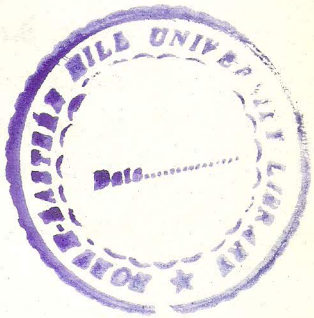
PHYSICOCHEMICAL CHANGES IN THE GLUCOCORTICOID RECEPTOR DURING DEVELOPMENT OF MICE



by

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Submitted in partial fulfillment of the requirement of the Degree of **Doctor of Philosophy** in **Biochemistry**

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I Md. Monsur Ahmed Borbhuiya, hereby declare that the subject matter of thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Biochemistry.

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Introduction

The period of time that an organism/species survives under a given environmental condition denotes the lifespan of that organism. In higher multicellular organisms, the onset of life begins with the fusion of the male and female gametes and ends with the death of the organism. This constitutes the whole lifespan that however, varies greatly among the different species. This makes it difficult to establish clear `chronological boundaries´ of the different stages of lifespan. Some authors have divided lifespan into two major periods: prenatal (before birth) and postnatal (after birth), taking into account the characteristic anatomical, physiological and biochemical features at each stage. The prenatal period encompasses the embryonic and fetal stages, whereas the postnatal period includes neonatal, infancy, adulthood and old age (Timiras, 1994). Others have defined lifespan as a continuum with development at one end, followed by the reproductive phase; other end being the senescence or aging, each stage showing a characteristic set of sequential events, regulatory mechanisms, rate and duration (Kanungo, 1994).

Developmental phase

This phase encompasses all the events taking place during prenatal period and extends to a considerable length of the postnatal life too. It is characterised by striking changes in the morphological, physiological, biochemical and psychological features, which lead to suitable specialization of various cells, tissues and organ systems of the body. These changes confer reproductive ability upon the organism, at a definite stage of development, which in many cases continues even after the attainment of reproductive capacity (Timiras, 1988).

Reproductive phase

The transition from the developmental to reproductive phase is characterised by the appearance of specialized structures and functions that confer reproductive ability to the organism. This enables the organism to reproduce its own kind that not only aid in the perpetuation but also in the evolution of species. This period is characterised by great functional stability: it connotes the attainment of optimal and integrated function of all body systems. At the molecular level, several genes that play an important and specific role in the development and maintenance of reproductive ability are now expressed. The duration of this phase of lifespan is more or less defined, especially in females. There is a direct correlation between the time taken to

reach reproductive maturity and the maximum lifespan of an organism (Hayflick, 1987).

Senescent phase

This is characteristic of all multicellular organisms and is illustrated by an overall decline in the bodily functions. This decline is quite noticeable during the latter stages of the reproductive phase and affects most tissues, organs and the overall physiological competence. An important feature at this stage of lifespan is the appreciable loss in the reproductive ability of organisms. During this phase, the ability to adapt to both internal and external stresses decrease, leading to a decline in the homeostatic balance. Thus aging, whereby a time-dependent drift from the optimum bodily functions are seen, was postulated to be pleiotropic in nature (Cutler, 1984). This states that the aging process is due to

- normal by-products of the living processes.
- evolutionary non-selected endogenous properties.

Two classes of pleiotropic aging have been proposed, one liked to energy metabolism, called the continuously acting biosenescent process (CABPs) and the other associated with developmental processes, named as the developmentally linked biosenescent process (DLBPs). Questions exist as to which of the two processes play a decisive role in the aging phenomenon.

Since numerous variability exists in the rate as well as the time of incipience of senescence among various species, it becomes difficult to attribute any specific parameter or a single `triggering event´ as being responsible for the aging of the organism. Moreover, scientific works over the years appear to implicate the events occurring during the developmental phase, that not only induces but accelerates the process of senescence (Walford, 1987). Our comprehension of the basis of senescence may therefore, be reinforced by detailed information on the developmental period of lifespan.

As mentioned earlier, the developmental phase is characterised by striking changes. These include an increase in the number and size of cells, their differentiation to perform specialized functions and the formation of organ systems. At the molecular level, an intricate display of genes is seen as some are switched on while others, shut off. This gives rise to a whole array of proteins that not only support organogenesis but also provides the organism with catalytic power to lead an independent existence. The developmental phase is also characterised by the significant influence of environment, both internal and external on adaptation made by the organism. These also play a considerable role in regulation and maintenance of homeostatic balance necessary for proper development.

Many of the present efforts taken to comprehend the process of development is directed towards understanding the age-related changes that transpire at all levels of the organism. These changes can be organized into several broad headings; they encompass but, a few of the well-documented phenomena that contribute to our knowledge of the developmental and subsequent aging processes. Experimental evidence shows quantitative changes in the tRNAs and aminoacyl-tRNA synthesis, responsible for the translation process, during development (Ilan and Patel, 1970; Mays *et al.,* 1979). Also, an augmentation in the fidelity of transcription and translation processes during development increases the chances of having proteins and enzymes with altered structural and functional properties (Lamb, 1977). Other workers however, contradicted such reports (Sharma & Patnaik 1982, Fleming *et al.,* 1986). The decline of immunologic competence and the concomitant increase in autoantibody production may contribute, significantly to the aging process (Goidl *et al.,* 1983; Nandy and Bennett, 1983). Other changes include age-related differences in the protein turnover rates (Adelman and Dekker, 1985; Richardson, 1985) and in the enzymes responsible for DNA repair (Tice, 1978). More recently, the effect of dietary restriction and its influence on the various cellular activities have provided some interesting evidence on the role of diet in the development and aging processes (Weindruch, 1991; Timiras, 1994).

Inspite of the voluminous work being present no clear cut evidence has emerged, so far, as to the exact cause(s) that could be implicated for the aging phenomenon. However, it is generally believed to be due to a gradual and simultaneous deterioration of one or more processes mentioned above. This gradual loss in the functional ability and homeostatic balance as a whole lays the ground for onset of senescence or aging. It is important to emphasize here that the changes mentioned above constitute a few amongst the many well-studied processes. Some of these changes warrant a brief discussion to underline their importance in the development and aging process.

Changes in gene expression

The structure and functional capacity of an organism are directed by information that is encoded in the genes contained in DNA, the genetic material of a living system. These genes direct the production of biomolecules that ultimately dictate the form and function of the organism throughout its lifespan. Three major types of alterations in gene expression occur with age:

i) actively expressed genes may undergo a gradual decrease in expression due to a decline in regulatory factors or due to a structural/organizational change in the chromatin.

ii) an increase in expression of those genes which are already being expressed.

iii) genes that undergo alternate expression and depression.

