49 lymphoma cells. Steroids 56:411-419.

Gametchu, B., and C.S. Watson. 2002. Correlation of membrane glucocorticoid receptor levels with glucocorticoid-induced apoptotic competence using mutant leukemic and lymphoma cells lines. J Cell Biochem 87:133-146.

Gessani, S., S. McCandless, and C. Baglioni. 1988. The glucocorticoid Dexamethasone inhibits synthesis of interferon by decreasing the level of its mRNA. J Biol Chem 263:7454-7457.

Giguere, V., S.M. Hollenberg, M.G. Rosenfeld, R.M. Evans. 1986. Functional domains of the human glucocorticoid receptor. Cell 46:645-52.

Godowski, P.J., S. Rusconi, R. Miesfeld, K.R. Yamamoto. 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325:365-368.

Godowski, P.J., D. Picard, K.R. Yamamoto. 1988. Signal transduction and transcriptional regulation by glucocorticoid receptor-LexA fusion proteins. Science 241:812-816.

Govindan, M.V., and C.E. Sekeris. 1978. Purification of two dexamethasone-binding proteins from rat-liver cytosol. Eur J Biochem 89:95-104.

Govindan, M.V. 1979. Purification of glucocorticoid receptors from rat liver cytosol. Preparation of antibodies against the major receptor proteins and application of immunological techniques to study activation and translocation. J Steroid Biochem 11:323-332.

Govindan, M.V., F. Pothier, S. Leclerc, R. Palaniswami, B. Xie. 1991. Human glucocorticoid receptor gene promoter-homologous down regulation. J Steroid Biochem Mol Biol 40:317-323.

Grandin, T. 1997. Assessment of stress during handling and transport. J Anim Sci. Jan:249-257.

Guidry, A.J., M.J. Paape, R.E. Pearson. 1976. Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids, and neutrophil phagocytosis in the cow. Am J Vet Res 37:1195-1200.

Hagerty, T., E. Fernandez, K. Lynch, S-S. Wang, W.W. Morgan, R. Strong. 2001. Interaction of a glucocorticoid-responsive element with regulatory sequences in the promoter region of the mouse tyrosine hyroxylase gene. J Neurochem 78:1379-1388.

Hanson, R.W., and L. Reshef. 1997. Regulation of phosphoenolpyruvate carboxykinase (GRP) gene expression. Annu Rev Biochem 66:586-611.

Harmon, J.M., H.J. Eisen, S.T. Bower, S.S. Simons, C.L. Langley, E.B. Thompson. 1984. Identification of human leukemic glucocorticoid receptors using affinity labeling and anti-human glucocorticoid receptor antibodies. Cancer Res 44:4540-4547.

Heck, S. M. Kullmann, A. Gast, H. Ponta, H.J. Rahmsdorf, P.Herrlich, A.C.B. Cato. 1994. EMBO J 13:4087-4095.

Henriksson, A., T. Almlof, J. Ford, I.J. McEwan, J.A. Gustafsson, A.P. Wright. 1997. Role of the Ada adaptor complex in gene activation by the glucocorticoid receptor. Mol Cell Biol 17:3065-3073.

Herrlich, P. 2001. Cross-talk between glucocorticoid receptor and AP-1. Oncogene 20:2465-2475,

Hittelman, A.B., D. Burakov, J.A. Iniguez-Lluhi, L.P. Freedman, M.J. Garabedian. 1999. Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. EMBO J 18:5380-8.

Hogan JS, Smith KL, Hoblet KH, Schoenberger PS, Todhunter DA, Hueston WD, Pritchard DE, Bowman GL, Heider LE, Brockett BL, et al. 1989. Field survey of clinical mastitis in low somatic cell count herds. J Dairy Sci 72:1547-1556.

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

Hollenberg, M.D. 1986. Mechanisms of receptor-mediated transmembrane signaling. Experientia 42:718-727.

Hollenberg, S.M., V. Giguere, P. Segui, R.M. Evans. 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 49:39-46.

Hollenberg, S.M., V. Giguere, R.M. Evans. 1989. Identification of two regions of human glucocorticoid receptor hormone binding domain that block activation. Cancer Res 49:2292s-2294s.

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

Hong, H., K. Kohli, A. Trivedi, D.L. Johnson, M.R. Stallcup. 1996. GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci 93:4948-4952.

Hong, H., B.D. Darimont, H. Ma, L. Yang, K.R. Yamamoto, M.R. Stallcup. 1999. An additional region of coactivator GRIP1 required for interaction with the hormone-binding domains of a subset of nuclear receptors. J Biol Chem 274:3496-3502.

Hope, I.A., and K. Struhl. 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell 46:885-894.

Houdebine, L-M, J. Djiane, I. Dusanter-Fourt, P. Martel, P.A. Kelly, E. Devinoy, J-L. Servely. 1985. Hormonal action controlling mammary activity. J Dairy Sci 68:489-500.

Howard, C.J., P. Sopp, K.R. Parsons, J. Finch. 1989. In vivo depletion of BoT4 (CD4) and of non-T4/T8 lymphocyte subsets in cattle with monoclonal antibodies. Eur J Immunol 19:757-764,

Huang, Y., and S.S. Simons. 1999. Functional analysis of R651 mutations in the putative helix 6 of rat glucocorticoid receptors. Mol Cell Endocrinol 158:117-130.

Huckaby, C.S., O.M. Conneely, W.G. Beattie, A.D. Dobson, M.J. Tsai, B.W. O'Malley. 1987. Structure of the chromosomal chicken progesterone receptor gene. Proc Natl Acad Sci 84:8380-8384.

Huizenga, N.A., J.W. Koper, P. de Lange, H.A. Pols, R.P. Stolk, H. Burger, D.E. Grobbee, A.O. Brinkmann, F.H. de Jong, S.W. Lamberts. 1998. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids *in vivo*. J Clin Endocrinol Metab 83:144-51.Hurley et al., 1991

Hurley, D.M., D. Accili, C.A. Stratakis, M. Karl, N. Vamvakopoulos, E. Rorer, K. Constantine, S.I. Taylor, G.P. Chrousos. 1991. J Clin Invest 87:680-686.

