National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

THE ROLE OF DNA-BINDING AND PHOSPHORYLATION ON NUCLEOCYTOPLASMIC TRAFFICKING OF THE GLUCOCORTICOID RECEPTOR

FAUSTINA N.A. SACKEY

Thesis submitted to the department of Biochemistry in partial fulfillment of the requirements for the degree of Doctor of philosophy

University of Ottawa
Ottawa, Ontario, Canada
January, 1995

ISBN 0-612-07861-2

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

To characterize the nuclear transfer defect in the mouse S49 lymphoma, I have used the Arg484-His and Tyr770-Asn single amino acid substituted glucocorticoid receptor (GR) mutants to examine the effects of four factors on nucleocytoplasmic (NC) transport (NCT): defective hormone binding, defective DNA binding, the GR antagonist RU486, and modulators of protein kinase and protein phosphatase activities. A quantitative indirect immunofluorescence assay was developed and used during G_0 to measure kinetics of nuclear import, cytoplasmic redistribution of nuclear receptors after hormone withdrawal and nuclear re-uptake of cytoplasmic redistributed receptors. I showed that the Tyr770-Asn GR mutant, defective in hormone binding, has no influence on nucleocytoplasmic transport. Kinetic studies with the Arg484-His and other DNA binding GR mutants demonstrated that the DNA binding function of the receptor is an important determinant for the persistence of GR in the nucleus following hormone treatment and withdrawal. I showed that detection of GR in the cytoplasm following hormone withdrawal occurs concomitantly with reformation of the 8-9S complex, a process we termed re-cycling. RU486, a potent GR antagonist (previously shown to block hyperphosphorylation of the receptor) potentiated apparent nuclear retention through an apparent block in receptor re-cycling. Further, this effect could be completely reproduced during withdrawal from agonist by treatment with protein kinase inhibitor, 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine (H-7). Conversely, phorbol ester treatment markedly enhanced the initial return of agonist treated nuclear receptors, but not RU486 treated GR, to the cytoplasm. These effects were independent of DNA binding of the receptor. This suggested that a 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-sensitive kinase activity is required for the re-cycling of GR following dissociation of hormone. In energy depletion experiments, using RU486- and H-7 treated cells, I showed that unliganded nuclear GR retained the ability to shuttle between nucleus and cytoplasm. Thus my results are consistent with re-incorporation of the receptor into an inactive 8-9S heat shock

proteins-complex (re-cycling), but not exit from the nucleus, being blocked by these two agents.

Kinetic measurements of loss of nuclear receptors to the cytoplasm after hormone withdrawal, using combined modulators such as forskolin/(TPA) or forskolin/okadaic acid (OA) demonstrated a cancellation effect on apparent nuclear retention of WT receptor. However, the Arg484-His GR mutant in both cases demonstrated either the effects of TPA alone or the forskolin alone respectively. These preliminary data provide evidence of different phosphorylation events playing an important role in DNA binding and subsequent transcriptional activity of GR.

DEDICATION

What shall I render to my God
for all His mercy's store ?
I'll take this gift He hath bestowed
And humbly ask for more.
(Charles Wesley, 1707-88)

To my sister, Felicia Sackey,
for all her love and encouragement
and whom by His grace
has lived to see this day.

ACKNOWLEDGMENTS

I wish to thank Dr. Y.A. Lefebvre, for her guidance throughout this project and to Dr. R.G.J. Haché who contributed much through discussions and critical evaluations.

I am also grateful to Dr. J. Bell for his help.

I would also like to thank the following professors for their help on specialized areas: Dr. Nuch Tanphaichitr, for her advice on Immunofluorescence, and Dr. George Wells, for his advice on Statistical Analysis.

I am thankful to Terry Reich (Rd Y.A Lefebvre's lab), Louis Pope (Dr. P. Walker's lab), Heather MacLeod (formerly in Dr. R.G.J Haché's lab), Ricardo Marius (Dr. J. Bell's lab), Diana Ferguson and Grazyna Ozorowska (Biomedical Communications, Civic Hospital), for their technical assistance.