These points emphasize the control of gene expression at the transcriptional level. However, regulations at the processing, transport, stability and translation of mRNA as well as post-translational events are no less significant. Cellular signals due to cell-cell interactions in controlling gene expression are also important (Lewin, 1995). However, during developmental phase most of the changes in gene expression are seen as a result of sequential activation and repression of genes (Caplan and Ordahl, 1978; Wilkins, 1986). A classic example of this intricate control of gene expression is seen in the changing hemoglobin composition patterns during the early developmental period in humans (Zuckerkandl, 1965) The four globin chains- α, ε, γ, β, each encoded by a separate gene, constitute the tetrameric hemoglobin. The molecular composition is $\alpha_2\varepsilon_2$ in the 1-2 month old fetus that is replaced by $\alpha_2\gamma_2$ (fetal hemoglobin, HbF) at a later stage of the fetal life. In the newborn, the hemoglobin is of $\alpha_2\beta_2$ (adult hemoglobin, HbA) type. This clearly demonstrates the sequential activation and repression of different genes and that too for various durations, thus providing greater functional significance to hemoglobin at each stage of development. Changes in protein pattern of the larval tissues in developing *Drosophila. melanogaster* are attributed to sequential changes in the expression of genes (Arking, 1991). Work by Kanungo and his co-workers have shown such developmental changes in the isoenzymes of lactate dehydrogenase and of the subunit composition of alanine aminotransferase during aging of rats (Kanungo and Singh, 1965; Kanungo and Patnaik, 1975). Others have reported changes in the types and amount of RNA, with development in different tissues of mice (Cutler, 1982; Richardson, 1985). Of importance to the process of development and aging are genes that play a significant role in imparting sexual maturity and reproductive ability to an organism. Among these are genes for the production of sex-hormones as well as other hormones that not only help the organism attain sexual competence but also help in the maintenance of homeostatic balance. The loss of this balance, as reported by various workers, is a result of reproduction and this in turn is implicated to alter the expression of genes and usher in the process of senescence (Wodinsky, 1977; Diamond, 1982; Medvedev, 1990). Moreover, the direct and/or indirect action of other factors such as nutrition and stress in regulating gene expression cannot be ruled out.

It is of interest to know that many cis-acting elements in the promoter/enhancer regions as well as transcription and trans-acting factors have, over the past few years, been implicated to play an indispensable role in regulating gene expression. The work of Strähle *et al.* (1988) and others have not only shown the role of these

elements but also provided an insight into the regulatory differences between species expressing similar genes (Kelsey *et al.*, 1987; Koopman *et al.*, 1989; Cavener, 1992).

Thus, the onset and maintenance of the various stages of lifespan of an organism depend to a considerable extent upon the duration, rate and regulatory mechanisms involved in gene expression. The work on human genetic diseases-Progeria and Werner's syndrome helped in reinforcing the above observations (Kanungo, 1994).

Changes in chromatin structure and composition

In cells, the genetic material, DNA is complexed with histone and non-histone proteins to form an ordered, compact structure called chromatin. Changes in chromatin organization were reported as early as 1967, taking into account the variability in the types and amount of mRNA during development (Yaffe and Fuchs, 1967). Other parameters to suggest changes in the chromatin structure with age have also been reported, for example, elevation of melting temperature (T_m), the decrease in salt extractable proteins, increase in single strand breaks and sensitivity to nuclease S1. Additional changes include chemical modifications, as exemplified by decrease in phosphorylation, acetylation, methylation and poly-ADP ribosylation of both histone and non-histone proteins (Kanungo, 1994). The above mentioned changes have been credited to a conformational change which leads to condensation of the overall chromatin structure with development and aging. DNase I and MNase digestion of the chromatin as well as nick translation of DNA confirmed such a conformational change during development and aging (Chaturvedi and Kanungo, 1983). All these changes ultimately effect the efficacy and rate at which genes are transcribed and can provide mechanism(s) to control gene expression at various stages of lifespan.

Change in hormones/hormone receptors

Hormones are molecules, synthesized and secreted from specialized group of cells, which are capable of transducing intra-/inter-cellular messages that influence a wide variety of cellular and metabolic processes (Zubay *et al.*, 1995). Neural hormones (neuro-transmitters) and hormones secreted by the endocrine, paracrine and autocrine glands are necessary for the proper functioning of almost every cell in the body and are responsible for providing diverse functional abilities to the organism. An important aspect of hormone action is its modulation of certain enzymes in a tissue- and age-specific manner which appears crucial for the maintenance and adaptive response of the organism (Kanungo, 1980; Sharma, 1988). The role of hormones in maintaining the effectiveness of homeostatic balance and adaptation to internal as well as external stresses is immense for proper growth and development of an

organism. Reports indicate a decline on both these accounts during the latter stages of lifespan, suggestive of apparent changes in the complex signaling mechanism conferred by these molecules. Data over the years have shown that tissue responsiveness depends upon the level of hormones and their receptors and also on the post-receptor events (Roth, 1981; 1989). Hence, any change in these parameters, may have profound influence on the process of development, growth, reproduction and aging.

Reports indicate significant changes in the level of certain hormones during early periods of development. A good example is that of glucocorticoids which appear in the fetal rats by the 19th. day of gestation and increase, significantly to reach adult levels by the 15th. day of postnatal age (Cohen, 1973; Martin *et al.,* 1977; Lu *et al.,* 1987). Similar observation was made in the avian system by Wise and Fyre (1973). After the attainment of adult values some hormones such as glucocorticoids, testosterone and serotonin do not show any further age-related changes. Others reported either an increase e.g., prolactin, GnRH, FSH, LH (Chakraborti *et al.,* 1976) or a decrease e.g., estrogen (Edman, 1983), ADH (Sladex *et al.,* 1981), dopamine and norepinephrine (Timiras *et al.,* 1985), aldosterone (Flood *et al.,* 1967) and DHEA (Orentreich *et al.*, 1984) with age.

The neuroendocrine system plays a notable role in the aging process and the importance of this system has over the years drawn considerable attention (Everitt and Walton, 1988; Timiras, 1991; Sharma, 1994). Changes in the neuroendocrine system is capable of altering various functional aspects of the organism. In this respect, the role of hormone receptors is central to the understanding of the control systems of the body and any age-related changes in the level or functional integrity of the receptors is likely to have diverse implications on the overall functioning of the organism.

It is now a well-documented fact that hormones exert their physiological effect either by way of receptors on the cell-surface or by intracellular receptors in target cells. For intracellular receptors, hormonal messages are transduced by the receptors themselves to the ultimate cellular centres. In the case of cell-surface receptors, the process of signal transmission extends to second and third messenger substances, which ultimately lead to an appropriate response. Furthermore, of late there is growing evidence of `cross-talk´ between the various constituents of signal transduction pathways and this provides even more complexity to the whole system (Sharma, 1993). Moreover, a sizeable body of evidence suggests an alteration in the tissue responsiveness to hormones during development and aging. This being true, such changes can be attributed to either change (a) in the receptor concentration, (b)

in binding affinity of hormone to receptor or (c) in other receptor properties including changes in post-receptor events. Much evidence is present on the alteration in various hormone receptor levels and also in the post-receptor events (Roth, 1989). Examples of some of the representative members of each group and changes if any, in the receptor-related processes are discussed below.

Membrane bound receptors

Neurotransmitters which include amines (acetylcholine, cathecholamines, dopamine), amino acids (glutamate, aspartate), peptides (enkephalins, somatostatin) and even gases (nitric oxide, carbon monoxide) are synthesized and secreted by specialized neurons of the nervous system. They along with their cognate receptors and a variety of second messengers constitute an important vehicle for neuronal communication. One of the most studied aspects of age-related changes in nervous system involves alterations in the hormone-receptor levels at the synapse (Giacobini, 1982; Strong *et al.*, 1991)

Adrenergic receptors

Cathecholamines (epinephrine and norepinephrine) are the ligands for adrenergic receptors. Age-related studies on the beta-adrenergic receptors in various species have been reported. In cerebral cortex of rats, a decline with age was observed (Misra *et al.*, 1980; Enna and Strong, 1981). However, in the cerebellum of rats, an age-related increase in β-adrenergic receptors is reported by Pittman *et al.* (1980). Decline in the receptor concentration in rat erythrocytes (Bylund *et al.*, 1977), human lymphocytes (Schocker and Roth, 1977), submandibular glands of rats (Piantanelli *et al.*, 1980) is also reported. Similar trends have likewise been reported for alpha-adrenergic receptors in the cerebral cortex (Misra *et al.*, 1980) and heart (Partilla *et al.*, 1982) of rats. Analogous to changes in the receptor levels, loss in the adenylate cyclase activity (an amplifier enzyme necessary for second messenger production) has also been reported (Schmidt and Thornberry, 1978)

Cholinergic receptors

Acetylcholine is the principal ligand for this class of receptor. Reports suggest an age-associated decline in acetylcholine receptors in the cerebral cortex of rat (Lippa *et al.*, 1981) and human (Perry, 1980). Observations for other tissues e.g., rat hippocampus (Nordberg and Winblad, 1981) and anterior pituitary (Avissar *et al.*, 1981) show similar trend. Contrary to these findings, reports by Davies and Verth (1978) and Strong *et al.* (1980) show no such age-related changes.