Iida, S., M. Gomi, K. Moriwaki, Y. Itoh, K. Hirobe, Y. Matsuzawa, S. Katagiri, T. Yonezawa, S. Tarui. 1985. Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. J Clin Endocrinol Metab 60:967-971.

Imai, E., J.N. Miner, J.A. Mitchell, K.R. Yamamoto, D.K. Granner. 1993. Glucocorticoid receptor-cAMP response element-binding protein interaction and the response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids. J Biol Chem 268:5353-5356.

Iniguez-Lluhi, J.A., D.Y. Lou, K.R. Yamamoto. 1997. Three amino acid substitutions selectively disrupt the activation but not the repression function of the glucocorticoid receptor N-terminus. J Biol Chem 272:4149-4156.

Iniguez-Lluhi, J.A. and D. Pearce. 2000. A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol Cell Biol 20:6040-6050.

Itani, O.A., K.Z. Liu, K.L. Cornish, J.R. Campbell, C.P. Thomas. 2002. Glucocorticoids stimulate human *sgk1* gene expression by activation of a GRE in its 5'-flanking region. Am J Physciol Endocrinol Metab 283:E971-E979.

Jabara, H.H., S.R. Brodeur, R.S. Geha. 2001. Glucocorticoids upregulate CD40 ligand expression and induce CD40L-dependent immunoglobulin isotype switching. J Clin Invest 107:371-378.

Jacob, J.B., R.J. Tempelman, P.M. Saama, P.M. Coussens, M.E. Kerhli Jr., A.E. Freeman, A.L. Kuck, B.R. Southey, J.L. Burton. 2003. Mutations in exon 2 of bovine glucocorticoid receptor genes associate with leukocyte sensitivity to glucocorticoids. (In preparation for submission to Anim Genet).

Jiang, Y., S.J. Triezenberg, J.D. Galla. 1994. Defective transcriptional activation by diverse VP16 mutants associated with a common inability to form open promote complexes. J Biol Chem 269:5505-5508.

Jiang, T., S. Liu, M. Tan, F. Huang, Y. Sun, X. Dong, W. Guan, L. Huang, F. Zhou. 2001. The phase-shift mutation in the glucocorticoid receptor gene: potential etiologic significance of neuroendocrine mechanisms in lupus nephritis. Clin Chim Acta 313:113-117.

Jima, B., J. Voltmann, S. Albinni, P. Stohlawetz, I. Schwarzinger, C.H. Gleiter, A. Rauch, H.G. Eichler, O.F. Wagner. 1997. Dexamethasone down-regulates the expression of L-selectin on the surface of neutrophils and lymphocytes in humans. Clin Pharmacol Ther 62:562-568.

Jobling, A.I., and R.C. Augusteyn. 2001. Is there a glucocorticoid receptor in the bovine lens? Exp Eye Res 72:687-694.

Jonat, C., H.J. Rahmsdorf, K-K Park, A.C.B. Cato, S.Gebel, H. Ponta, P. Herrlich. 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (fos/jun) activity by glucocorticoid hormone. Cell 62:1189-1204.

Kahsar-Miller, M., R. Azziz, E. Feingold, S.F. Witchel. 2000. A variant of the glucocorticoid receptor gene is not associated with adrenal androgen excess in women with polycystic ovary syndrome. Fert Steril 74:1237-1240.

Kakidani, H., and M. Ptashne. 1988. GAL4 activates gene expression in mammalian cells. Cell 52:161-167.

Kalinyak, J.E., R.I. Dorin, A.R. Hoffman, A.J. Perlman. 1987. Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J Biol Chem 262:10441-10444.

Kamada AK, Spahn JD, Surs W, Brown E, Leung DY, Szefle. 1994. Coexistence of glucocorticoid receptor and pharmacokinetic abnormalities: factors that contribute to a poor response to treatment with glucocorticoids in children with asthma. J Pediatr 124:984-986,

Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S-C. Lin, R. A. Heyman, D.W. Rose, C.K. Glass, M.G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85:403-414.

Kansas, G.S. 1996. Selectins and their ligands: current concepts and controversies. Blood 88:3259-3287.

Karl, M., S.W.J. Lamberts, S.D. Detera-Wadleigh, I.J. Encio, C.A. Stratakis, D.M. Hurley, D. Accili, G.P. Chrousos. 1993. Familial glucocorticoid resistance caused by splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 76:683-689.

Karl, M., G. Von-Wichert, E. Kempter, D.A. Katz, M. Reincke, H. Monig, I.U. Ali, C.A. Stratakis, E.H. Oldfield, G.P. Chrousos, H.M. Schulte. 1996a. Nelson's syndrome associated with a somatic frame shift mutation in the glucocorticoid receptor gene. J Clin Endocrinol Metab 91:124-9.

Karl, M., S.W. Lamberts, J.W. Koper, D.A. Katz, N.E. Huizenga, T. Kino, B.R. Haddad, M.R. Hughes, G.P. Chrousos. 1996b. Cushing's disease preceded by generalized glucocorticoid resistance clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Am Physicians 108:296-307.

Kawakami, K., C. Scheidereit, R.G. Roeder. 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc Natl Acad Sci 85:4700-4704.

Kehrli, M.E., Jr., J.L. Burton, B.J. Nonnecke, and E-K. Lee. 1999. Effects of stress on leukocyte trafficking and immune responses: implications for vaccination. *Advances in Veterinary Medicine Vol. 41: Veterinary Vaccine and Diagnostics.* R.D. Schultz (ed.). pp 61-81. Academic Press, New York, NY.

Kehrli, M.E., Jr., and J.A. Harp. 2001. Immunity in the mammary gland. Vet Clin North Am Food Anim Pract 17:495-516.

