Syndromes of Glucocorticoid Resistance

Moderator: George P. Chrousos, MD; *Discussants*: Sevilla D. Detera-Wadleigh, PhD, and Michael Karl, MD

■ **Glucocorticoid resistance results from the partial, albeit apparently generalized, inability of glucocorticoids to exert their effects on target tissues. The condition is associated with compensatory increases in circulating pituitary corticotropin and cortisol, with the former causing excess secretion of both adrenal androgens and adrenal steroid biosynthesis intermediates with salt-retaining activity. The manifestations of glucocorticoid resistance vary from chronic fatigue (perhaps a result of glucocorticoid deficiency in the central nervous system) to various degrees of hypertension with or without hypokalemic alkalosis or hyperandrogenism, or both, caused by increased cortisol and other salt-retaining steroids and adrenal androgens, respectively. In women, hyperandrogenism can result in acne, hirsutism, menstrual irregularities, oligoanovulation, and infertility; in men, it may lead to infertility and in children, to precocious puberty. Different molecular defects, such as point mutations or a microdeletion of the highly conserved glucocorticoid receptor gene, alter the functional characteristics or concentrations of the intracellular receptor and appear to cause glucocorticoid resistance.**

The extreme variability in the clinical manifestations of glucocorticoid resistance and its mimicry of many common diseases can be explained by the overall degree of glucocorticoid resistance, differing sensitivity of target tissues to mineralocorticoids or androgens or both, and perhaps different biochemical defects of the glucocorticoid receptor, with selective resistance of certain glucocorticoid responses in specific tissues. The various different symptoms of classic glucocorticoid resistance and the theoretical potential of this condition to appear surreptitiously emphasize the importance of the glucocorticoid receptor in the pathogenesis of human disease.

Ann Intern Med. 1993;119:1113-1124.

An edited summary of a Clinical Staff Conference held on 30 September 1992 at the Amphitheater, Building 10, Bethesda, Maryland. The conference was sponsored by the National Institute of Child Health and Human Development, National Institutes of Health.

Authors who wish to cite a section of the conference and specifically indicate its author may use this example for the form of reference:

Detera-Wadleigh S. The glucocorticoid receptor gene, p 1119. In: Chrousos GP, moderator. Syndromes of glucocorticoid resistance. Ann Intern Med. 1993;119:1113-1124.

Dr. George P. Chrousos (Developmental Endocrinology Branch, National Institute of Child Health and Human Development [NICHD], National Institutes of Health [NIH], Bethesda, Maryland): Glucocorticoids have an important role in human physiology, and almost every tissue in the human body is affected by them (1). Glucocorticoids are crucial for the integrity of central nervous system function and for the maintenance of cardiovascular and metabolic homeostasis (2). Increased secretion of glucocorticoids during stress is also pivotal in altering central nervous system function (3), in preventing the inflammatory and immune response systems from over-reacting (4, 5), and in adjusting energy expenditures (6), all changes that improve chances for survival. Given this array of life-sustaining functions, the complete inability of glucocorticoids to exert their effects on target tissues would be incompatible with life; therefore, only syndromes of partial or incomplete glucocorticoid resistance exist. Several patients or members of kindreds with partial forms of this disease have been described (6–18); they show a wide spectrum of clinical symptoms and an interesting set of pathophysiologic mechanisms (Table 1). Recent advances in our understanding of the mechanism of action of glucocorticoids at the molecular level have allowed a glimpse into the pathophysiology of resistance and have increased our awareness of the potential involvement of this condition in the pathogenesis of human disease.

Familial Glucocorticoid Resistance: The Syndromes

Pathophysiology

An elaborate feedback system regulates glucocorticoid homeostasis. Of principal importance is the ability of glucocorticoids to exert negative feedback on secretion by the hypothalamic paraventricular nucleus of corticotropin-releasing hormone and arginine vasopressin, on secretion by the anterior pituitary of corticotropin, and on suprahypothalamic centers that control the activity of the hypothalamic-pituitary-adrenal axis (3, 19–21). This complex system is activated in states of generalized glucocorticoid resistance and produces compensatory increases in corticotropin and cortisol secretion (Figure 1). Although the increased cortisol concentrations in most affected persons appear to compensate adequately for the inability of target tissues to respond to glucocorticoids, the excess corticotropin secretion results in the increased production of intermediate compounds in adrenal steroidogenesis that have salt-retaining (mineralocorticoid) activity (such as deoxycorticosterone and corticosterone [8]) and in the enhanced secretion of adrenal androgens (such as Δ4-androstenedione, dehydroepiandrosterone, and dehydroepiandrosterone-sulfate [12]).

Table 1. Glucocorticoid Resistance in Humans: Reported Patients and Kindreds

Authors, year	Patients	Clinical Features	Receptor Defect
Vingerhoeds et al, 1976	Father and son	Hypertension and hypokalemic alkalosis, asymptomatic	Not done
Chrousos et al, 1982, 1983	Family 1, 10 affected members		Decreased affinity
Iida et al, 1985	Mother and son	Mild hypertension	Decreased number
Brönnegard et al, 1986	Mother and son	Fatigue	Thermolability
Lamberts et al, 1986, 1987	Family 2, 3 men, 1 woman	Asymptomatic, hyperandrogenism	Decreased number
Nawata et al, 1987	Woman	Asymptomatic	Decreased affinity, decreased DNA binding
Vecsei et al, 1989	Woman	Mild hypertension, hyperandrogenism	Not done
Jaursch-Hancke et al, 1989	Woman	Hyperandrogenism	Decreased number
Malchoff et al, 1990	Boy	Isosexual precocity	Decreased affinity
Lamberts et al, 1992	2 men, 3 women, all unrelated	Mild hypertension, hyperandrogenism	Decreased number, decreased affinity
Werner et al, 1992	6 women, unrelated	Fatigue, hyperandrogenism, obesity	Decreased number, decreased affinity, thermolability

Clinical Presentation

The clinical presentation of patients with glucocorticoid resistance is summarized in Table 1 and is related to the pathophysiology described in Figure 1. Generally, clinical manifestations of glucocorticoid deficiency were not present in most of the patients, and most of the patients evaluated in the context of family studies were asymptomatic despite biochemical indices of excessive cortisol production. The chief complaint of members of one particular family and an additional unrelated patient, however, was chronic fatigue, which might indicate inadequate compensation by the increased glucocorticoids in certain resistant target tissues, perhaps parts of the central nervous system or the muscles. In several patients, the increased concentrations of cortisol, deoxycorticosterone, and corticosterone (all steroids known to have inherent mineralocorticoid activity) caused signs of mineralocorticoid excess, such as hypertension and hypokalemic alkalosis. Finally, increased levels of adrenal androgens caused signs of androgen excess, including masculinization in women, with manifestations affecting the skin (acne and hirsutism) and the reproductive system (oligomenorrhea, oligoanovulation, and infertility). Also, early and excessive adrenarche was associated with precocious puberty, and interference of adrenal androgens on the feedback regulation of follicle-stimulating hormone caused abnormal spermatogenesis in men with the syndrome (17). This abnormal spermatogenesis, however, could also be explained by corticotropin-induced intratesticular growth of adrenal rests, a phenomenon known to occur in classic and "late-onset" congenital adrenal hyperplasia (22).