Dopaminergic receptors

Age-related decrease in dopamine receptors have been reported in the corpus striatum of rabbits (Thal *et al.,* 1980), mice (Marquis *et al.,* 1981) and rats (Roth, 1982). However, an increase in dopamine receptors with age, in the retina of rats is reported by Riccardi *et al.* (1981). Consequently, dopamine induced adenylate cyclase activity has been observed to decrease with age (Puri and Volicer, 1977).

Since neurotransmitters convey chemical information among neurons, any change in the signal transmission pathway via receptor-mediated processes will be directly reflected in altered physiological functions with age. Decrease in peripheral motor system, loss of memory and sensory functions as seen in Parkinson's and Alzheimer's diseases are manifestations often associated with the aging process (Timiras, 1994).

Insulin receptors

Insulin is one of the important hormones responsible for the regulation of carbohydrate metabolism, maintenance of glucose level and other metabolic processes involving gluconeogenesis, lipogenesis, protein synthesis and general growth in mammalian system (Norman and Litwack, 1987). Receptors for insulin are known to be present in a variety of tissues. Reports suggest a tissue- and species-specific alteration in the receptor concentration with age. Rosenbloom *et al.* (1976) reported an increase in the receptor number in human skin fibroblasts. However, most reports suggest a decrease in insulin receptor number, e.g., in rat liver (Pagano *et al.,* 1981), adipose tissues (Olefsky, 1975) and in human erythrocytes (Dons *et al.*, 1981). Goldfine (1987), however, suggests that the number and affinity of insulin receptors does not change, but the possibility of defects in the cascade of post-receptor reactions is being actively investigated upon (Caro, 1987; Fink *et al.*, 1983). Pathophysiological conditions e.g., increased incidence of diabetes and its associated disorders may reflect changes in receptor and/or post-receptor events with age (Bolinder *et al.*, 1983; Green, 1986)

Gonadotropin receptors

Gonadotropins (FSH and LH), secreted by the anterior pituitary control the sex steroid hormone production and reproductive function in both sexes. No change in the receptor concentration has been reported in the interstitial cells and ovaries in rats (Steger and Huang, 1983). On the other hand, Pirke *et al.* (1978) have observed a decline in the gonadotropin receptors in rat testes and leydig cells, respectively.

Intracellular hormone receptors

Thyroid hormone receptors

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), play an indispensable role in affecting a variety of biochemical reactions, in controlling the basal metabolic activity and also in regulating the growth and development. Receptors for thyroid hormone, primarily for T_3, is present in many tissues- liver, brain, lung, kidney and anterior pituitary, the receptor being present predominantly in the nucleus and also non-specifically, in the cytoplasm, mitochondria and plasma membrane (Eberhardt *et al.*, 1978). The biological actions of the hormone occur primarily through nuclear binding and stimulation of protein synthesis. Reports on age-related changes in this receptor are few. A decrease with age in the brain and liver of rats have also been reported (Margeuity *et al.*, 1985). However, no such change in the rat brain and liver was reported by Cutler (1981). The level of thyroid hormones, conversion of T_4 to T_3 and the rate of secretion of T_4 from the thyroid gland also affect the receptor concentration with age.

Androgen, Estrogen and Progesterone receptors

Androgens (testosterone and 5α-dihydrotestosterone) are primarily responsible for the differentiation, growth, maturation and maintenance of male reproductive organs. They are also responsible for the development of secondary sex characteristics and behavioral manifestations related to muscularity. The androgen receptor is present in a number of tissues- testes, prostrate, seminal vesicles, epididymis, kidney, liver, uterus, brain and pituitary. Studies on most of the tissues show a decline in the receptor numbers with age, e.g., in rat cerebral cortex and testes, ventral and lateral prostrate (Haji *et al.*, 1981), and in liver (Roy *et al.*, 1974).

The estrogens and progesterone are responsible for the development and maintenance of female reproductive system. Gessel and Roth (1981), and Kaur and Thakur (1991) observed a decrease in rat uterine receptors with age. Also, an age-related decrease in estrogen receptor concentration in cerebral cortex and amygdala of rats and in mouse brain cortex has been reported (Kanungo *et al.*, 1975; Roselli *et al.*, 1993; Asaithambi *et al.*, 1997). However, progesterone receptors show no such age-related decline in rat (Saiuddin and Zassenhaur, 1979).

Glucocorticoid receptors

Glucocorticoids partake in a number of crucial metabolic reactions, especially those occurring in the liver, muscle, adipocytes and brain. These processes are important during the developmental period and more so during periods of stress (Sapolsky *et al.*, 1986). Glucocorticoid receptors are ubiquitous in their distribution and studies reveal an early developmental increase in the receptor number upto about 15-20

days of postnatal age in rats (Henning, 1978). Thereafter a gradual decrease is seen in most tissues, e.g., in rat liver (Kalimi, 1984), kidney (Sharma and Timiras, 1988), brain (Kitkari *et al.*, 1984; Pfeiffer *et al.*, 1991), pituitary (Pfeiffer *et al.*, 1991), skeletal and cardiac muscles (Mayer *et al.*, 1981; Sharma and Timiras, 1987). Keeping in mind our interest in this receptor, a brief discussion on the recent advances in the study of receptor structure, mechanism of action and also the tissue- and age-specific changes, associated with receptor during development, is given below.

Inspite of the voluminous work being present, no clear cut consensus have emerged which could possibly explain the alterations in various aspects of hormone action during development and aging. Moreover, recent data adds to the overall complexity by suggesting age-dependent alterations that may occur at every step of the signal transduction process.

Structure and function of glucocorticoid receptor

Glucocorticoids are synthesized and secreted by the adrenal cortex under the control of hypothalamus-pituitary axis. At cellular level, most known effects of glucocorticoids are mediated by a ~94 kDa intracellular protein, the glucocorticoid receptor (GR) (Evans, 1988). Till the mid-sixties the concept of receptor for hormones was unclear. However, the availability of radioactive hormones (Jensen *et al.*, 1966), capable of selective binding to receptors provided a method of studying these proteins and their mechanism of action in greater detail. Glucocorticoid receptor was first detected in the thymus (Munck and Brinck-Johnsen, 1968) and since then its presence in almost all the tissues of mammals has been confirmed. During the last three decades, use of classical techniques and more recently the use of powerful molecular genetic tools have provided much information on the receptor structure and other details of its action mechanism. Glucocorticoid receptor belongs to a phylogenetically conserved superfamily of nuclear hormone receptors, that also include other steroid receptors, thyroid hormone receptors, oncogene products and the recently discovered `orphan receptors´, whose ligand requirement has not yet been identified (Laudet *et al.*, 1991; Mangelsdorf *et al.*, 1995). This family constitutes the largest known group of eukaryotic transcription factors and it is of interest to know that glucocorticoid receptor is the first transcription factor to be isolated and studied in detail.