Keightley, M-C., and P.J. Fuller. 1994. Unique sequences in the guinea pig glucocorticoid receptor induce constitutive transactivation and decrease steroid sensitivity. Mol Endocrinol 8:43f1-439.

Keightley, M-C., and P.J. Fuller. 1995. Cortisol resistance and the guinea pig glucocorticoid receptor. Steroids 60:87-92.

Kelm, S.C., A.E. Freeman, M.E. Kehrli, Jr., B.R. Southey, J.L. Burton, and A.L. Kuck. Genetic and phenotypic variation for immune response traits by Holstein bulls within an immunosuppression model. In review.

Kino, T. A. Vottero, E. Charmadari, G.P. Chrousos. 2002a. Familial/sporadic glucocorticoid resistance syndrome and hypertension. Ann NY Acad Sci 970:101-111.

Kino, T., A. Gragerov, O. Slobodskaya, M. Tsopanomichalou, G.P. Chrousos, G.N. Pavlakis. 2002b. Human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr induces transcription of the HIV-1 and glucocorticoid-responsive promoters by binding directly to p300/CBP coactivators. J Virol 76:9724-9734.

Kitraki, E., C. Kittas, F. Stylianopoulou. 1997. Glucocorticoid receptor gene expression during rat embryogenesis. An *in situ* hybridization study. Differentiation 62:21-31.

Ko, H.S., P. Fast, W. McBride, L.M. Staudt. 1988. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. Cell 55:135-144.

Kolla, V., N.M. Robertson, G. Litwack. 1999. Identification of a mineralcorticoid/glucocorticoid response element in the human Na/K ATPase α1 gene promoter. Biochem Biophys Res Comm 266:5-14.

Konig, H., H. Ponta, H.J. Rahmsdorf, P. Herrlich. 1992. Interference between pathway-specific transcription factors glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation *in vivo*. EMBO J 11:2241-2246.

Koper, J.W., R.P. Stolk, P. de Lange, N.A.T.M. Huizenga, G-J. Molijn, H.A.P. Pols, D.E. Grobbee, M. Karl, F.H. de Jong, A.O. Brinkmann, S.W.J. Lamberts. 1997. Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Hum Genet 99:663-668.

Korte, S.M. 2001. Corticosteroids in relation to fear, anxiety, and psychopathology. Neurosci Biobehav Rev 25:117-142.

Kraft, N., A.J. Hodgson, J.W. Funder. 1979. Glucocorticoid receptor and effector mechanisms a comparison of the corticosensitive mouse with the corticoresistant guinea pig. Endocrinol 104:344-349.

Krett, N.L., S. Pillay, P.A. Moalli, P.R. Greipp, S.T. Rosen. 1995. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res 55:2727-2729.

Kuhn, L.A., J.H. Griffin, C.L. Fisher, J.S. Greengard, B.N. Bouma, F. Espana, J.A. Tainer. 1990. Elucidating the structural chemistry of glycosaminoglycan recognition by protein C inhibitor. Biochemistry 87:8506-8510.

Kunzendorf, U., T.H. Tran, S. Bulfone-Paus. 1998. The Th1-Th2 paradigm in 1998: law of nature or rule of exception. Nephrol Dial Transplant 13:2445-2448.

Laako, T., and P. Fraser. 2002. Rapid changes in the lymphopoietic and granulopoietic compartments of the marrow caused by stress levels of corticosterone. Immunol 105:111-119.

Lahiri, D. K., S. Bye, J. I. Nurnberger, Jr., M. E. Hodes, M. Crisp. 1992. A non-organic and non-enzymatic extraction method gives higher yields of genomic DNA from whole-blood samples than do nine other methods tested. J Biochem Biophys Methods. 25:193-205

Lamberts, S.W., D. Poldermans, M. Zweens, F.H. de Jong. 1986. Familial cortisol resistance differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 63:1328-1333.

Lamberts, S.W., J.W. Koper, P. Biemond, F.H. den Holder, F.H. de Jong. 1992. Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol Metab 74:313-321.

Lee, E-K., and M.E. Kehrli, Jr. 1998. Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. Am J Vet Res 59:37-43.

Lethimonier, C., G. Flouriot, O. Kah, B. Ducouret. 2002. The glucocorticoid receptor represses the positive autoregulation of the trout estrogen receptor gene by preventing the enhancer effect of a C/EBPβ-like protein. Endocrinol 143:2961-2974.

Liden, J., I. Rafter, M. Truss, J-A. Gustafsson, S. Okret. 2000. Glucocorticoid effects on NF-κB binding in the transcription of the ICAM-1 gene. Biochem Biophys Res Comm 273:1008-1014.

Lietzke, S.E., R.D. Scavetta, M.D. Yoder, F.A. Jurnak. 1996. The refined three-dimensional structure of pectate lyase E from *Erwinia chrysanthemi* at 2.2 A resolution. Plant Physiol 111:73-92.

Linder, M.J., and E.B. Thompson. 1989. Abnormal glucocorticoid receptor gene and mRNA in primary cortisol resistance. J Steroid Biochem 32:243-249.

Liu, W., A.G. Hillmann, J.M. Harmon. 1995. Hormone-independent repression of AP-1-inducible collagenase promoter activity by glucocorticoid receptors. Mol Cell Biol 15:1005-1013.

Long, A.D., and C.H. Langley. 1999. The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. Gen Res 9:720-731.

Lopez, J.F., H. Akil, S.J. Watson. 1999. Role of biological and psychological factors in early development and their impact on adult life. Biol Psychiatry 46:1461-1471.

Luisi, B.F., W.X. Xu, Z. Otwinowski, L.P. Freedman, K.R. Yamamoto, P.B. Sigler. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497-505.

Lutje, V., and S.J. Black. 1991. Cellular interactions regulate the *in vitro* response of bovine lymphocytes to ovalbumin. Vet Immunol Immunopath 28:275-288.

Ma, J., and M. Ptashne. 1987. A new class of yeast transcriptional activators. Cell 51:113-119.