These clinical manifestations were not reported in all patients with symptomatic glucocorticoid resistance, and the clinical presentation varied even within families. Two main explanations can be given for this: First, the degree of resistance, as indicated by the compensatory increases of cortisol production, has generally differed among patients, with associated variability in the production of mineralocorticoid and androgen compounds and, therefore, differing effects resulting from excess production. Second, the sensitivity of target tissues to mineralocorticoids and androgens can also vary among persons, resulting in unequal responsiveness to similar increases of circulating steroids. The factors responsible for the different sensitivity of target tissues to these hormones could be individual differences in 1) the activity of key hormone-inactivating or -activating en-

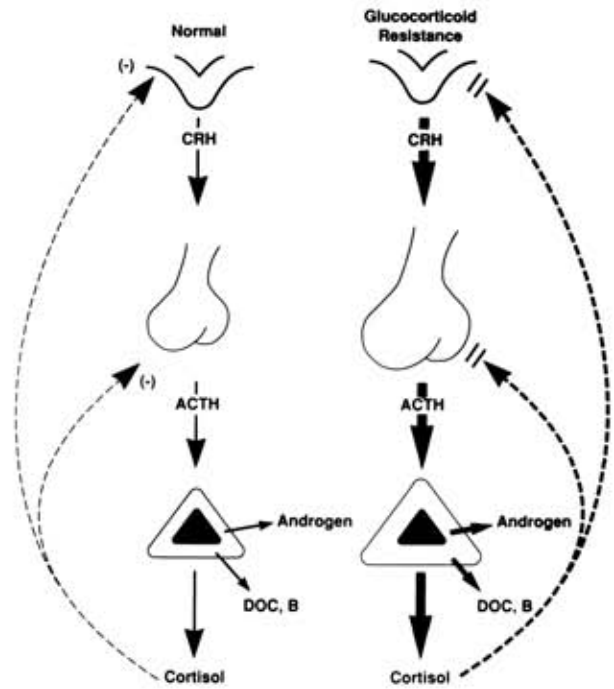

Figure 1. Pathophysiologic mechanism of glucocorticoid resistance. The elaborate negative feedback mechanisms responsible for maintaining glucocorticoid homeostasis compensate for the insensitivity of tissues to glucocorticoids by resetting the hypothalamic-pituitary-adrenal axis at a higher level. Thus, corticotropin-releasing hormone (*CRH*), adrenocorticotropin (*ACTH*), and cortisol secretion are increased. The compensatory increase in ACTH production causes increased secretion of glucocorticoid precursors with mineralocorticoid activity (DOC, deoxycorticosterone; B, corticosterone) and increased secretion of several adrenal androgens.

zymes, such as 11-beta-ketosteroid reductase in the kidney, which inactivates cortisol, deoxycorticosterone, or corticosterone (23), or 5α-reductase and 17-ketosteroid dehydrogenase in the skin, which convert adrenal androgens to more potent metabolites (24); or 2) the cascades of the mineralocorticoid and androgen-receptor transduction systems, which can vary at different steps (25, 26). In addition, other genetic or epigenetic factors, such as insulin resistance or upper body (abdominal) obesity or both, may influence the clinical manifestations of the syndrome by altering pituitary and gonadal function and peripheral steroid metabolism.

Table 2. Syndromes of Glucocorticoid Resistance

Clinical presentation
Apparently normal glucocorticoid function
Asymptomatic
Chronic fatigue (glucocorticoid deficiency?)
Mineralocorticoid excess
Hypertension
Hypokalemic alkalosis
Androgen excess
Female masculinization
Acne, hirsutism, menstrual irregularities (oligomenorrhea), oligoanovulation, infertility
Precocious puberty
Abnormalities in spermatogenesis
Diagnostic evaluation
No clinical evidence of the Cushing syndrome
Increased indices of cortisol production
Plasma total and free cortisol, normal plasma cortisol-binding globulin concentration
24-hour urinary free cortisol or 17-hydroxysteroid excretion
Cortisol production rate
Resistance to dexamethasone
Full-dose response
One or two doses
Maintenance of a normal circadian and a stress-responsive pattern of hypothalamic-pituitary-adrenal axis activity, albeit at a higher level
Diurnal plasma cortisol
Insulin tolerance test
Target tissue studies (circulating leukocytes, cultured lymphoblasts, cultured skin fibroblasts)
Functional characteristics of glucocorticoid receptor: concentration, affinity, stability, nuclear and DNA-binding properties
In vitro bioassays: defective glucocorticoid-induced inhibition of [^{3}H]-uridine incorporation, [^{14}C]-2-deoxyglucose uptake, or lectin-induced transformation in leukocytes; induction of aromatase activity or down-regulation of glucocorticoid receptor messenger RNA expression in cultured skin fibroblasts
Molecular studies

Diagnostic Evaluation

Of major importance is the definition of appropriate criteria for the correct diagnosis of glucocorticoid resistance and its differentiation from the many diseases it mimics clinically or biochemically. These criteria are summarized in Table 2. First, the patient should not have clinical evidence of the Cushing syndrome, including the phenotypic changes and biochemical consequences of hypercortisolism. Second, indices of increased cortisol production should be present, such as increased 24-hour urinary free cortisol levels or increased 17-hydroxysteroid per gram creatinine excretion, increased plasma total and free cortisol concentrations, or an increased rate of cortisol production. Third, glucocorticoid resistance should be present as determined by dexamethasone testing using either a complete dose-response test, as previously described (8), or by using one or two doses of the compound. The results of this test should be validated against the plasma levels of dexamethasone, because absorption or metabolism of this synthetic glucocorticoid may vary from person to person. Finally, the hypothalamic-pituitary-adrenal axis should retain its normal circadian rhythmicity and its responsiveness to stressors such as hypoglycemia; however, its basal and stimulated activities should be higher than those of normal persons.

Although the single-dose overnight dexamethasone suppression test may be appropriate for screening patients with potential generalized glucocorticoid resistance, its high false-positive rate of 15% to 20% makes measurement of 24-hour urinary free cortisol excretion preferable, if this syndrome is suspected. Levels above 100% (twofold) of the upper normal range are suggestive of glucocorticoid resistance or the Cushing syndrome (27). Levels between the upper normal range and the 100% limit, however, are compatible with these diagnoses, along with a host of other states characterized by mild hypercortisolism (27, 28).