Special thanks go to Dr. V. Mezl for the supporting role he played in helping me to secure admission into Graduate Studies.

I am grateful to the Ghana government for 4 yrs. of financial support, Dr. Y.A. Lefebvre, for providing additional 12 months of support, the LOEB Research Institute and Graduate School, for short term financial assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

NUCLEIC ACIDS/SPECIFIC SEQUENCES

DNA--Deoxyribonucleic Acid

rRNA--Ribosomal Ribonucleic Acid

tRNA--Transfer Ribonucleic Acid

mRNA--Messenger Ribonucleic Acid

ATP--Adenosine Triphosphate

ADP--Adenosine Diphosphate

GTP--Guanine Trinucleotide Phosphate

cAMP--Cyclic Adenosine Monophosphate

GRE--Glucocorticoid Response Element

BUFFERS

TE--Tris-EDTA

TEG--Tris-EDTA-Glucose

TEN--Tris-EDTA-NaCl

TN--Tris-Sodium Chloride

PBS--Phosphate Buffered Saline

TSB--Transformation and Storage Buffer

FUNCTIONAL PROTEINS/DOMAINS/PROTEIN COMPLEXES

SHR/THR--Steroid Hormone/ Thyroid Hormone Receptor

GR-- Glucocorticoid Receptor

ER--Estrogen Receptor

PR--Progesterone Receptor

AR--Androgen Receptor

MR--Mineralocorticoid Receptor

RAR--Retinoic Acid Receptor

WT--Wild Type Receptor

mWT--mouse Wild Type Receptor

hWT--human Wild Type Receptor

hsps--Heat Shock Proteins

PKA--Protein Kinase A

PP-1--Protein Phosphatase 1

PP2A--Protein Phosphatase 2A

mAbs--Monoclonal Antibodies

WGA--Wheat Germ Agglutinin

snRNP--Small Nuclear Ribonucleo-Protein

NLS--Nuclear Localization Signals

NE--Nuclear Envelope

NPC--Nuclear Pore Complex

PROPERTIES OF PROTEINS

SBC--Steroid Binding Capacity

GBC--Glucocorticoid Binding Capacity

CHEMICALS

$MgCl_2$--Magnesium Chloride

$MgSO_4$--Magnesium Sulphate

NaOH--Sodium Hydroxide

DMSO--Dimethyl Sulphoxide

NaCl--Sodium Chloride

HCl--Hydrocholic Acid

EDTA--Ethylene Diamine-Tetraacetic Acid

FITC--Fluorescein Isothiocyanate

BSA--Bovine Serum Albumin

CHD--Cycloheximide

ONPG--O–Nitrophenyl-β-D-Galacto-Pyranoside

X-gal--5-Bromo-4-chloro-3-Indolyl-β-D-Galactopyranoside

TPA--12-O-Tetradecanoyl-Phorbol-13-Acetate

FCS--Fetal Calf Serum

H-7--1-(5-Isoquinolinyl-sulphonyl)-2-Methylpiperazine

OA--Okadaic Acid

Dex--Dexamethasone

TECHNIQUES

SDS-PAGE--Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

HRSEM--High Resolution Scanning Electron Microscopy

TEM--Transmission Electron Microscopy

MEASUREMENTS

OD--Optical Density

rpm--Revolutions Per Min

DEFINITIONS

CACCC box:- Cytidine rich sequence found upstream of certain genes to which a factor binds to enhance the transcriptional activity. In the case of glucocorticoid inducible genes in which this sequence is found, it is located immediately upstream of a cluster of factor binding sequences which includes the glucocorticoid receptor binding site called the glucocorticoid response element (GRE).

GR activation:- The dissociation of hsps from GR which results in the conversion of the receptor from hormone binding-heat shock protein-heteromeric form (8-9S heterocomplex) to a non-hormone binding or DNA-binding form (occupied 4S receptor), usually accompanied by a change in conformation of the receptor (transformation).

GR inactivation:- The reverse process of activation, i.e. the conversion of the GR from non-steroid binding, free unliganded state to the GR-heat shock protein heterocomplex (8-9S) which is able to re-bind ligand.