Maden, A.P., and D.B. DeFranco. 1993. Bidirectional transport of glucocorticoid receptors across the nuclear envelope. Proc Natl Acad Sci 90:3588-3592.

Madsen, S.A., P.S.D. Weber, J.L. Burton. 2002. Altered expression of cellular genes in neutrophils of periparturient dairy cows. Vet Immunol Immunopath 86:159-175.

Malchoff, D.M., A. Brufsky, G. Reardon, P. McDermott, E.C. Javier, C.H. Bergh, D. Rowe, C.D. Malchoff. 1993. A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 91:1918-25.

Malkoski, S.P., and R.I. Dorin. 1999. Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. Mol Endocrinol 13:1629-1644.

Maroder, M., A.R. Farina, A.Vacca, M.P. Felli, D. Meco, I. Screpanti, L. Frati, A. Gulino. 1993. Cell-specific bifunctional role of jun oncogene family members on glucocorticoid-receptor-dependent transcription. Mol Endocrinol 7:570-584.

Maughan, W.Z., C.R. Bisho, T.Aa.Pryor, J.W. Athens. 1973. The question of cycling of the blood neutrophil concentrations and pitfalls in the statistical analysis of sampled data. Blood 41:85-91.

McEwan, I.J., A.P. Wright, K. Dahlman-Wright, J. Carlstedt-Duke, J.A. Gustafsson. 1993. Direct interaction of the τ1 transactivation domain of the human glucocorticoid receptor with the basal transcriptional machinery. Mol Cell Biol 13:399-407.

McEwan, I.J., T. Almlof, A.C. Wikstrom, A.P. Wright, J.A. Gustafsson. 1994. The glucocorticoid receptor functions at multiple steps during transcription initiation by RNA polymerase II. J Biol Chem 269:25629-25636.

McEwan, I.J., K. Dahlman-Wright, T. Almlof, J. Ford, A.P. Wright, J.A. Gustafsson. 1995. Mechanisms of transcription activation by nuclear receptors: studies on the human glucocorticoid receptor τ1 transactivation domain. Mutat Res 333:15-22.

McEwan, I.J. 2000. Steroid receptor co-activators and the remodeling of chromatin. Biochem Soc Trans 28:369-373.

McKay, L.I., and J.A. Cidlowski. 1998. Cross-talk between nuclear factor-κB and the steroid hormone receptors: mechanisms of mutual antagonism. Mol Endocrinol 12:45-56.

Mendonca, B.B., M.V. Leite, M. De Castro, T. Kino, L.L.K. Elias, T.A.S. Bachega, I.J.P. Arnhold, G.P. Chrousos, C. Latronico. 2002. Female pseudohermaphroditism caused by a novel homozygous missense mutation of the GR gene. J Clin Endocrinol Metab 87:1805-1809.

Metchnikov, I.I. 1908. Nobel lecture: On the present state of the question of immunity in infectious diseases. *Nobel Lectures*, Physiology or Medicine 1901-1921. http://www.nobel.se/medicine/laureates/1908/metchnikov-lecture.html.

Meyer, T., D.B. Starr, J. Carlstedt-Duke. 1997. The rat glucocorticoid receptor mutant K461A differentiates between two different mechanisms of transrepression. J Biol Chem 272:21090-21095.

Milhon, J., K. Kohli, M.R. Stallcup. 1997. Genetic analysis of the N-terminal end of the glucocorticoid receptor hormone binding domain. J Steroid Biochem Molec Biol 51:11-19.

Miller, J., A.D. McLachlan, A. Klug. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J 4:1609-1614.

Miller, A.H., R.L. Spencer, J. Hassett, C. Kim, R. Rhee, D. Ciurea, F. Dhabhar, B. McEwen, M. Stein. 1994. Effects of selective type I and II adrenal steroid agonists on immune cell distribution. Endocrinology 135:1934-1944.

Miner, J.N., and K.R. Yamamoto. 1992. The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Gene Dev 6:2491-2501.

Minton, J.E. 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. J Animal Sci July:1891-1898.

Mitchell, G., J. Hattingh, M. Ganhao. 1988. Stress in cattle assessed after handling, after transport, and after slaughter. Vet Rec J Br Vet Assoc London 20:201-205.

Mitchell, P.J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371-378.

Mitchell, B.M., and R.C. Webb. 2002. Impaired vasodilation and nitric oxide synthase activity in glucocorticoid-induced hypertension. Biol Res Nursing 4:16-21.

Moalli, P.A., and S.T. Rosen. 1994. Glucocorticoid receptors and resistance to glucocorticoids in hematologic malignancies. Leuk Lymphoma 15:363-374.

Morrison, N., and J. Eisman. 1993. Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. J Bone Miner Res 8:969-975.

Muller, M., and R. Renkawitz. 1991. The glucocorticoid receptor. Biochim Biophys Acta 16:171-182.

Nakagawa, M., G.P. Bondy, D. Waisman, D. Minshall, J.C. Hogg, S.F. van Eeden. 1999. The effect of glucocorticoids on the expression of L-selectin on polymorphonuclear leukocytes. Blood 93:2730-2737.

Nakai, Y., T. Usui, T. Tsukada, H. Takahashi, J. Fukata, M. Fukushima, K. Senoo, H. Imura. 1991. Molecular mechanisms of glucocorticoid inhibition of human proopiomelanocortin gene transcription. J Steroid Biochem Mol Biol 40:301-306.

Nanda, A.S., H. Dobson, W.R. Ward. 1990. Relationship between an increase in plasma cortisol during transport-induced stress and failure of oestradiol to induce a luteinising hormone surge in dairy cows. Res Vet Sci London July:25-28.

Nawata, H., K. Sekiya, K. Higuchi, K. Kato, H. Ibayashi. 1987. Decreased deoxyribonucleic acid binding of glucocorticoid-receptor complex in cultured skin fibroblasts from a patient with the glucocorticoid resistance syndrome. J Clin Endocrinol Metabl 65:219-226.