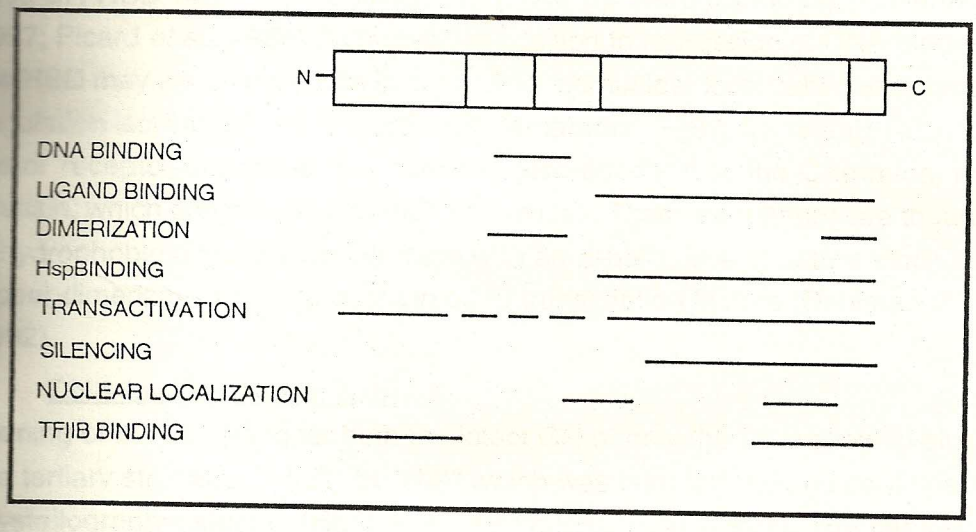
GR gene structure

The ability to clone genomic genes transcribing for GR in mouse and in humans has revealed a complex organization of the GR gene. The gene is about ~60 kb in size and the translated portion is found to be distributed among 8 exons for mouse GR. A

single exon of about 1230 nucleotides codes for the N-terminal end of the receptor protein whereas the DNA- and steroid-binding domains are encoded by more than one separate exons. It has a promoter region similar to that of a housekeeping gene with multiple transcription initiation sites (Encio and Detera-Wadleigh, 1991; Cole TJ *et al.*, 1993).

Domain structure of GR

The use of classical proteolytic analysis of intact GR as well as the recent advances in amino acid sequencing, mutation/deletion studies, and expression of chimeric GR have provided much information about the structural and functional organization of the GR protein (Vedeckis, 1983; Rusconi and Yamamoto, 1987; Evans, 1988; Muller and Renkawitz, 1991). GR has a characteristic three domain structure similar to that of other members of the steroid receptor superfamily A schematic diagram of the GR domains and their functional attributes is given in the figure below.



Functional Domains of Glucocorticoid Receptor

Hormone binding domain (HBD)

The first step in the glucocorticoid action mechanism, is its binding to high affinity intracellular receptors present in the cytoplasm of target cells. The portion of GR responsible for ensuring proper binding of the ligand is located at the carboxyl terminus, encompassing about one third of the receptor molecule and is referred to as the hormone binding domain (HBD) (Godowski *et al.*, 1987)). Proteolytic analysis revealed a 16 kDa fragment (Thr^{537} to Arg^{673}) which binds steroid but, with a much lowered affinity than the intact molecule. The HBD must be properly folded to have a high affinity binding site and this is helped by the presence of other regions in the GR itself and through the association of GR with the components of the protein

folding system (Xu *et al.*, 1996). This system , termed `foldosome´ consists of two molecules of 90 kDa heat shock proteins (hsp90), a molecule of hsp70, hsp56 (an immunophillin) and a 23 kDa acidic protein (Pratt , 1993; Stancato *et al*, 1996). The HBD region is rich in amino acids, cysteine and methionine and several of them, e. g. $cys^{638, 665}$ in human GR, met^{622} and $cys^{640, 656, 661, 674}$ in rat GR and the associated intramolecular S-S linkages are involved in high affinity steroid binding (Yu *et al.*, 1995; Simons and Pratt, 1995). These studies revealed the three dimensional structure of HBD to consist of a conformationally flexible pocket where the binding of ligand occurs with maximum hydrophobic interaction. Recent studies also attribute the role for HBD as a repressor of transactivation. Deletion of the HBD yields a molecule whose ability to undergo transactivation equals almost that of an intact GR and that too in the absence of the hormone ligand. Transactivation of chimeric construct with rat GR HBD and E1a is repressed in the absence of hormone. Other fusion proteins with GR-HBD have their activity controlled by steroid binding (Hollenberg *et al.*, 1987; Picard *et al.*, 1988). Moreover, in addition to repression of DNA-binding activity, the HBD may also have a role in controlling the nuclear localization and transcriptional regulation activity of GR (Picard and Yamamoto, 1987; Lanz and Rusconi, 1994). Major receptor dimerization function is also ascribed to the C-terminal end of this domain, which contains leucine-rich sequences. These sequences are thought to form a hydrophobic dimerization interface with an α-helix, in a structure similar to leucine zipper dimerization motif present in other transcription factors (Dahlman-Wright *et al.*, 1992).

DNA-binding domain (DBD)

Cloning of cDNA coding for both the intact GR or only the DBD allowed elucidation of the tertiary structure, initially by NMR which was later refined and confirmed by X-ray crystallographic studies (Härd *et al.*, 1990; Luisi *et al.*, 1991). The central portion of the GR, rich in basic residues constitutes the DBD, which is highly conserved among the steroid receptor superfamily. The DBD is globular in structure and divided into two distinct motifs, each contributing a `zinc-finger´- in which a zinc ion is co-ordinated to four cysteine residues with a tetrahedral geometry. The peptide loop thus formed is structurally similar to the *Xenopus* transcription factor, TFIIIA, where a zinc ion is co-ordinated by two cysteines and two histidines. Site directed mutagenesis revealed the absolute necessity of seven out of eight cysteines for proper receptor function (Schena *et al*, 1989). The first motif in the three dimensional DBD structure, called the P-box, contains the first Zn-finger; the peptide loop here starts with a short segment of antiparallel β-sheet and ends with an α-helical conformation. The constitution of the second Zn-finger, called the D-box is similar. The two α-helices lie perpendicular to one another and these along with the β-sheet helps to orient not

only the residues that contact the phosphate backbone but also the DBD to fit into the major groove of the double helical DNA (Wright *et al.*, 1993). The two motifs, though identical structurally, play distinct roles in the steroid action mechanism. To P-box is attributed the role of maintaining the fidelity of binding to cognate glucocorticoid response element (GRE) while the D-box has a role to play in the receptor dimerization process (Tsai and O´Malley, 1994).