Assuming proper procedures and techniques, the syndrome of generalized glucocorticoid resistance should be easily distinguished on biochemical grounds from the chronic fatigue syndrome, essential hypertension, hyperaldosteronism, idiopathic hirsutism, polycystic ovarian disease, female infertility, precocious puberty, or male infertility, because none of these conditions is associated with hypercortisolism. It may be difficult to distinguish this syndrome in its mild biochemical form (increases in urinary free cortisol up to 100% of the upper normal range) from other mild forms of hypercortisolism, such as mild or early Cushing syndrome with a lack of or borderline manifestations of hypercortisolism, hypercortisolemic melancholic depression, anorexia nervosa, chronic active alcoholism, and hypercortisolism in persons who exercise heavily (27, 28). In the case of mild or early Cushing syndrome, the absence of circadian rhythmicity and lack of responsiveness of plasma cortisol during an insulin tolerance test should aid the physician in diagnosing this condition. The patient's clinical course, which is frequently characterized by progressive deterioration and development of the classic Cushing phenotype, should allow eventual differentiation. The history or concurrent symptomatology and the dependence of the hypercortisolism on the actual state of the condition should differentiate glucocorticoid resistance from the other states.

Therapy

Synthetic glucocorticoids with minimal intrinsic mineralocorticoid activity, such as dexamethasone, provide a rational treatment for familial glucocorticoid resistance. These patients should receive oral doses of dexamethasone, usually ranging between 1 and 3 mg/d, that are clearly pharmacologic for normal persons but are

equivalent to glucocorticoid replacement for the glucocorticoid-resistance state. Dexamethasone suppresses corticotropin and, therefore, endogenous cortisol, deoxycorticosterone, corticosterone, and adrenal androgen secretion, thus correcting the excess mineralocorticoids and androgens and their effects on these patients (8, 17). Long-term daily dexamethasone dosage should be individualized and titrated to normalize adrenal mineralocorticoid and androgen secretion and to control the clinical manifestations of the disease.

Glucocorticoid Receptors

As early as 1980, it was suggested that glucocorticoid resistance represented a defect in the intracellular cascade of events from the entrance of the glucocorticoid into the cell to its final effect on cellular function. By that time, it was known that most glucocorticoid effects were exerted by interaction with a finite number of intracellular glucocorticoid receptors, proteins with profound modulatory effects on gene transcription once they came into specific contact with their ligand. In addition to their ability to bind the hormone, these receptors recognized specific sites on the chromatin and could bind to nuclei and to naked DNA. Even before the structure of the glucocorticoid receptors was known, studies done in patients with glucocorticoid resistance showed alterations in the functional characteristics of their receptors. Several tissues from the first reported kindred with glucocorticoid resistance had normal concentrations of glucocorticoid receptors but decreased affinity for glucocorticoids (8). The stability of these receptors, as well as their gross molecular size, was normal (29), the latter suggesting that a point mutation of the glucocorticoid receptor gene was responsible for the defective interaction between the receptor and the steroid. Subsequently, additional kindreds and individual patients whose glucocorticoid receptors were also characterized by decreased binding affinities, low concentration, thermolability, or defective DNA binding were described as well (*see* Table 1).

The cloning of complementary DNA (cDNA) from the human glucocorticoid receptor in 1985 (30) allowed the deduction of its primary structure, definition of its functional domains (31), and cloning of its gene (32), and thus permitted further studies on the molecular mechanisms of glucocorticoid resistance. The glucocorticoid receptor also served as the prototypic member of a large superfamily of ligand-activated receptors homologous to the v-*erb*A oncogene, including not only the steroid and sterol family of receptors but also the receptors for triiodothyronine and retinoic acid, as well as others whose ligands have not yet been determined (25).

The primary structure of the human glucocorticoid receptor gene and the glucocorticoid receptor protein with its functional domains are shown schematically in Figure 2, panels A and B, respectively, and its homologies to other receptors of the steroid and sterol family are shown in Figure 2, panel C. The 777 amino acid protein (*panel B*) has three main functional domains: the ligand-binding domain (which lies in the carboxyterminal portion of the protein), the DNA-binding domain (which is in the center), and the aminoterminal domain, which was initially characterized by its property of providing antigenic epitopes for the generation of anti-receptor antibodies and hence was called the "immunogenic domain." Further characterization of the functional domains of the receptor allowed not only better recognition of the sequences responsible for steroid binding and DNA recognition but also showed a number of domains representing other functions involved in the cascade of events, from ligand binding to modulation of gene transcription (33–41). Current knowledge about the location of these domains is shown schematically under the primary structure of the glucocorticoid receptor in Figure 2 (*panel B*). Further discussion of these functions and their potential involvement in the pathogenesis of glucocorticoid resistance will follow.

A gross similarity exists among the structures of members of the steroid and sterol family of receptors, with most of the homologies occurring primarily in the DNA-binding domains and, secondarily, in the ligand-binding domains (*see* Figure 2, *panel C*). This similarity explains both the known existing cross-reactivities of ligand binding between the natural steroids and their receptors and explains the sharing of common DNA sequences in the promoter regions or enhancers of steroid-regulated genes with which these receptors interact. Relevant to glucocorticoid resistance is the virtual 100% cross-reactivity of the mineralocorticoid receptor for cortisol (42). Normally, the kidney mineralocorticoid receptor is partially protected from the mineralocorticoid effects of circulating cortisol by the intracellular enzyme 11-ketosteroid reductase in the cells of the distal convoluted and proximal collecting tubules; this enzyme converts 11-beta-hydroxylated compounds, such as cortisol, into inactive 11-ketosteroids, such as cortisone (23). The presence of increased amounts of circulating cortisol as well as deoxycorticosterone and corticosterone in patients with glucocorticoid resistance appears to overwhelm the protective enzyme and produces effects of mineralocorticoid excess in some patients. The mineralocorticoid receptor appears to have further relevance to glucocorticoid resistance; recent work suggests that some of the negative feedback effects of glucocorticoids on the hypothalamic-pituitary-adrenal axis are mediated by the mineralocorticoid receptor of the hippocampus, a major restraining influence on the activity of this axis (3).