Newton, R. 2000. Molecular mechanisms of glucocorticoid action: what is important? Thorax 55:603-613.

Nishio, Y., H. Isshiki, T. Kishimoto, S. Akira. 1993. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of a rat α1-acid glycoprotein gene via direct protein-protein interaction. Mol Cell Biol 13:1854-1862.

Nobukuni, Y., C.L. Smith, G.L. Hager, S.D. Detera-Wadleigh. 1995. Characterization of the human glucocorticoid receptor promoter. Biochemistry 34:8207-8214.

Nonnecke, B.J., J.L. Burton, M.E. Kehrli, Jr. 1997. Associations between function and composition of blood mononuclear leukocyte populations from Holstein bulls treated with dexamethasone. J Dairy Sci 80:2403-2410.

Nord, M., T.N. Cassel, H. Braun, G. Suske. 2000. Regulation of the Clara cell secretory protein/uteroglobin promoter in lung. Ann NY Acad Sci 923:145-165.

Norman, C., M. Runswick, R. Pollock, R. Treisman. 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell 55:989-1003.

Oakley, R.H., M. Sar, J.A. Cidlowski. 1996. The human glucocorticoid receptor β isoform. Expression, biochemical properties, and putative function. J Biol Chem 271:9550-9559.

Oakley, R.H., J.C. Webster, M. Sar, C.R. Parker, J.A. Cidlowski. 1997. Expression and subcellular distribution of the β-isoform of the human glucocorticoid receptor. Endocrinology 138:5028-5038.

Oakley, R.H., C.M. Jewell, M.R. Yudt, D.M. Bofetiado, J.A. Cidlowski. 1999. The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action. J Biol Chem 274:27857-27866.

Okret, S., J. Carlstedt-Duke, O. Wrange, K. Carlstrom, J.A. Gustafsson. 1981. Characterization of an antiserum against the glucocorticoid receptor. Biochim Biophys Acta 677:205-219.

Okret, S., L. Poelliinger, Y. Dong, J.A. Gustafsson. 1986. Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Porc Natl Acad Sci 83:5899-5903.

Oldham, G., and C.J. Howard. Suppression of bovine lymphocyte responses to mitogens following *in vivo* and *in vitro* treatment with dexamethasone. 1992. Vet Immunol Immunopathol 30:161-77.

Oro, A.E., S.M. Hollenberg, R.M. Evans. 1988. Transcriptional inhibition by a glucocorticoid receptor-β-galactosidase fusion protein. Cell 55:1109-1114.

Paape, M.J., W.D. Schultze, A.J. Guidry, W.M. Kortum, B.T. Weinland. 1981. Effect of an intramammary polyethylene device on the concentration of leukocytes and immunoglobulins in milk and on the leukocyte response to *Escherichia coli* endotoxin and challenge exposure with *Staphylococcus aureus*. Am J Vet Res 42:774-783.

Paape, M.J., K. Shafer-Weaver, A.V. Capuco, K. van Oostveldt, C. Burvenich. 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv Exp Med Biol 480:259-277.

Palmer, L.A., and J.M. Harmon. 1991. Biochemical evidence that glucocorticoid-sensitive cell lines derived from the human leukemic cell line CCRF-CEM express a normal and a mutant glucocorticoid receptor gene. Cancer Res 51:5224-5231.

Pan, L., D.B. Mendel, J. Zurlo, P.M. Guyre. 1990. Regulation of the steady state level of FcγRI mRNA by IFN-γ and dexamethasone in human monocytes, neutrophils, and U-937 cells. J Immunol 145:267-275.

Panarelli, M. C.D. Holloway, R. Fraser, J.M.C. Connell, M.C Ingram, N.H. Anderson, C.J. Kenyon. 1998. Glucocorticoid receptor polymorphism, skin vasoconstriction, and other metabolic intermediate phenotypes in normal human subjects. J Clin Endocrinol Metab 83:1846-1852.

Parrelli, J.M., N. Meisler, K.R. Cutroneo. 1998. Identification of a glucocorticoid response element in the human transforming growth factor beta 1 gene promoter. Intl J Biochem Cell Biol 30:623-627.

Pearce, D., and K.R. Yamamoto. 1993. Mineralcorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science 259:1161-1165.

Pedersen, S.B., M. Jonler, B. Richelsen. 1994. Characterization of regional and gender differences in glucocorticoid receptors and lipoprotein lipase activity in human adipose tissue. J Clin Endocrinol Metab 78:1354-1359.

Periyasamy, S., and E.R. Sanchez. 2002. Antagonism of glucocorticoid receptor transactivity and cell growth inhibition by transforming growth factor-β through AP-1-mediated transcriptional repression. Intl J Biochem Cell Biol 1310:1-15.

Perretti, M., and A. Ahluwalia. 2000. The microcirculation and inflammation: site of action for glucocorticoids. Microcirculation 7:147-161.

Pfahl, M. 1993. Nuclear receptor/AP-1 interaction. Endo Rev 14:651-658.

Pitzalis, C., N. Pipitone, M. Perretti. 2002. Regulation of leukocyte-endothelial interactions by glucocorticoids. Ann NY Acad Sci 966:108-118.

Pollock, J.M., and M.D. Welsh. 2002. The WC1+ γδ T-cell population in cattle: a possible role in resistance to intracellular infection. Vet Immunol Immunopathol 89:105-114.

Preisler, M.T., P.S.D. Weber, R.J. Tempelman, R.J. Erskine, H. Hunt, J.L. Burton. 2000a. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. Am J Vet Res 61:14-19.

Preisler, M.T., P.S.D. Weber, R.J. Tempelman, R.J Erskine, H. Hunt, and J.L. Burton. 2000b. Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient Holstein cows. J Dairy Sci 83:38-47.

Prufer, K., and J. Barsony. 2001. Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. Mol Endocrinol 16:1738-1751.