Modulatory/immunologic domain

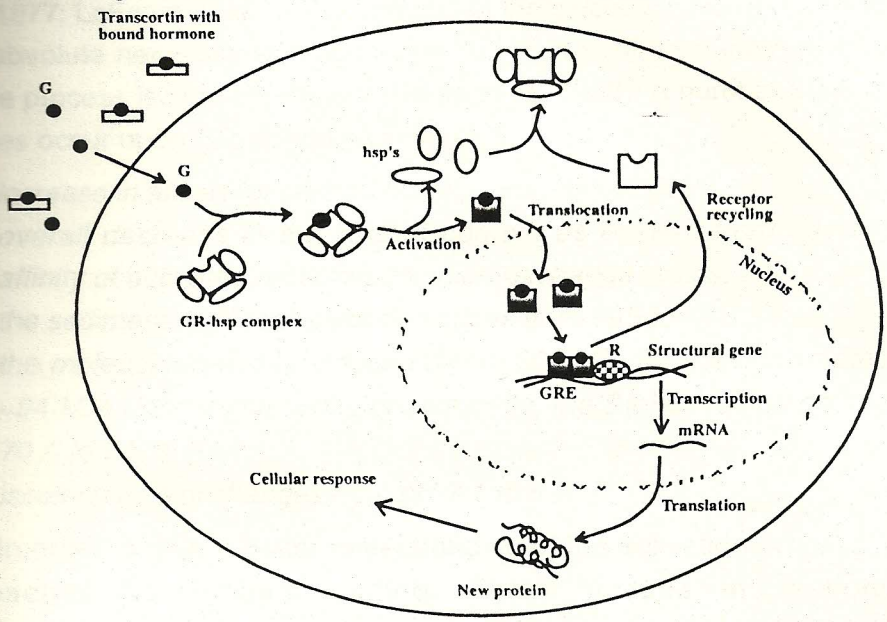
The other important portion of GR is the N-terminal region which is responsible for synergistic activation of transcription from adjacent binding sites. The tau1 and tau2 regions in hGR have been implicated in the transactivation process, the tau1 region contributing over 90% of the receptors' activity. Other functional constituents of this region are nuclear localization signals and the immunogenic epitopes. Most of the known antibodies generated against GR are from the antigenic epitopes localized in the N-terminal of the receptor protein (Dahlman-Wright, 1994; Cidlowski *et al.*, 1990)

Glucocorticoid action mechanism

The discovery of intracellular receptors for glucocorticoids in early 70s was followed by extensive work to decipher not only the role of the receptor in signal transduction process but also in understanding the exact mechanism of glucocorticoid action. Although a lot of questions remain unanswered, as yet, work over the years have contributed a lot and a number of schemes/models have been proposed for the glucocorticoid action mechanism.

Glucocorticoids synthesized and released from the adrenal cortex (zona fasciculata) is transported to the target organs by a protein- corticosteroid binding globulin (CBG) present in the blood. Most of the glucocorticoid released exists in a dynamic equilibrium between the bound and free form. Since glucocorticoid is lipophillic, the unbound hormone enters the target cell by a process of free diffusion and binds with high affinity and specificity to its cognate receptors (Brann *et al.*, 1995). The unliganded GR resides in the cytoplasm, where it exists as a large multiprotein complex consisting of a molecule of GR, two molecules of hsp90, a molecule each of hsp70 and hsp56 and other smaller proteins (Pratt, 1993; Czar *et al.*, 1994; Webster *et al.*, 1994). The association of other proteins especially the hsp90s is necessary to help the receptor attain high steroid binding affinity, prevent nuclear translocation by masking the nuclear localization signals and subsequent transactivation. Upon hormone binding, a conformational change occurs leading to the dissociation of all the receptor associated protein molecules. The hormone-bound receptor then translocates to the nucleus, where binding of the receptors directly to specific response element(s) or to other proteins, e.g., components of AP-1 signaling

pathway, lead to modulation of target gene activity. The nuclear bound receptors are subsequently degraded or cycled back to the cytoplasm (Litwack, 1988; Bamberger *et al.*, 1996). A schematic diagram representing the events in the glucocorticoid action mechanism is given below.



Glucocorticoid action mechanism

G, glucocorticoid; GR, glucocorticoid receptor; hsp, heat shock protein; GRE, glucocorticoid response element; R, RNA polymerase

Activation/Transformation

The process of activation (transformation) has been the subject of intense investigations over the years. However, the process remains ill defined till date although, many details of the events at molecular level have been elucidated in the recent past. As mentioned, the unliganded (unactivated) GR is present as a aporeceptor in association with a number of non-hormone binding proteins that enable the receptor to be in a conformational state capable of ligand binding. Activation is a response to an increase in the glucocorticoid concentration that drives ligand binding to the GR and consequently causes a conformational change in the HBD. This leads to the dissociation of all the protein components of the aporeceptor complex, a process termed activation or transformation (Truss and Beato, 1988; Hutchison *et al.*, 1993; Tsai and O´Malley, 1994). This process occurs *in vivo* under physiological conditions and is rate-limiting for nuclear binding, the entire process representing a normal step in signal transduction pathway (Munck and Foley, 1979;

Markovic and Litwack, 1980; Htun *et al.*, 1996). While the hormone binding under cell free conditions occurs at 2-4 °C, activation is achieved at elevated temperatures of about 25-37 °C (Milogram *et al.*, 1979). Activation could also be achieved *in vitro*, by other means, e.g., dilution, acidic pH, high salt concentration, gel filtration, etc. (Goidl *et al.*, 1977; LeFevre *et al.*, 1979). Binding of the glucocorticoid hormone to its receptor is an absolute necessity to attain activation by any of the above mentioned factors and the process is time dependent (Denis *et al.*, 1988). A number of physicochemical changes occur upon GR activation in vitro-

- *increase in affinity for purified nuclei, chromatin or DNA.*
- *overall decrease in the charge content as evident from decreased binding affinity of activated receptors to anion-exchange resins.*
- *the sedimentation coefficient decreases from ~9S to ~4S.*
- *the molecular mass is reduced from ~300 kDa for the unactivated receptor to ~94 kDa upon activation. Consequently, the Stokes radius decrease from 60-70 Å to 20-26 Å.*
- *isoelectric point changes from pH 7.1 to 6.1.*

In order to gain a better understanding of the activation process, a number of approaches have been applied. These include interactions between unactivated/activated receptors with isolated nuclei, DNA, DNA-cellulose, etc. Consequently, several compounds have been identified that either enhance or inhibit the above mentioned interactions (Grody *et al.*, 1982; Moudgil *et al.*, 1984). Among these are transition metal ions, namely, molybdate and tungstate, thiol-modifying agents, e.g., N-ethylmaleimide. In addition, several endogenous heat-stable cytoplasmic and nuclear factors, phosphorylation/dephosphorylation processes have been identified that may have an important role in the glucocorticoid action mechanism.

Modulators of GR activation process

Initial studies on the use of molybdate reported it to be a stabilizer of glucocorticoid receptors (Nielsen *et al*, 1977), but was subsequently shown to block the *in vitro* activation process (Nishigori and Toft, 1980). However, no inhibitory effects are seen on the already activated hormone-receptor complexes. These effects were found to be reversible upon removal of molybdate from the system. The effects of tungstate and vanadate were also reported to be similar, albeit tungstate appeared to be a more effective agent for blocking receptor activation (Moudgil *et al.*, 1984). The ability to block the activation process allowed workers to purify the unactivated receptor complexes and study the structure and function in greater detail (Wrange *et al.*, 1986; Grandics *et al.*, 1984). Several workers have also reported the inhibitory effects of

thiol-modifying agents like N-ethyl maleimide, suggesting a possible role for thiol group(s) in the activation and/or DNA-binding processes of the glucocorticoid receptor (Simons and Pratt, 1995).