A wealth of new information on the molecular mechanisms of action of glucocorticoids has led to the refinement of the initial model, which continues to be rapidly modified, and to new explanations for the pathophysiology of glucocorticoid resistance. The cascade of events leading to the modulation of gene transcription is as follows (*see also* Appendix Table 1 for a glossary of genetic terms): The inactivated ligand-free glucocorticoid receptor is found in the cytoplasm associated with other proteins, such as heat-shock proteins 90 (37) and 70 (43) and immunophilin of the FK506/rapamycin binding type (44), in the form of a hetero-oligomer. It appears that the receptor is anchored to heat-shock protein 90 through sites present in its steroid-binding domain (*see* Figure 2, *panel B*). This special interaction with heat-shock protein 90 appears to facilitate the binding of the steroid to the receptor and to increase its

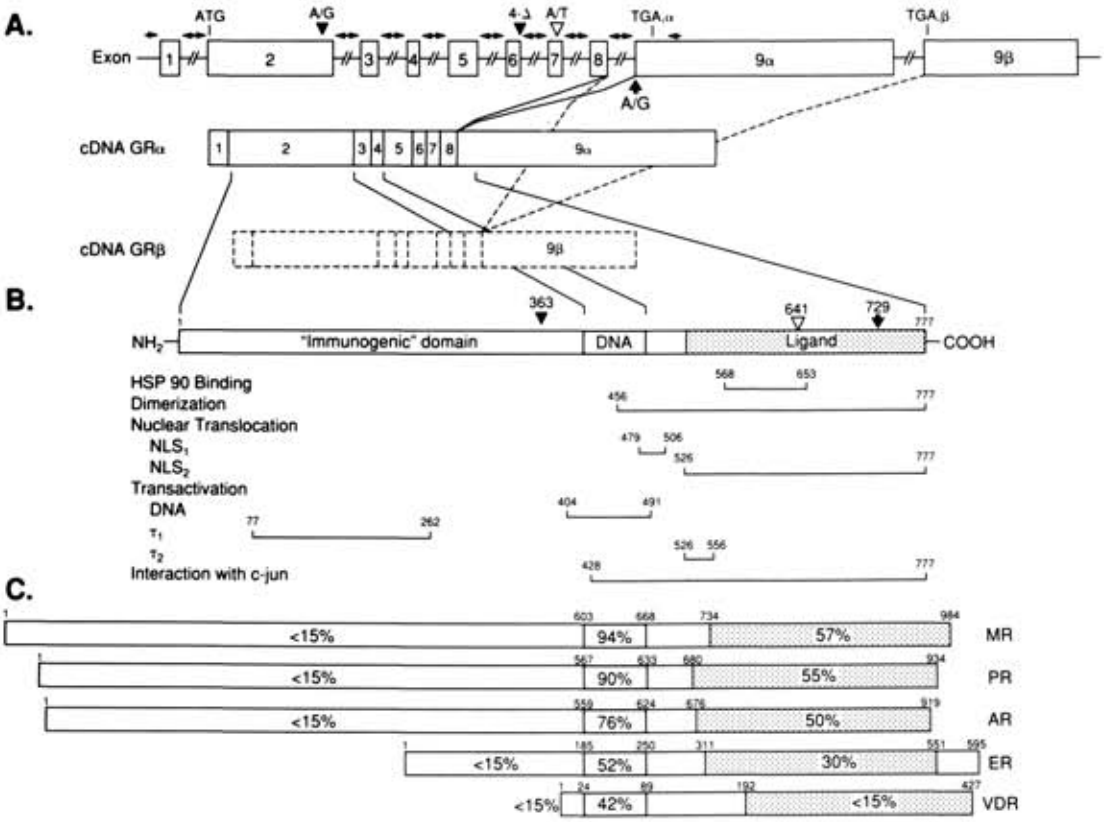

Figure 2. Genomic and cDNA and protein structure of the human glucocorticoid receptor, its functional domains, and its homologies to other steroid and sterol hormone receptors. ▽ indicates the position of the pathogenetic mutation in the first kindred from the National Institutes of Health (NIH) (64); ▼ indicates the 4-basepair deletion and conservative mutation in the second NIH family (65); and the black arrowhead indicates the pathogenetic mutation in the family reported by McDermott and colleagues (66). **Panel A.** The gene consists of 10 exons of variable lengths numbered 1 to 8, and 9α and 9β. Exon 2 codes for the amino terminal domain; exons 3 and 4 code for the DNA-binding domain; and exons 5 to 9α code for the ligand-binding domain. The steroid-binding glucocorticoid receptor is glucocorticoid receptor α ($GR\alpha$). Glucocorticoid receptor β ($GR\beta$) is produced by alternative splicing and does not bind glucocorticoids, and its functional importance is obscure. **Panel B.** The three main domains of the human glucocorticoid receptor are represented in a linear model, as originally defined. Subsequent in vitro mutagenesis studies have assigned these and other functions to various regions of the receptor protein, as indicated underneath the schematic representation of the receptor. Numbers correspond to amino acids in the primary sequence of the receptor. HSP = heat-shock protein; NLS = nuclear localization sequences. **Panel C.** Homologies of the five other classes of steroid and sterol receptors to the glucocorticoid receptor expressed as percent identity in primary sequence (AR = androgen receptor; ER = estrogen receptor; MR = mineralocorticoid receptor; PR = progesterone receptor; and VDR = $1,25(OH)_2$ vitamin D_3 receptor).

effectiveness in eliciting a glucocorticoid response (45). This binding results in the release of the steroid-receptor complex from heat-shock protein 90 and in the unmasking of domains responsible for dimerization, nuclear localization, DNA binding, and transactivation (36) (*see* Figure 2, *panel B*).

The dimerization domains are found in the steroid-binding domain. In theory, receptor homodimers can be present both in the cytoplasm and in the nucleus. Monomers or dimers from ligand-bound receptors appear to cross into the nucleus through recognition of their nuclear localization domains or nuclear localization sequences by proteins of the nuclear pore (33). One of these sequences is homologous to that of the SV40 T antigen and other proteins also known to translocate into the nucleus. Heat-shock protein 70 and intracellular fibrils might participate in the alignment of receptors and their translocation through the nuclear pore (46).

Inside the nucleus, the ligand-bound glucocorticoid receptor exerts its genomic effects on transcription in at least three potential ways. First, in the form of a homodimer, it interacts with specific DNA sequences, the glucocorticoid-responsive elements, in the promoter or enhancer regions of glucocorticoid-responsive genes (40, 47). The DNA-binding domain of the receptor consists of two protein loops held together by a zinc atom, the "zinc fingers," which selectively interact with the glucocorticoid-responsive elements (30, 48). The ligand-bound receptor homodimers appear to stabilize the polymerase II initiation complex of regulated genes, perhaps by binding directly or through another protein to ancillary factor TFIIB, whose interaction with the partial initiation complex may be the rate-limiting step of transcription (49). The degree of gene transactivation induced by the receptor homodimer may be modulated by separate regulatory elements in the DNA and their specific transcription factors (50). Examples of these are the CACCC box and the glucocorticoid modulatory element and their binding factors (51, 52). Although this mechanism has been studied mostly in enhancement systems where the receptor dimer interacts with "positive" glucocorticoid-responsive elements, glucocorti-