Ray, K.P., S. Farrow, M. Daly, F. Talabot, N. Searle. 1997. Induction of the E-selectin promoter by interleukin-1 and tumor necrosis factor α, and inhibition by glucocorticoids. Biochem J 328:707-715.

Ray, A., and K.E. Prefontaine. 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. Proc Natl Acad Sci 91:752-756.

Reichardt, H.M., K.H. Kaestner, J. Tuckermann, O. Kretz, O. Wessely, R. Bock, P. Gass, W. Schmid, P. Herrlich, P. Angel, G. Schutz. 1998. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93:531-541.

Reichardt, H.M., K. Horsch, H-J. Grone, A. Kolbus, H. Beug, N. Hynes, G. Schutz. 2001. Mammary gland development and lactation are controlled by different glucocorticoid receptor activities. Eur J Endo 145:519-527.

Refojo, D., A.C. Liberman, F. Holsboer, E. Arzt. 2001. Transcription factor-mediated molecular mechanisms involved in the functional corss-talk between cytokines and glucocorticoids. Immunol Cell Biol 79:385-394.

Reiger, M.K., D.L. DeWitt, M.S. Schindler, W.L. Smith. 1993. Subcellular localization of prostoglandin endoperoxide synthase-2 in murine 3T3 cells. Arch Biochem Biophys 301:439-444.

Reul, J.M., P.T. Pearce, J.W. Funder, Z.S. Krozowski. 1989. Type I and type II corticosteroid receptor gene expression in the rat: effect of adrenalectomy and dexamethasone administration. Mol Endocrinol 3:1674-1680.

Rice, D.W., and D. Eisenberg. 1997. A 3D-1D substitution matrix for protein fold recognition that includes predicted secondary structure of the sequence. J Mol Biol 267:1026-1038.

Riollet, C., P. Rainard, B. Poutrel. Cells and cytokines in inflammatory secretions of the bovine mammary gland. 2000a. Adv Exp Med Biol 480:247-258.

Riollet, C., P. Rainard, B. Poutrel. 2000b. Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* α-toxin. Inflamm Res 49:486-496.

Riollet, C., P. Rainard, B. Pontrel. 2001. Cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection. J Dairy Sci 84:1077-1084.

Robyr, D., and A.P. Wolfe. 1998. Hormone action and chromatin remodeling. Cell Mol Life Sci 54:113-124.

Rosen, S.D., and C.R. Bertozzi. 1994. The selectins and their ligands. Curr Opin Cell Biol 6:663-673.

Rosewicz, S., A.R. McDonald, B.A. Maddux, I.D. Goldfine, R.L. Miesfeld, C.D. Logsdon. 1988. Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. J Biol Chem 263:2581-2584.

Rosmarin, A.G., R. Levy, D.G. Tenen. 1992. Cloning and analysis of the CD18 promoter. Blood 79:2598-2604.

Rosmond, R., Y.C. Chagnon, M. Chagnon, L. Perusse, C. Bouchard, P. Bjorntorp. 2000. A polymorphism of the 5'-flanking region of the glucocorticoid receptor gene locus is associated with basal cortisol secretion in men. Metabolism 49:1197-1199.

Rosmond, R., C. Bouchard, P. Bjorntorp. 2001. Tsp509I polymorphism in exon 2 of the glucocorticoid receptor gene in relation to obesity and cortisol secretion: cohort study. BMJ 322: 652-653.

Roth, J.A., and M.L. Kaeberle. 1982. Effect of glucocorticoids on the bovine immune system. J Am Vet Med Assoc 180:894-901.

Ruiz, M., U. Lind, M. Gafvels, G. Eggertsen, J. Carlstedt-Duke, L. Nilsson, M. Holtmann, P. Stierna, A-C. Wikstrom, S. Werner. 2001. Characterization of two novel mutations in the glucocorticoid receptor gene in patients with primary cortisol resistance. Clin Endocrinol 55:363-371.

Sackey, F.N.A., C.S. Watson, B. Gametchu. 1997. Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis. Am J Physiol 273:E571-E583.

Sadowski, I., J. Ma, S. Triezenberg, M. Ptashne. 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature 335:563-564.

Sakai, D.D., S. Helms, J. Carlstedt-Duke, J-A. Gustafsson, F.M. Rottman, K.R. Yamamoto. 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Gene Dev 2:1144-1154.

Sakurai, A., T. Miyamoto, L.J. DeGroot. 1992. Cloning and characterization of the human thyroid hormone receptor β1 gene promoter. Biochem Biophys Res Commun 185:78-84.

Schalm, O.W., J. Lasmanis, E.J. Carroll. 1964. Effects of pre-existing leukocytosis on experimental coliform (*Aerobacter aerogenes*) mastitis in cattle. Am J Vet Res 25:83-89.

Scheidereit, C., S. Geisse, H.M. Westphal, M. Beto. 1983. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. Nature 304:749-752.

Scheinman, R.I., A.Gualberto, C.M. Jewell, J.A. Cidlowski, A.S. Baldwin, Jr. 1995. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. Mol Cell Biol 15:943-953.

Schena, M., L.P. Freedman, K.R. Yamamoto. 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Gene Dev 3:1590-1601.

Schmitt, J., and H.G. Stunnenberg. 1993. The glucocorticoid receptor hormone binding domain mediates transcriptional activation in vitro in the absence of ligand. Nucleic Acids Res 21:2673-2681.

Schreiber, S.L. 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 251:283-287.

Schule, R., M. Muller, C. Kaltschmidt, R. Renkawitz. 1988. Many transcription factors interact synergistically with steroid receptors. Science 242:1418-1420.

Schule, R. P. Rangarajan, S. Kliewer, L.J. ransone, J. Bolado, N. Yang, I.M. Verma, R.M. Evans. 1990. Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. Cell 62:1217-1226.

Scott, D.K., P-E. Stromstedt, J-C. Wang, D.K. Granner. 1998. Further characterization of the glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. The role of the glucocorticoid receptor-binding sites. Mol Endocrinol 12:482-491.