The possible role of pyridoxal-5´-phosphate (Vit B_6), as an endogenous modulator has also been investigated by many workers (DiSorbo *et al.*, 1980; Allgood *et al.*, 1990). Vit B_6. is an essential, water-soluble vitamin required for normal growth and development. Several biochemical properties if the GR is influenced by Vit B_6 including molecular conformation, polyanion binding, surface charge and susceptibility to exogenous proteolysis (Cidlowski, 1980; O´Brien and Cidlowski, 1981). In addition, subcellular localization and the DNA-binding capacity are also affected. Vit B_6 deficiency in experimental animals caused an increased translocation of GR from cytoplasm to nucleus, whereas the opposite was observed under conditions of elevated Vit B_6 concentration (Holley *et al.*, 1983; Bruce and Vessal, 1987). Vit B_6 also decreases the transcriptional efficiency of target genes by GR but the exact mechanism(s) of the process are not yet clear (Allgood *et al.*, 1993; Tully *et al.*, 1994).

Many other natural compounds, named as glucocorticoid action biomodulators, have been identified. These include compounds which are potent activators of Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C), e.g., 1,2-racemic dioctanoyl glycerol (1,2-DG), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), guanisine 3´-diphosphate. epidermal growth factors (EGF) and interleukins. Based on these and other observations, a possible role for protein kinase C in glucocorticoid action mechanism has been proposed (Sharma, 1991).

Phosphorylation/dephosphorylation of GR

The role of phosphorylation/dephosphorylation in regulating the activities of many enzymatic and non-enzymatic proteins is well known, however, the persuasive importance of such processes on the functional ability of the GR is not very clear. Phosphorylation of the GR has been studied in a number of systems and it is seen that the basally phosphorylated GR becomes hyperphosphorylated, mostly on serine residues concomitantly with or shortly after dissociation from hsp complex, during the activation process (Hu *et al*, 1994). In most of these studies, phosphorylation has been shown to play a role in the binding of hormone to the receptor. Recently, Vivanco *et al.* (1995) proposed that changes in transcriptional activity of rat GR is due to alterations in receptor phosphorylation. The generation of `null receptor´ in ATP-depleted cells, which has low affinity for hsp and the hormone ligand, led researchers to propose that there exists both hormone-binding/non-binding GR forms depending on the phosphorylation status of receptor (Bodwell *et*

al, 1993). Moreover, the use of phosphatase inhibitors that stabilized the hormone-receptor complex and prevented its dissociation, pointed to the possibility that inactivation of receptors may result from dephosphorylation. The role of dephosphorylation in the activation process of glucocorticoid receptor was also studied. Alkaline phosphatase was reported to enhance the activation process whereas phosphatase inhibitors e.g., molybdate, tungstate and fluoride were seen to inhibit this process (Matic and Trajkovic, 1986; Schmidt and Litwack, 1982). The role of dephosphorylation has, however, been questioned and evidence presented to show that there is no dephosphorylation of GR during activation process (Mendel *et al.*, 1987, 1990; Tienrungroj *et al.*, 1987).

The role of protein kinase(s) and phosphatase(s) which mediate in the above processes has not yet been clearly defined. However, the role of protein kinase C in phosphorylation of GR seems interesting. Stimulation of cAMP-dependent protein kinase A (PKA) pathway has also been reported to augment GR activity in response to glucocorticoids. This change probably does not involve alterations in phosphorylation pattern of GR and is likely to be mediated by phosphorylation of factors interacting with the receptor (Rangarajan *et al.*, 1992; Moyer *et al.*, 1993; Reisfeld and Vardimon, 1994).

Thus, a consistent pattern of enhancement or inhibition of GR function via phosphorylation/dephosphorylation processes is lacking. It may be possible that the phosphorylation status of GR determines its subcellular localization rather than its overall activity (Orti *et al.*, 1993; Borror *et al.*, 1995).

Nuclear translocation of GR

Once the glucocorticoid receptors are transformed (activated) they translocate to the nucleus. The actual process of GR translocation has evaded numerous investigations and no conclusive evidence has been forwarded to explain the actual mechanism. It is known that the size of the GR is considerably larger than that of the nuclear pores to allow for passive diffusion (Lang *et al.*, 1986). A transport system, reported to be located in the nuclear membrane, called `transportosome´, is responsible for the facilitated transport of GR. In addition, Pratt (1993) has proposed a role for hsp90 along with the intracelluar microtuble system in the nuclear transport of receptor. This proposal is based on several observations that cited the association of hsp90 with GR and also of GR with tubulin (Wikstrom *et al.*, 1987; Sanchez *et al.*, 1988; Pratt, 1990). Moreover, observations suggest the co-translocation of GR and hsp90 to the nucleus under normal as well as stressed conditions. Howell *et al.* (1990) also reported the association of GR with nuclear envelopes, using [^{3}H] dexamethasone-mesylate affinity-labeling and immunological

techniques. They showed that this association of GR with nuclear envelopes is hormone responsive, suggesting that hormone binding causes the exposure of certain nuclear localization domains (signals) that helps in targeting of receptors to the nucleus. In support of the above observation, two nuclear localization signals (NLS), characterized by abundance of basic amino acids lysine and arginine, have been identified in the rat GR (Picard and Yamamoto, 1987). These signals probably permit interaction of activated GR with the nuclear transport machinery. The first nuclear localization signal (NL1) located at the C-terminal of GR, between amino acids 497-524 and has a short region of homology to the nuclear localization signal of SV40-T antigen. The NL2 lies within the HBD (Aa 525-795) and is probably masked by the bound hsps, since its function is revealed only upon hormone binding and subsequent dissociation of the hetero-oligomeric protein complex. Further support for the facilitated transport of GR into the nucleus came from an interesting observation by Htun *et al.* (1996), who used a chimeric protein to study the translocation process. A mutant form of the green fluorescent protein (GFP) was fused to GR, the unliganded chimera was then observed to reside in the cytoplasm that translocates to the nucleus only in response to glucocorticoid. The translocation process was ligand-, time- and dose-dependent and required energy, showing it to be a facilitated process.

Glucocorticoid response elements

Upon translocation to the nucleus, the hormone bound GR binds to specific DNA sequences, usually located 100-300 bp in the 5´-flanking regions of glucocorticoid regulated genes. These sequences, responsive to glucocorticoids, are termed glucocorticoid response elements (GREs) (Beato, 1989). Gene transfer techniques have helped in the identification of such GREs in many glucocorticoid inducible genes and any deletion or mutation of such sequences eliminated hormonal control (Yamamoto, 1985). It was also shown that this short oligonucleotide sequence is responsible for conferring glucocorticoid responsiveness, even to a heterologous promoter (Strahle *et al.*, 1987). The GRE has been identified as a 15-mer, consisting of two short inverted repeats separated by three nucleotides- 5´GGTACAnnnTGTTCT3´. Such consensus DNA sequences have been observed for progesterone, mineralocorticoid, androgen, estrogen as well as for other members of steroid and non-steroid receptors. (Beato, 1989; Lucas and Granner, 1992). Recent work, also suggests that the mere availability of a GRE in isolation is not enough and the presence of multiple copies of GRE or its association with other cis-elements, constituting a complex unit- termed glucocorticoid response unit (GRU), is necessary for mediating the effect of glucocorticoids (Lucas and Granner, 1992). In

addition several other factors play a very significant role in the process of DNA-binding, a brief mention of a few will emphasize the complexity involved in regulating gene activity by glucocorticoids.

Receptor dimerization

Increasing evidence suggests that the glucocorticoid receptor binds to GRE as a homodimer (Kumar and Chambon, 1988; Hard *et al.*, 1990). This dimerization occurs prior to DNA-binding and the dimers interact with the GRE in a head-to-head configuration, each receptor contacting a single arm of the palindromic GRE. This observation is supported by studies on the interaction between DBD of GR with GRE using NMR, X-ray crystallography and other biochemical techniques (Hard *et al.*, 1990; Luisi *et al.*, 1991). As mentioned earlier, the dimerization motifs are located in the DBD but in absence of glucocorticoids are masked by the HBD. The role of hormone in DNA-binding is thus, seen as an agent that induces a conformational change in the receptor molecule leading to exposure of its dimerization function apart from NLS thereby, allowing high affinity binding to GRE and subsequent modulation of the cognate gene expression.