GR desensitization after transactivation:- A mechanism involving dephosphorylation and dissociation of ligand from the receptor, and dissociation of protein-protein interactions with other transcriptional factors with which it interacts on the transcription machinery; two forms of receptors are generated as products in different phosphorylation states, the unliganded 4S receptor and the null receptor.

Null receptor:- A form of GR that differs from the unliganded 4S receptor in having only two out of the three phosphate content and remains tightly associated with the nuclear matrix.

GR recycling/cycling:- Nucleo-cytoplasmic turnover of the receptor.

Recycled GR:- Cytoplasmic GR observed after hormone withdrawal.

GR shuttling:- The constant and transient migration of receptors between nucleus and cytoplasm.

GR Re-cycling:- A process that includes re-incorporation of the 4S unliganded receptor into an inactive hsp-containing complex that is competent to re-bind ligand.

GR 'apparent nuclear occupancy/retention':- Receptor molecules that appear to be retained in the nucleus but may in fact be available for shuttling between nucleus and cytoplasm.

Trans-modulation:- Loss of the receptor signaling function due to a factor which brings about a reduction in the ligand binding response, often called uncoupling.

Down-regulation/trans-repression:- Regulatory mechanisms leading to a loss of receptor number. When two distinct proteins reciprocally oppose one anothers' activity through down regulation, it is referred to as trans-repression.

I. INTRODUCTION

A. Molecular Mechanisms of the Action of Glucocorticoids and Glucocorticoid Resistance

Glucocorticoids are an important component of chemotherapy regimens used in the treatment of hematopoietic malignancies including lymphomas, leukemias, thymomas and multiple myelomas[1-3]. The mechanism of glucocorticoid hormone action has been studied extensively in leukemias and lymphomas using both human and mouse cell lines as model systems[4-11]. Several studies have shown that the glucocorticoids mediate their effects through the glucocorticoid receptor (GR), with which glucocorticoids form complexes which translocate into the nucleus, with subsequent modulation of target gene transcription and finally, cytolysis of the cell[1-11].

The GR is a trans-acting sequence specific transcriptional regulatory factor, and serves as a prototypic member of a large super-family of ligand activated receptors referred to as steroid/thyroid hormone receptors (SHR/THR)[12-14]. This superfamily also include retinoic acid receptors (RAR), as well as other members whose ligands have not yet been determined (orphan receptors)[12]. The cloning of complementary DNA (cDNA) from human and mouse GR allowed the deduction of their primary structure, definition of their functional domains and the cloning of their genes, and thus permitted further studies on the molecular mechanisms of steroid resistance.

The primary structure of mouse GR protein with its functional domains is shown schematically in Figure 1[14]. The 783 amino acid protein has four main functional domains; 1) an N-terminal region,

which is variable in amino acid sequence among different steroid receptors (residues 1-426 in mouse GR). It was initially characterized by its property of providing antigenic epitopes for the generation of anti-receptor antibodies and has now been suggested to have a transactivation function; 2) a highly conserved DNA-binding domain of about sixty-five amino acid residues (residues 427-492 in mouse) characterized by two cysteine zinc finger motifs which are required for interaction with specific sequences on the DNA called the glucocorticoid response element (GRE); 3) a hinge region, (residues 493-537 in mouse) containing one of the two nuclear localization signals (NLS) which direct the accumulation of the receptor to the nucleus; and 4) a carboxy terminal hormone binding domain (residues 538-783 in mouse) which in the absence of ligand associates with heat shock proteins (hsps) 90, 59 and 70 in the form of a heterocomplex and appears to maintain the receptor in a cytoplasmic inactive state [15-20]. In this state of GR the NLSs are masked and it renders the receptor competent for hormone binding.