Screpanti I, S. Morrone , D. Meco, A. Santoni, A. Gulino, R. Paolini, A. Crisanti, B.J. Mathieson, L. Frati. 1989. Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17 β-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. J Immunol 142:23378-3383.

Segal, J.A., J.L. Barnett, D.L. Crawford. 1999. Functional analyses of natural variation in Sp1 binding sites of a TATA-less promoter. J Mol Evol 49:736-749.

Sevi, A., L. Taibi, M. Albenzio, A. Muscio, S. Dell'Aquila, F. Napolitano. 2001. Behavioral, adrenal, immune, and productive responses of lactating ewes to regrouping and relocation. J Anim Sci 79:1457-1465.

Sharif, S., B.A. Mallard, B.N. Wilkie, J.M. Sargeant, H.M. Scott, J.C.M. Dekkers, K.E. Leslie. 1998a. Associations of the bovine major histocompatibility complex DRB3 (*BoLa-DRB3*) with production traits in Canadian dairy cattle. Anim Genet 30:157-160.

Sharif, S., B.A. Mallard, B.N. Wilkie, J.M. Sargeant, H.M. Scott, J.C.M. Dekkers, K.E. Leslie. 1998b. Associations of the bovine major histocompatibility complex DRB3 (*BoLa-DRB3*) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Anim Genet 29:185-193.

Sigler, P.B. 1988. Transcriptional activation. Acid blobs and negative noodles. Nature 333:210-212.

Simons, S.S., J.G. Pumphrey, S. Rudikoff, H.J. Eisen. 1987. Identification of cysteine 656 as the amino acid of hepatoma tissue culture cell glucocorticoid receptors that is covalently labeled by dexamethasone 21-mesylate. J Biol Chem 262:9676-9680.

Simons, S.S., F.D. Sistare, P.K. Chakraborti. 1989. Steroid binding activity is retained in a 16-kDa fragment of the steroid binding domain of rat glucocorticoid receptors. J Biol Chem 264:14493-14497.

Simons, S.S., Jr. 1994. Function/activity of specific amino acids in glucocorticoid receptors. Vit Horm 49:49-130.

Sivo, J., A.D. Politis, S.N. Vogel. 1993. Differential effects of interferon-γ and glucocorticoids on FcγR gene expression in murine macrophages. J Leuk Biol 54:451-457.

Slater, E.P., O. Rabenau, M. Karin, J.D. Baxter, M. Beato. 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol Cell Biol 5:2984-2992.

Smale, S.T. 1997. Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochim Biophys Acta 20:1-2.

Smith, V. G., L.A. Edgerton, H.D. Hafs, E.M. Convey. 1973. Bovine serum estrogens, progestins, and glucocorticoids during late pregnancy, parturition, and early lactation. J Anim Sci 36:391-396.

Smith, C.L., and G.L. Hammond. 1992. Hormonal regulation of corticosteroid-binding globulin biosynthesis in the male rat. Endocrinol 130:2245-2251.

Smith, P.J., D.J. Cousins, Y-K. Jee, D.Z. Staynov, T.H. Lee, P. Lavender. 2001. Suppression of granulocyte-macrophage colony-stimulating factor expression by glucocorticoids involves inhibition of enhancer function by the glucocorticoid receptor binding to composite NF-AT/activator protein-1 elements. J Immunol 167:2502-2510.

Starr, D.B., W. Matsui, J.R. Thomas, K.R. Yamamoto. 1996. Intracellular receptors use a common mechanism to interpret signaling information at response elements. Gene Dev 10:1271-1283.

Sternberg, M.J.E., Ed. 1996. Protein Structure Prediction, A Practical Approach. Oxford University Press, New York, NY.

Strahle, U., A. Schmidt, G. Kelsey, A.F. Stewart, T.J. Cole, W. Schmid, G. Schutz. 1992. At least three promoters direct expression of the mouse glucocorticoid receptor gene. Proc Natl Acad Sci 89:6731-6735.

Struhl, K., C.J. Brandl, W. Chen, P.A. Harbury, I.A. Hope, S. Mahadevan. 1988. Transcriptional activation by yeast GCN4, a functional homolog to the jun oncoprotein. Cold Spring Harb Symp Quant Biol 53:2701-2709.

Sturm, R.A., G. Das, W. Herr. 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Gene Dev 2:1582-1599.

Subramaniam, N., W. Cairns, S. Okret. 1997. Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene. DNA Cell Biol 16:153-163.

Subramaniam, N., W. Cairns, S. Okret. 1998. Glucocorticoids repress transcription from a negative glucocorticoid response element recognized by two homeodomain-containing proteins, Pbx and Oct-1. J Biol Chem 273:23567-23574.

Takahasi, L.K. 1996. Glucocorticoids and the hippocampus: developmental interactions facilitating the expression of behavioral inhibition. Mol Neurobiol 13:213-226.

Tasset, D., L. Tora, C. Fromental, E. Scheer, P. Chambon. 1990. Distinct classes of transcriptional activating domains function by different mechanisms. Cell 62:1177-1187.

Tempelman, R.J., P.M. Saama, A.E. Freeman, S.C. Kelm, A.L. Kuck, M.E. Kehrli, Jr., and J.L. Burton. 2002. Genetic variation in bovine neutrophil sensitivity to glucocorticoid challenge. Acta Agric Scand 52:189-202.

Theriault A, E. Boyd, S.B. Harrap, S.M. Hollenberg, J.M. Connor. 1989. Regional chromosomal assignment of the human glucocorticoid receptor gene to 5q31. Hum Genet 83(3):289-91.

Tjian, R., and T. Maniatis. 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell 77:5-8.

Tomita, M., D.D. Brandon, G.P. Chrousos, A.C. Vingerhoeds, C.M. Foster, D. Fowler, D.L. Loriaux, M.B. Lipsett. 1986. Glucocorticoid receptors in Epstein-Barr virus-transformed lymphocytes from patients with glucocorticoid resistance and a glucocorticoid-resistant New Yorld primate species. J Clin Endocrinol Metab 62:1145-1154.