Interaction of GR with transcription machinery

The process of transcription is a complicated phenomena, involving a large number of factors which constitute the basal transcription machinery. Many trans-acting factors, including glucocorticoid receptor interact either directly or indirectly with the transcription machinery to modulate gene expression. The formation of transcription initiation complex is a sequential process and is a prerequisite for proper transcription of any gene. The activated GR is thought to either stimulate the formation and/or stabilization of this complex or help in the recruitment of preformed complexes at glucocorticoid responsive gene promoters. The role of tau1 and tau2 domains of GR in the above process(s) is currently under investigation (Truss and Beato, 1993; Onate *et al.*, 1995; Beato and Sanchez-Pacheco, 1996).

Synergism between different cis-acting elements

Many steroid response elements, including GREs are present in multiple copies or in conjugation with other cis-acting elements (Tsai and O'Malley, 1994). Mutation(s) in one of the GREs or in the adjacent cis-element produce a drastic decline in the promoter activity, suggesting the possibility of synergistic interaction between adjacent response elements in the transcription process. In the case of GR, it has been observed that binding of one receptor-dimer facilitates the binding of a second one thereby, allowing both the complexes to bind with higher affinity/specificity thus, promoting increased transcription (Schmid *et al.*, 1989). Such synergistic interactions probably through protein-protein interactions outside of DBD, between GR dimers

and other proteins like CCAAT- and CACCC-binding factors, NF1 or SP1 motifs have also been reported (Bamberger *et al.*, 1996; Ricousse *et al.*, 1996). The consequence of such synergistic interactions is not clear however, speculations about its role in nucleosome disruption that will provide access to other transcription factors is being researched upon.

Arrangement of cooperative binding sites

The magnitude of transcriptional regulation is also affected by the location of GREs relative to transcription initiation site. It has been reported that GREs located too far upstream of the TATA box have insignificant contribution on transcription as compared to those located just upstream of the initiation site (Bradshaw *et al.*, 1988; Schatt *et al.*, 1990). This distance-related synergism shows a cyclic pattern, with a period of about 10 bp which corresponds to one turn of the double helix, suggesting stereospecific requirement for protein-protein interactions. However, the relative arrangement of GREs and other cis-elements (either upstream or downstream of initiation site) showed no change in transcriptional ability of the respective genes (Schule *et al.*, 1988).

Alteration in chromatin structure

Genetic analysis using modern techniques has revealed a widespread involvement of the structural organization of DNA/chromatin in regulating gene expression (Weintraub, 1985). DNase I sensitivity of mouse mammary tumor virus (MMTV) promoter increases upon glucocorticoid administration, showing an alteration in the chromatin organization in the vicinity of glucocorticoid receptor binding sites (Zaret and Yamamoto, 1984). This change is believed to be due to either displacement or rearrangement of nucleosomes over GRE, causing a relaxation in the chromatin structure. This relaxation facilitates the binding of other transcriptional factors including nuclear factor, NF1 and the octamer transcription factor, OTF1 to MMTV promoter (Beato *et al.*, 1995). Furthermore, it has been shown that GR is able to bind naked DNA as well as reconstituted chromatin with equal affinity. In contrast, NF1 binds efficiently only with free DNA suggesting a requirement for structural relaxation in the chromatin structure to enable efficient binding of NF1. Also, secondary modifications of histones in the chromatin, e.g., acetylation, methylation in response to GR binding may be involved in such a displacement/rearrangement process (Bresnick *et al.*, 1990). It has also been reported that the specificity of DNA-binding by GR increases as the DNA obtains a higher ordered structure. This, probably augments the ability of GR to discriminate between specific- and random-binding sites in the genome (Perlman, 1992). Overall, the chromatin structure influences gene activity in three

ways- (i) by enhancing receptor-factor(s) interaction to promote transcription, (ii) by preventing the binding of trans-acting factors that can inhibit gene transcription and (iii) by masking the unwanted genes by packing them into a heterochromatin.

Role of other proteins in GR-DNA interaction

Recently, several proteins have been identified which possibly mediates in the GR-dependent transcription process. A set of proteins, termed GRIP (glucocorticoid receptor interacting protein) have been isolated, these do not influence basal promoter activity but does enhance GR induction of target genes (Eggart *et al.*, 1995). Similar receptor interacting proteins for estrogen, termed ERAP and T_3, termed TRAP have also been reported (Beato and Sanchez-Pacheco, 1996). Another protein called steroid receptor coactivator-1 (SRC), which stimulates trans-activation of all the steroid receptors have, recently been characterized (Onate *et al.*, 1995). Protein components of the SN1/SNF complex in yeast and its human homologs are reported to potentiate GR-mediated transactivation process (Bamberger et., 1996)

The above mentioned factors and their many possible interactions with GR, somehow stimulate the transcription machinery leading to induction of several glucocorticoid responsive genes, e.g., mouse mammary tumor virus, human metallothionein II_A, chicken lysozyme, growth hormone, maloney murine sarcoma virus, rat tyrosine aminotransferase, rat tryptophan oxygenase, phosphoenol pyruvate carboxykinase and α_1-acid glycoprotein. However, glucocorticoids are also involved in negative regulation of some physiologically relevant genes, e.g., α-subunit of glycoprotein hormone, rat prolactin, α-fetoprotein, urokinase, collagen, stromylesin and pro-opiomelanocortin (Beato *et al.*, 1989; Lucas and Granner, 1992). Two components, relevant to negative regulation have received much attention lately- one is the presence of negative GREs and the other is the presence of certain GR-binding proteins which interfere with the transcription process. Evidence for the presence of negative GRE came from the work of Sakai *et al.* (1988). Genetic analysis of the negative GREs did not yield a clear cut consensus, however a 15 nucleotide long sequence analogous to +ve GRE have been proposed. This sequence, which is an imperfect copy of the +ve GRE has the following arrangement, 5´-ATYACNNNTNTGATCN-3´. It allows high affinity binding of the activated GR, but appears to force the GR into a conformation that exposes domains which silence transcription. These sequences are orientation-independent and serve as a negative enhancer, the binding of GR may either block or in some way alter the action of other transcription factors that function through an overlapping site. The other mechanism mentioned above probably, involves the components of non-steroid hormone action pathways interacting with the steroid action component(s), the so called "cross-talk"

in signal transduction. Of particular interest is the role of AP-1 and NF-κB in interfering with the transcriptional activity of genes regulated by GR. Interaction of GR with the components of AP-1 (Fos/Jun) leads to the formation of a complex unable to bind DNA, consequently down regulating target genes (Tsai and O'Malley, 1994). This interaction takes place even in the absence of the DBD of GR, suggesting a role for other domains in this process. Moreover, the induction of either of the AP-1 components, responsive to phorbolesters leads to repression of GR expression. Similarly, the induction of GR represses the expression of AP-1 components. This mutual repression requires the N-terminal sequences of GR and the basic leucine zipper region of Jun suggesting conformational change as the basis of altered activity. Similar interaction between p65, one of the transcriptionally active subunits of NF-κB (an important element of the inflammatory response) and GR have also been reported (Beato *et al.*, 1995).

The above observations clearly emphasize the complexity involved in the transcriptional regulation of genes by glucocorticoid receptors and also the persuasive role steroid hormones play in a vast number of physiologic and pathologic processes.