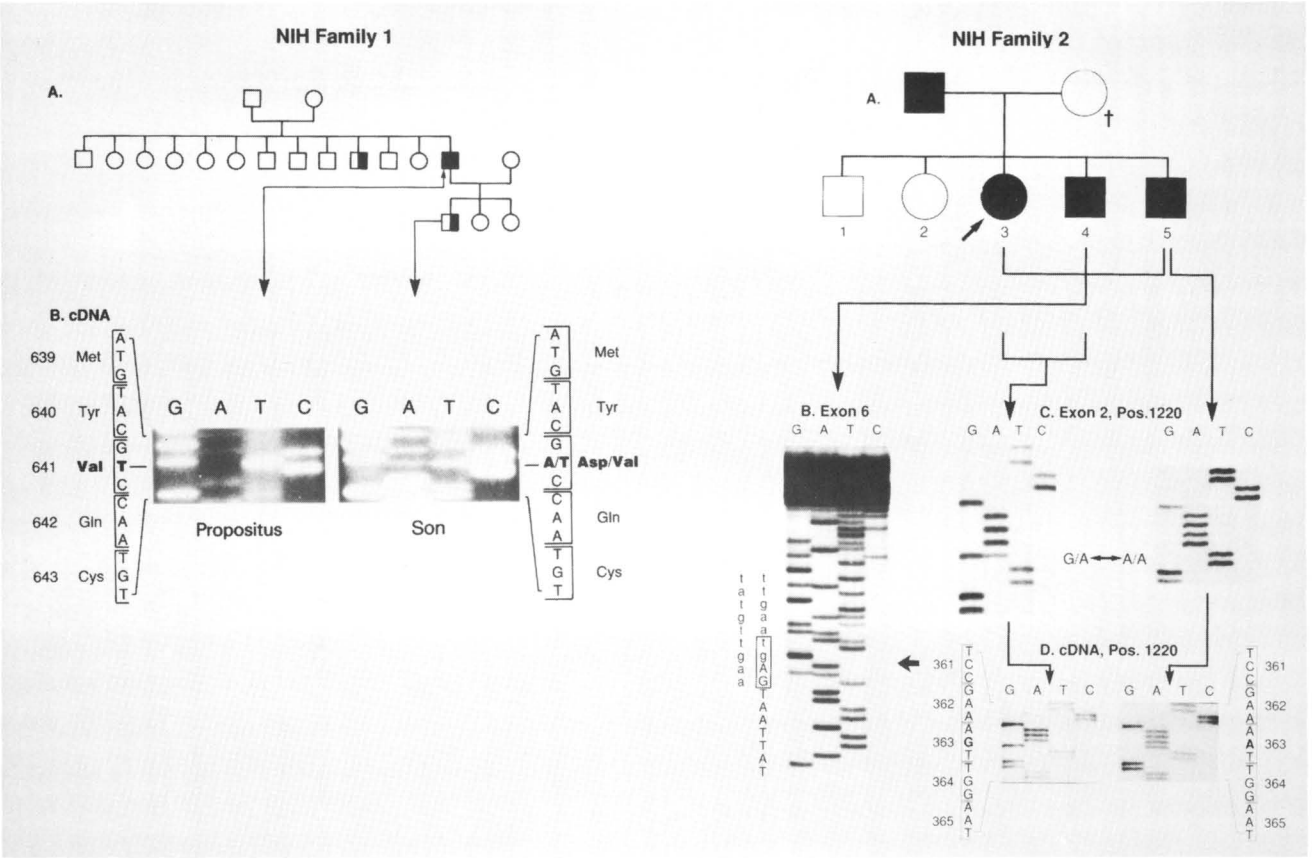

Figure 3. Molecular studies of the glucocorticoid receptor in families 1 and 2 studied at the National Institutes of Health. Left, Panel A. Family 1 pedigree showing autosomal codominant transmission of the glucocorticoid resistance (black symbol indicates symptomatic; half-black symbol indicates biochemically affected only). **Left, Panel B.** Nucleotide sequence of the glucocorticoid receptor cDNA from the propositus and his asymptomatic but biochemically affected son. The propositus has T in place of A at nucleotide 2054, changing the aspartate codon GAC normally present at position 641 to the valine codon, GTC. Both A and T are present in the son's sequence, suggesting heterozygosity. The cDNA sequence of the asymptomatic but biochemically affected brother was identical to that of the son. From Hurley and colleagues (64); reproduced with permission. **Right, Panel A.** Family 2 pedigree showing autosomal-dominant segregation of patients with glucocorticoid resistance (black symbols, affected; white symbols, unaffected). **Right, Panel B.** Sequences of the glucocorticoid receptor alleles at the 3′-donor splice junction of exon 6 in the proposita and her glucocorticoid-resistant brothers. The boxed nucleotides depict the 4-basepair sequence deleted in one allele. The arrow on the right indicates the exon-intron boundary in the normal allele. The autoradiogram shows the sequence of the normal and the affected allele: the 4-basepair deletion in one allele, including the last bases of the exon and the first nucleotides of the intron, results in double bands. **Right, Panel C.** Nucleotide sequences of glucocorticoid receptor–exon 2 genomic DNA of the proposita and her affected brothers. On the left side, the proposita and one of her affected brothers were heterozygous for a mutation at codon 363 (cDNA position 1220) with a guanine (G) replacing the wild-type adenine (A) in one allele. On the right side, the second affected brother was homozygous for the wild-type sequence. **Right, Panel D.** Analysis of glucocorticoid receptor gene transcripts by direct sequencing of PCR-amplified cDNA obtained by reverse transcription of total RNA. On the left side, analysis of the proposita's cDNA showed only the sequence bearing G at position 1220, whereas transcripts with the wild-type sequence were not detectable. The base substitution results in a conservative amino acid substitution from asparagine to serine. On the right side, in contrast, the second affected brother had only the wild-type sequence. From Karl and colleagues (65); reproduced with permission.

coids may also repress other genes through the same (53) or different, "negative" glucocorticoid-responsive elements (54). Second, negative regulation of transcription can result from the interaction of the glucocorticoid receptor dimer with part of the responsive element of another transcription factor, which can be displaced or hindered from properly exerting its transcription-promoting effect. Such an example is the inhibition by glucocorticoids of glycoprotein hormone α-subunit gene transcription induced by cyclic AMP-responsive element-binding protein (55). Third, another major molecular path through which glucocorticoids seem to exert part of their antigrowth and anti-inflammatory effects appears to be the formation of intranuclear complexes of the glucocorticoid-bound receptor with c-jun, which prevents this transcription factor from exerting the ubiquitous activating and growth-promoting effects produced by its interaction as a c-jun/c-fos heterodimer with its DNA-responsive element, the AP1 site (38–40).

This model of glucocorticoid actions at the cellular level is still evolving and many crucial questions remain. Thus, phosphorylation and dephosphorylation may participate in the activation or inactivation, recycling, and turnover of the receptor (56). Many earlier rapid effects of glucocorticoids, such as changes in intracellular cyclic GMP and calcium levels and in membrane potential, may take place by as-yet-undefined mechanisms (57, 58). Finally, the mechanism or mech-

anisms by which glucocorticoids alter the translatability and stability of specific messenger RNAs (mRNA), an important unequivocal function of these hormones that has been observed with several genes, are still unclear (59, 60).