Tonegawa, S. 1988. Somatic generation of immune diversity. Biosci Rep 8:3-26.

Tsai, M.J. and B.W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-86.

Tseng, Y-T., J.P. Stabila, T.T. Nguyen, B.G. McGonnigal, J.A. Waschek, J.F. Padbury. 2001. A novel glucocorticoid regulatory unit mediates the hormone responsiveness of the β1-andrenergic receptor gene. Mol Cell Endocrinol 181:165-178.

Vanden Berghe, W., E. Francesconi, K. de Bosscher, M. Reschone-Rigon, G. Haegeman. 1999. Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-κB-dependent mechanism. Mol Pharmacol 56:797-806.

van der Stoep, N., E. Quinten, P.J. van den Elsen. 2002. Transcriptional regulation of the MHC class II *trans*-activator (CIITA) promoter II: identification of a novel regulatory region in the 5'-untranslated region and an important role for cAMP-responsive element binding protein 1 and activating transcription factor-1 in CIITA-promoter III transcriptional activation in B lymphocytes. J Immunol 169:5061-5071.

van Eijk, M.J.T., J.A. Stewart-Haynes, H.A. Lewin. 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Anim Genet 23:483-496.

van Tilborg, M.A., A.M. Bonvin, K. Hard, A.L. Davis, B. Maler, R. Boelens, K.R. Yamamoto, R. Kaptein. 1995. Structure refinement of the glucocorticoid receptor-DNA binding domain from NMR data by relaxation matrix calculations. J Mol Biol 247:689-700.

Vedeckis, W.V. 1983. Limited proteolysis of the mouse liver glucocorticoid receptor. Biochemistry 22:1975-1983.

Vottero A, Kino T, Combe H, Lecomte P, Chrousos GP. 2002. A novel, C-terminal dominant negative mutation of the GR causes familial glucocorticoid resistance through abnormal interactions with p160 steroid receptor coactivators. J Clin Endocrinol Metab 87(6):2658-67.

Walsh, C.T., L.D. Zydowsky, F.D. McKeon. 1992. Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. J Biol Chem 267:13115-13118.

Weaver, J.U., G.A. Hitman, P.G. Kopelman. 1992. An association between a BclI restriction fragment length polymorphism of the glucocorticoid receptor locus and hyperinsulinaemia in obese women. J Mol Endocrinol 9:295-300.

Weber, P.S., S.A. Madsen, G.W. Smith, J.J. Ireland, J.L. Burton. 2001. Pretranslational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet Immunol Immunopathol 83:213-40.

Webster, J.I., L. Tonelli, E.M. Sternberg. 2002. Neuroendocrine regulation of immunity. Annu Rev Immunol 20:125-163.

Weinberger, C., S.M. Hollenberg, M.G. Rosenfeld, R.M. Evans. 1985. Domain structure of human glucocorticoid receptor and its relationship to the v-erb-a oncogene product. Nature 318:670-672.

Whitworth, J.A., C.G. Schyvens, Y. Zhang, G.J. Mangos, J.J. Kelly. 2001. Glucocorticoid-induced hypertension: from mouse to man. Clin Exp Pharm Phys 28:993-996.

Williams, C.L., J.M. Peterson, R.G. Villar, T.F. Burks. 1987. Corticotropin-releasing factor directly mediates colonic responses to stress. Am J Physiol 253:G582-G586.

Wilckens, T., and R. De Rijk. 1997. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 18:418-424.

Wingett, D., K. Forcier, C.P. Nielson. 1996. Glucocorticoid-mediated inhibition of RANTES expression in human T lymphocytes. FEBS Letters 398:308-311.

Wissink, S., E.C. van Heerde, M.L. Schmitz, E. Kalkhoven, B. van der Burg, P.A. Baeuerle, P.T. van der Saag. 1997. Distinct domains of the RelA NF-κB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. J Biol Chem 29:22278-22284.

Wohlt, J.E., M.E. Allyn, P.K. Zajac, L.S. Katz. 1994. Cortisol increases in plasma of Holstein heifer calves from handling and method of electrical dehorning. J Dairy Sci Dec:3725-3729.

Wolberger, C. 1998. Combinatorial transcription factors. Curr Opin Genet Dev 8:552-559.

Wright, A.P., I.J. McEwan, K. Dahlman-Wright, J.A. Gustafsson. 1991. High level expression of the major transactivation domain of the human glucocorticoid receptor in yeast cells inhibits endogenous gene expression and cell growth. Mol Endocrinol 5:1366-1372.

Yamamoto, K.R., B.D. Darimont, R.L. Wagner, J.A. Iniguez-Lluhi. 1998. Building transcriptional regulatory complexes: signals and surfaces. Cold Spring Harbor Symp Quant Biol LXIII:587-598.

Yang Yen, H.F., J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, M. Karin. 1990. Transcriptional interference between c-jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 62:1205-1215.

Yao, J., S.E. Aggrey, D. Zadworny, J.F. Hayes, and U. Kuhnlein. 1996. Sequence variation in the bovine growth hormone gene characterized by single-strand conformation polymorphism (SSCP) analysis and their association with milk production traits in Holsteins. Genetics 144:1809-1816.

Yu, P-W., L.A. Schuler, M. Kehrli, L. Mattocks, B.J. Nonnecke and C.J. Czuprynski. 1997. Effects of dexamethasone treatment on IL-1 receptor mRNA levels *in vivo*. J Leuk Biol 62:401-404.

Yudt, M.R., and J.A. Cidlowski. 2002. The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. Mol Endocrinol 16:1719-1726.

Zandi, E., I. Galli, U. Dobbeling, S. Rusconi. 1993. Zinc finger mutations that alter domain interactions in the glucocorticoid receptor. J Mol Biol 230:124-136.

Zhong, J., J. Ashraf, E.B. Thompson. 1990. The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. Mol Cell Biol 10:5580-5585.