GR cycling

An interesting feature of the glucocorticoid signal transduction pathway is the nucleo-cytoplasmic cycling of GR. It was observed that the DNA bound hormone-receptor complexes get dislodged following gene modulation, the free receptors then migrate back to the cytoplasm where they are mostly recycled or sometimes degraded (Munck and Holbrook, 1988). Receptor recycling can also be gauged from the observation of the so-called `null' receptors, which are either newly translated ones or most likely, are those recycled from the nucleus. These null receptors need to be associated with the components of the protein folding system to attain a conformation suitable for steroid binding. This cycle is ATP-dependent and the involvement of phosphorylation/dephosphorylation steps cannot be ruled out (Mendel *et al.*, 1990; Bohen, 1995). Observations have suggested the presence of export signals similar to nuclear localization signals, that help in receptor recycling.

Regulation of glucocorticoid responsiveness

Tissue responsiveness to glucocorticoids is dependent upon a number of parameters: (i) the concentration of free hormone available to a cell, (ii) the intracellular receptor concentration and (iii) the concentration and availability various factors integral to the transduction of the hormonal signal. The regulation of plasma and tissue glucocorticoid concentration is achieved mainly by the interplay between hypothalamo-hypophyseal system and has been relatively well studied and

understood. This concentration is also influenced by the plasma and tissue levels of corticosteroid-binding globulin (CBG), which are themselves under complex regulatory control (Orth *et al.*, 1992; Dhabhar *et al.*, 1993). An interesting mechanism by which kidney cells in the distal tubules are rendered unresponsive to physiological concentrations of glucocorticoids, is achieved by the presence of high levels of 11β-hydroxysteroid dehydrogenase (11β-HSD). In these cells, the incoming glucocorticoids are rapidly metabolized by this enzyme, thus rendering the cells primarily mineralocorticoid responsive. The expression level and/or activity of this enzyme in different tissues may therefore, modulate glucocorticoid responsiveness (Funder *et al.*, 1988; Brown *et al.*, 1996). A transporter protein, recently reported by Kralli *et al.* (1995) that actively and specifically exports glucocorticoids can regulate intracellular levels of this steroid.

There is a tissue- and age-specific variation in the GR expression level, thymus being the tissue showing the highest number of receptors per cell (Miller *et al.*, 1990). A number of factors have been reported to cause such a change- glucocorticoids themselves appear to be the most potent regulator and has been shown to cause down-regulation of its receptor in many cell lines and tissues from animals and humans. This regulation is probably at the transcriptional level through the inhibition of GR mRNA generation, by interfering with the mediators of transcriptional activation of the GR gene, AP-1 and/or AP-2 (Burnstein and Cidlowski, 1992; Barrett *et al.*, 1996). Inhibition can also occur via binding of activated GR to sites within the coding DNA and/or mRNA, thus reducing mRNA stability and translatibility. Moreover, in the presence of glucocorticoids, the half-life of the GR protein has been reported to be reduced (Burnstein *et al.*, 1990; McIntyre and Samuels, 1985). The effects of estrogens, neurotransmitters and of heterozygous microdeletions in an exon-intron splice site in one GR allele, on GR expression add to the complexity of GR transcription and expression (Bamberger *et al.*, 1996).

The structural integrity of the HBD, and hence, the hormone-binding affinity also determines the potency of GR as a transcriptional regulator. The role of hsp90 in assembly, folding and maintenance of the GR HBD in a ligand-friendly, high-affinity conformation is vital. The intracellular levels of hsp90 and its structural integrity can influence GR function. In yeast low levels of hsp90 leads to impaired signal transduction and in the thymus, which is highly sensitive to glucocorticoids, hsp90 levels are also high (Hu *et al.*, 1994; Bohen, 1995).

In addition, numerous other endogenous factors have been identified that affect the various stages of this signal transduction process and any intra-/inter-individual variations in one or more of these factors can, therefore, have profound influence on

the GR activity. Many of these factors have already been discussed before and a few need a brief mention here to stress their possible importance in glucocorticoid responsiveness. A heat-stable stimulator protein and a inhibitory modulator, a 1500 Da phosphoglyceride that binds both the GR and hsp and stabilize the complex has been reported (Schmidt *et al.*, 1985; Bodine and Litwack, 1988). Also, variations in a tissue- and age-specific manner of the phosphorylation/dephosphorylation, nuclear translocation, DNA/GRE binding and many other factors associated with these processes can play an important role in regulating GR activity (Orti *et al.*, 1993; Hsu and DeFranco, 1995; Beato *et al.*, 1995). An important, physiologically relevant observation made recently shows the presence of two isoforms of the GR- GRα and GRβ that are generated by alternate splicing of the primary transcript. These two isoform proteins have the first 727 amino acids in common but differ only in the C-terminus with replacement of the last 50 amino acids by a unique 15 amino acid in GRβ. This difference renders the GRβ unable to bind hormone and is thus, transcriptionally inactive. The GRβ is located primarily in the nucleus and its over expression led to an antagonizing effect on the GRα activity, causing upto 90% reduction in reporter gene activity. The mechanism of this inhibitory effect is proposed to occur via occupation of GRE target sites by GRβ/GRβ homodimers or GRα/GRβ heterodimers. These block the binding of GRα/GRα homodimers, necessary for GR-mediated transcription (Hollenberg *et al.*, 1985; Encio and Detera-Wadleigh, 1991; Oakley *et al.*, 1996)

The thorough understanding of the mechanisms regulating glucocorticoid sensitivity in target tissues, necessitates the determination of relative amounts of all these factors and the regulatory processes, if any, which control their expression/production levels. Many pathophysiological states are potentially associated with tissue-specific and/or acquired glucocorticoid resistance or hypersensitivity (Bamberger *et al.*, 1996). Therefore, such studies will help in our understanding the actual mechanisms involved in glucocorticoid sensitivity of target tissues.

All the above observations aptly accentuate the importance of glucocorticoid receptor-mediated signal transduction process in the growth, development and in the maintenance of the overall homeostatic balance in higher organisms. Keeping in view the data generated over the years on GR structure and action mechanism, we decided to direct our attention to the study of physicochemical changes in the receptor during the postnatal development of male mice.

The work was divided into the following phases:

i) *Determination of age- and tissue-specific concentration of the GR at various postnatal ages (10- to 60-day), to see changes, if any, in the level of GR. Such changes could also be due to alterations in the receptor's affinity for the ligand. Therefore, scatchard analysis of the binding data was performed to see if there is any change in the dissociation constant, which might correlate to changes in the receptor level.*

ii) *The activation process of the receptor, by temperature and salt, at two ages (10- and 60-day) and in two different tissues (liver and kidney) were studied, using DNA-cellulose and purified nuclear binding assays, to assess the age- and tissue-related changes. Furthermore, nuclear-exchange assays were performed to determine the role of nucleus in GR binding. The effects of various inhibitors (molybdate, tungstate and N-ethylmaleimide) of the activation process were studied to observe the tissue- and age-specific sensitivity of GR to these inhibitors. Salt extraction of the nuclear bound hormone-receptor complexes was performed to see the strength of receptor-chromatin interaction at two ages. Moreover, DNase I digestion of the nuclear chromatin was done to ascertain the change in chromatin organization, which might have a significant role to play in tissue responsiveness to glucocorticoids.*

iii) *Finally, other properties of the unactivated and activated glucocorticoid receptor, e.g., molecular weight, stoke's radii and charge content were determined to see if there is any age-related change in these parameters. Also, the antigenic property of the unactivated receptor was assessed by immunoadsorption technique.*