The Glucocorticoid Receptor Gene

Dr. Sevilla Detera-Wadleigh (Clinical Neurogenetics Branch, National Institute of Mental Health, NIH, Bethesda, Maryland): Given the many functions served by a single glucocorticoid receptor protein, it is not surprising that the structure of its gene, located on chromosome 5 (61), is quite complex; it consists of 10 different exons (45) (*see* Figure 2, *panel A*). One exon codes for the immunogenic domain, 2 for the DNA-binding domain (1 for each zinc finger), and 5 for the steroid-binding domain, which carries most of the identified functions (*see* Figure 2, *panel B*). Alternative splicing appears to result in the synthesis of another receptor form, glucocorticoid receptor β, which does not bind glucocorticoid and whose function is unknown; it might form heterodimers with the classic glucocorticoid receptor α and might prevent or alter one or more of its many effects. A similar thyroid hormone receptor isoform, thyroid receptor α_2, exists; it does not bind triiodothyronine but instead it forms heterodimers with ligand-binding isoforms from thyroid receptor α_1 and β, and it alters the effects of the latter (62). Finally, because expression of the glucocorticoid receptor gene might be crucial for understanding states of glucocorticoid resistance resulting from abnormal gene regulation, the 5′ regulatory region of this gene should be studied. The human glucocorticoid receptor gene has similarities with several housekeeping genes in that it has no TATA or CAAT boxes but instead has at least 17 SP1 transcription factor sites in a region of approximately 3 kilobases. Several other putative regulatory elements, including half-sites for glucocorticoid-responsive elements, two "negative" glucocorticoid receptor elements, and an AP1 site have been identified, although it is not yet clear whether these elements are active (63; Encio and Detera-Wadleigh. Unpublished results).

Molecular Mechanisms of Glucocorticoid Resistance

Dr. Michael Karl (Developmental Endocrinology Branch, NICHD, NIH, Bethesda, Maryland): The molecular basis of familial glucocorticoid resistance has been elucidated in three kindreds (64–66). Two of the three kindreds were investigated at NIH (64, 65). Point mutations and a gene microdeletion in the glucocorticoid receptor have been identified as causing familial glucocorticoid resistance. The cDNA from affected members of the family reported by Vingerhoeds and colleagues (7) in 1976 (*see* Table 1) was sequenced to find potential mutations altering the affinity of the receptor for the steroid (64). Affected members had a thymidine for adenine substitution at cDNA position 2054 in the ligand-binding region of the receptor (Figures 2 and 3). This base change resulted in a nonconservative amino acid substitution from aspartate, an acidic amino acid, to the hydrophobic valine at codon 641. The position of this amino acid residue is adjacent to cysteine 638. Its corresponding residue in the rat glucocorticoid receptor, cysteine 656, is intimately involved in hormone binding (67). Therefore, an alteration in the ligand-binding cavity of the receptor, such as the nonconservative amino acid change described, might explain the low affinity of the receptor observed in this kindred.

The propositus, who presented with severe mineralocorticoid excess, was homozygous for the mutation; his brother and son, who were affected only biochemically, were heterozygous. When the DNA sequences of the glucocorticoid receptor were examined in normal persons, no such alteration was found. The mutated receptor was then tested in an in vitro chloramphenicol transferase assay. The wild-type or the mutant plasmid were separately cotransfected into COS-7 cells with the reporter plasmid pMTVCAT. The latter carries the promoter and enhancer region of the mammary tumor virus, which is responsive to glucocorticoids coupled to the structural chloramphenicol transferase gene. When the chloramphenicol transferase gene expression induced by dexamethasone was assessed by measuring the acetylation of chloramphenicol to acetylchloramphenicol, the nucleotide substitution resulted in a low-affinity, dysfunctional glucocorticoid receptor, thus confirming the pathogenic importance of the mutation detected.

The second family investigated at NIH had been previously reported by Lamberts and colleagues (12, 17) in 1986 and 1992 (*see* Table 1). Circulating leukocytes from the glucocorticoid-resistant members of this kindred had only 50% of the normal concentrations of glucocorticoid receptors (14). In contrast to the first family, the affinity of the receptor in these patients was normal. It is interesting to note that direct sequencing of the proposita's and of one of her affected brother's glucocorticoid-receptor cDNA (which was amplified using the polymerase chain reaction [PCR]), showed a point mutation at position 1220 (65) (*see* Figures 2 and 3), where guanine substituted for the wild-type adenine. The cDNA sequence of the second affected brother was normal. This mutation did not segregate with the disorder but rather resulted in a conservative amino acid substitution, from asparagine to serine, at codon 363 of the glucocorticoid receptor. Located in the aminoterminal region of the receptor, the mutation was downstream of the τ_1 transactivation domain (*see* Figure 2, *panel B*). Asparagine 363 is conserved at the corresponding positions of the rat and mouse glucocorticoid receptor residues 383 and 371, respectively (68, 69).

The functional importance of the G_{1220} substitution on gene transcription was tested by introducing the mutation into a glucocorticoid receptor expression vector. No difference was noted in the ability of the wild-type and the Ser^{363} mutant receptor to activate chloramphenicol transferase expression, thus indicating that the substitution of Asn for Ser^{363} had no major impact on the ability of the glucocorticoid receptor to activate transcription of its target genes and was, therefore, not responsible for the glucocorticoid insensitivity in this family. This finding was additionally confirmed by the

disclosure of guanine at position 1220 in the unaffected sister of the proposita.

The finding of guanine only at cDNA position 1220 provided evidence for the expression of only one of the two glucocorticoid receptor alleles in the affected members of this family, because their parents were not consanguineous. To address this hypothesis, the gene structure of the glucocorticoid receptor had to be investigated for alterations resulting in the expression of only one of the two receptor alleles. Each of the nine exons of the glucocorticoid receptor gene α from the proposita and from her two affected brothers was amplified by PCR using primers located in the 5′ and 3′ flanking intron regions and was directly sequenced (45, 65). As expected, when exon 2 from the proposita and from one of her brothers was sequenced, a guanine and an adenine base were identified at the second position of codon 363, which corresponds to cDNA position 1220. Thus, as hypothesized above, the proband and one of her affected brothers were heterozygous for this mutation, whereas the second affected brother was homozygous for the wild-type sequence in codon 363. This analysis of the exon-intron boundaries of the glucocorticoid receptor gene disclosed the defect responsible for the end-organ insensitivity to glucocorticoids in this family: a 4-basepair deletion identified at the 3′ boundary of exon and intron 6 in all three affected persons studied (*see* Figures 2 and 3). The deletion removed a donor splice site in one of the two glucocorticoid receptor alleles. When we amplified, using PCR, and examined exon 6 and the exon 6–intron 6 boundary in the two unaffected siblings of the proband, we found the wild-type sequence. This observation was consistent with the conclusion that the 4-basepair deletion segregated with glucocorticoid resistance.

Mutations or deletions involving the first two nucleotides of an intron disrupt normal splicing, the process of removing introns and joining exons in transcribed RNA (70). Aberrant mRNA variants generated in such cases are probably susceptible to nuclease degradation, which precludes the production of mature, translatable mRNA and therefore excludes the expression of the encoded protein. This hypothesis might explain why the glucocorticoid receptor concentrations in affected members of the second family were 50% of normal.

Thus, in the affected members of this family, translation of the glucocorticoid receptor protein appeared to originate solely from templates of correctly spliced precursor mRNA transcribed from the allele without the 4-base-pair deletion, thereby suggesting that the deletion and the point mutation were located on different alleles. Only the cDNA species, the reverse-transcribed product of the patients' mRNA with guanine at position 1220, was detected in the proposita and in one of her affected brothers. The transcripts of the allele harboring the wild-type adenine in the second position of codon 363 together with the boundary deletion at exon–intron 6 seemed to be rapidly degraded, possibly by endogenous nucleases. No cDNA molecules derived from the allele with the 4-basepair deletion could be detected, thus suggesting that mature mRNA derived from this allele was not present and therefore not translated into glucocorticoid receptor protein.

An additional family studied by Malchoff and colleagues (16) (*see* Table 1) had a guanine-to-adenine point mutation in cDNA position 2317 (66) (*see* Figure 2). This mutation predicted the substitution of a valine residue 729 by an isoleucine within the ligand-binding domain; it also led to decreased affinity and apparently compromised the functional ability of the glucocorticoid receptor.

Implications and Future Directions

Dr. George P. Chrousos: Glucocorticoid receptor genes and cDNAs from additional patients with generalized glucocorticoid resistance are currently being studied. Given the large number of sequencing studies that need to be done to determine the defect, even with the assistance of symmetric and asymmetric PCR, we recommend the following procedure to ascertain the diagnosis and to study the mechanisms of glucocorticoid resistance (*see* Table 2). First, clinical and biochemical studies as discussed in the section on "Diagnostic Evaluation" should be done, both in suspected patients and their families, even though sporadic cases caused by new mutations may exist. Increased urinary free cortisol excretion, resistance of cortisol responsiveness to dexamethasone, and a clear circadian rhythm of plasma cortisol concentrations all need to be present to establish the diagnosis of generalized, classic glucocorticoid resistance. Second, studies to characterize the concentration, affinity, stability, and nuclear or DNA-binding properties of the receptor in circulating leukocytes, cultured skin fibroblasts, or Epstein-Barr virus–transformed B lymphocytes (lymphoblasts) should be done. Even if all these functional features of the receptor are normal, a "postreceptor" defect related to transactivation is not excluded. Glucocorticoid dose-response bioassays, which measure end points such as inhibition of ^{3}[H]-uridine incorporation, [^{14}C]-2-deoxyglucose uptake, or lectin-induced transformation by circulating leukocytes (71–73), and induction of aromatase activity (74) or down-regulation of the glucocorticoid receptor mRNA by cultured skin fibroblasts (18) may show postreceptor defects resulting in decreased transactivation function. Third, if a defect in glucocorticoid receptor structure or function is suspected, potential alterations might be screened using newly developed PCR-assisted techniques. These techniques include denaturing gradient gel electrophoresis using a guanine-cytidine clamp (75) and chemical or enzymatic mismatch cleavage (76), which could suggest what fragment of examined DNA carried a mutation and thus what fragment should be sequenced. Finally, once a structural defect has been determined, its deleterious effect on receptor function should be confirmed using in vitro mutagenesis and using standardized assays that examine the ability of the mutant glucocorticoid receptor to activate gene expression and to produce an effect, such as chloramphenicol transferase activity.

Because of the difficulty in diagnosing the syndrome of familial generalized glucocorticoid resistance and because of its variable clinical presentation (including chronic fatigue, mild hypertension, and hyperandrogenism), many patients with these manifestations might

have this condition. Accurate prevalence estimates of generalized glucocorticoid resistance do not currently exist, because measurements of urinary free cortisol excretion and dexamethasone tests are not normally done in patients with these conditions. The closest estimate we have is from a recent study (18) in which 7 of 420 consecutive patients presenting to an adrenal disorders clinic had bonafide generalized glucocorticoid resistance with functional abnormalities of their glucocorticoid receptors in cultured skin fibroblasts. These abnormalities included decreased receptor concentration or affinity, or both, thermolability, and defective down-regulation of glucocorticoid receptor mRNA expression by glucocorticoids. One of these patients had chronic fatigue, 1 had obesity and amenorrhea, 1 had hirsutism and chronic fatigue, and 4 had hirsutism alone. The 5 patients with glucocorticoid resistance who had hirsutism represented 14% of the 35 women with this diagnosis seen in the clinic. This is higher than the 5% to 7% incidence of "late onset" congenital adrenal hyperplasia with 21-hydroxylase deficiency in women with hirsutism (22). Thus, screening women with hyperandrogenism by obtaining measurements of urinary free cortisol excretion per 24 hours may be cost effective at this time.

Acquired forms of generalized incomplete glucocorticoid resistance may also exist. In a recent study (77), patients with the acquired immunodeficiency syndrome (AIDS) were described as having symptoms similar to patients with Addison disease (weakness, weight loss, hypotension, hyponatremia, and intense mucocutaneous melanosis) and as having increased plasma corticotropin and cortisol levels, as well as peripheral mononuclear leukocyte glucocorticoid receptors with reduced affinity for dexamethasone. Further studies should be done in patients suspected of having acquired glucocorticoid resistance, and the mechanism(s) of such a state should be examined.

Finally, research on the mechanism of action of glucocorticoids at a cellular or molecular level or both and on the profound effects of glucocorticoids on central nervous system function and on the immune response could have a substantial impact on society. Given the complexity of glucocorticoid receptor regulation and the varying effect of potential mutations, glucocorticoid resistance states may exist that are not generalized but that influence a limited number of tissues or functions. Thus, a mutation might not affect regulation of the hypothalamic-pituitary-adrenal axis (in which case the patients would not be classified as having glucocorticoid resistance by the classic biochemical criteria) but might affect the action of the hormone in some other system, such as the dopaminergic mesocortical or mesolimbic system (responsible for pleasure and reward phenomena) or the norepinephrine–locus ceruleus system (responsible for sustaining proper arousal [28, 78–80]). In both instances, emotional disorders characterized, respectively, by stimulus or drug-seeking behaviors and chronic fatigue could result despite normal cortisol production.

If only part of the immunosuppressive effects of glucocorticoids were exerted because of immune system–specific resistance, we would expect phenomena resulting from an unrestrained immune system, such as autoimmune, allergic, or inflammatory diseases. Potential examples are rheumatoid arthritis and glucocorticoid-resistant asthma, a severe form that starts early in life and does not respond to glucocorticoid therapy. Rheumatoid arthritis is associated with normal or decreased plasma cortisol concentration and a decrease in the concentrations of glucocorticoid receptors in circulating peripheral leukocytes to approximately 50% of control levels (81–83). In this disease, the reponse to glucocorticoid treatment can be predicted by an in vitro cell-mediated immune assay (84). Glucocorticoid-resistant asthma is associated with normal hypothalamic-pituitary-adrenal axis function and normal glucocorticoid receptor concentration and affinity in peripheral leukocytes but with abnormal suppression of several cellular immune functions by glucocorticoids in vitro or in vivo (85–87).

The mirror image of these phenomena would be expected in a nongeneralized glucocorticoid hypersensitivity syndrome. The generalized form of this condition has recently been described (88), and "super" receptors have been artificially created in vitro (89). Extricating pathologic mutations of the glucocorticoid receptor associated with normal hypothalamic-pituitary-adrenal axis activity and proving their pathogenic potential in diseases characterized by nongeneralized glucocorticoid resistance or hypersensitivity will be an interesting and provocative challenge.

Appendix Table 1. Glossary of Genetic Terms

AP1 site	Specific DNA sequence binding the c-jun and c-fos heterodimer. The latter are transcription factors that mediate the effect of kinase C activation on gene transcription.
CACCC box	Nucleotide sequence that binds specific transcription factors, which modify the actions of glucocorticoids.
CAAT box	DNA sequence associated with the promoter region of genes.
C-fos	Transcription factor as a heterodimer with c-jun; the heterodimers bind to AP1 sites.
Chemical or enzymatic mismatch methods	Used in the separation of closely related DNA sequences; chemicals or enzymes are used that recognize different base sequences.
C-jun	Transcription factor acting as a heterodimer with c-fos; the heterodimers bind to AP1 sites.
Codon	Three nucleotides whose precise order specifies 1 of the 20 amino acids. Special codons that do not code for any amino acid act as stop signals.
Complementary DNA	DNA synthesized from RNA in test tubes using an enzyme called reverse transcriptase.
Cos-7	Cultured monkey kidney cells that contain few or no glucocorticoid receptors.

Continued on following page

Appendix Table 1. *(Cont'd)*

Cyclic AMP-responsive element binding protein	Transcription factor mediating the effects of cAMP on gene transcription.
Donor splice site	RNA sequence recognized by RNA-processing enzymes during splicing.
Downstream versus upstream	5' to 3' versus 3' to 5' (upstream) direction in DNA; aminoterminal to carboxyterminal versus carboxyterminal to aminoterminal direction in proteins.
DNA denaturation	Conversion of double-stranded DNA into single-stranded DNA.
Enhancer	A short region of DNA that augments the transcriptional activity of DNA from a distance.
Exon	A coding section of a gene retained at its mRNA after splicing of the initial mRNA transcripts.
Expression vector	A plasmid or phage whose DNA contains an active but easily controlled promoter upstream from the insertion of a gene of interest. After induction of the promoter, the protein of interest can be produced in measurable amounts.
Guanine-cytidine–clamped denaturing gradient gel electrophoresis	A method for separating fragments of denatured DNA based on single base differences. Fragments are forced to run through a gel by placing them in an electric field. The speed at which they move depends on their size and charge.
Glucocorticoid modulatory element	Brief DNA sequence that binds specific transcription factors, which modify the actions of glucocorticoids.
Glucocorticoid-responsive elements	Brief DNA sequences recognized by the glucocorticoid receptor.
Heat-shock proteins 70 and 90	Families of essential, highly conserved proteins of approximate molecular weight 70 or 90 kd, respectively.
Immunophilins	Highly conserved proteins that bind immunosuppressant agents, such as FK 506, rapamycin, or cyclosporin.
Intron	A noncoding section of a gene that is removed from RNA transcripts before they are translated in cells from higher organisms.
Mammary tumor virus	Its promoter contains four glucocorticoid-responsive elements; it has been used in conjunction with the chloramphenicol acetyl transferase gene to evaluate the activity of glucocorticoid receptors.
Microdeletion	Deletion of a small number of nucleotides from a gene.
Nuclear localization domains or sequences	Brief amino acid sequences associated with the potential of a protein to enter the nucleus.
Oligonucleotide	A short piece of DNA or RNA containing 3 or more nucleotides. The oligonucleotides used in gene cloning are generally less than 100 nucleotides long.
Point mutation	A change of only one nucleotide pair in a DNA molecule.
Polymerase chain reaction (PCR)	A test tube reaction in which a specific region of DNA is amplified many times by repeated synthesis of DNA using DNA polymerase and specific primers that define the ends of the amplified region. Symmetric PCR yields an end-product that is double stranded; asymmetric PCR yields an end-product that is single stranded for direct sequencing.
Polymerase II initiation complex	RNA polymerase II and several ancillary factors called transcription factors (TF) II A–F join together to initiate and complete transcription of a gene.
Primer	A piece of DNA or RNA that provides an end to which DNA polymerase can add nucleotides.
Reporter plasmid	A plasmid carrying a promoter with one or more responsive elements in conjunction with a gene whose product can be readily detected.
Reverse transcriptase	Enzyme catalyzing the reaction for converting RNA to DNA.
RNA polymerase II	The enzyme complex responsible for making mRNA from DNA. RNA polymerase binds at specific nucleotide sequences (promoters) in front of genes. It then moves along a gene to catalyse production of the complementary RNA molecule.
RNA splicing	The process of removing regions from RNA. The removed regions are called introns, and the regions spliced together are called exons.
Southern hybridization (southern blotting)	A method for transferring DNA from an agarose or acrylamide gel to nitrocellulose paper; this is followed by hybridization to a radioactive probe.
SP1 site	DNA sequence associated with the promoter region of genes and binding the SP1 transcription factor.
TATA box	DNA sequence associated with the promoter region of genes, perhaps an anchor for an ancillary factor of the RNA polymerase II enzyme.
Transactivation	Ability of a protein (transcription factor) to modulate the transcription rate of a gene.
Transactivation domain (τ_1, τ_2)	Portions of a transcription factor necessary for modulating the transcription rate of a gene.
Transcription	Transcription involves making an RNA molecule using the information encoded in the DNA. RNA polymerase II is the enzyme complex that executes this conversion of information.
v-*erb*A	Oncogene first described in the avian erythroblastosis virus; homologous to the T_3 receptor.

Requests for Reprints: George P. Chrousos, MD, NIH, Building 10, Room 10N262, Bethesda, MD 20892.

Current Author Addresses: Drs. Chrousos and Karl: NIH, Building 10, Room 10N262, Bethesda, MD 20892.
Dr. Detera-Wadleigh: NIH, Building 10, Room 3C 108, Bethesda, MD 20892.

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