The cascade of events leading to the modulation of target gene transcription is as follows: The association of the cytoplasmic unliganded GR with hsps renders the receptor inactive with repression of its nuclear localization, dimerization, DNA-binding, and transcription regulatory activities[21]. Ligand binding to the receptor results in dissociation of the hsps, which results in a conformational change. Subsequently, there is a rapid and efficient translocation of the hormone-receptor complex to the nucleus[22,23]. Inside the nucleus, the ligand bound GR exerts its genomic effects on transcription in at least three potential ways. First, in the form of a homodimer, it interacts with a specific cis-acting

transcriptional regulatory sequence (GRE) in the promoter or enhancer regions of glucocorticoid responsive genes[13,24]. This interaction appears to stabilize the polymerase II initiation complex of regulated genes, perhaps by binding directly to the complex or through another protein ancillary factor, TFIIB[23], whose interaction with the partial initiation complex may be the rate-limiting step of transcription. 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. Examples of these are the CACCC box and the glucocorticoid response element together with their binding factors[25,26]. Although this mechanism has been studied mostly in enhancement systems where the receptor dimer interacts with "positive" GREs, glucocorticoids may also repress other genes through the same or different, "negative" GREs[27,28]. Second, negative regulation of transcription can result from interaction of the GR dimer with part of the responsive element of another transcription factor, which can be displaced or hindered from properly exerting its transcription promoting effect. An example is the inhibition by glucocorticoids of glycoprotein hormone α-subunit gene transcription induced by cAMP-responsive element binding protein (CREB)[29]. Third, another major molecular path through which glucocorticoids seem to exert part of their anti-growth 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 interaction of a c-*jun*/c-*fos* heterodimer with its DNA-responsive element, the AP1 site[30-32].

As mentioned above, some glucocorticoid-responsive cell lines undergo GR mediated cytolysis upon hormone treatment[1-6,33]. GR mutants have been isolated from various murine lymphoma (S49 lymphoma, P1798 lymphosarcoma and WEHI-7) cells lacking responsiveness to hormone induced cytolysis[34]. These receptor mutants have been generated through naturally occurring spontaneous events mainly from cells which were originally sensitive to the growth inhibitory effect of the hormone. The frequency by which these variants arise is in the order of 10^{-6} to 10^{-5}. The resulting mutant GRs have a variety of molecular defects such as point mutations or microdeletion of a highly conserved region of the GR genes with a subsequent alteration of either the level of expression of the receptor or its functional characteristics. This results in a significant fraction of thymocytes becoming resistant to the cytolytic effects of glucocorticoids.

Approximately 80-90 % of the resistant variants with altered receptor that have been examined thus far lack glucocorticoid binding activity altogether (receptorless, r^-). The remaining 10-20 % of the variants exhibit aberrant nuclear transfer properties (nuclear transfer decreased, nt^-; or nuclear transfer increase, nt^i). Resistant clones have also been isolated from murine S49 and human cultured T-cell acute lymphoblastic leukemia cell lines (CEM-1) termed 'deathless' in which the glucocorticoid resistance has no association with a receptor defect and has been suggested to have resulted from a post-receptor lesion in a steroid sensitive cell line[35,36].

In addition to the natural mutations, some studies have shown that steroid resistance could be drug induced. For example, in combination therapies, antitumor drugs such as mitomycin C, might

induce glucocorticoid resistance probably because of its alkylating properties. Alkylating agents have the property of intercalating nucleic acids in DNA[37,38], which may result in mutations in the expressed gene.

These functional differences of receptor variants account for the fact that while a positive correlation exists between GR concentration or status in normal and lymphoma/leukemic cells to predict clinical response in certain cases many patients with high levels of GR binding activity fail to respond to therapy[1-3]. Thus during diagnosis of lymphoid and breast cancers[39], steroid receptor binding activity is a necessity but in some cases not a sufficient index for the determination of steroid responsiveness.

B. Nucleocytoplasmic Exchange of Proteins

1. *Function of Components Involved in Nuclear Import of Proteins*

a) Structure and function of the nuclear envelope

Molecular exchanges between nucleus and cytoplasm occur continuously in eukaryotic cells by passage across the nuclear envelope (NE) through nuclear pores. The NE is a double membrane system that is continuous with the endoplasmic reticulum (EDR) and forms the boundary of the nucleus. It consists of three main components; an outer and inner nuclear membrane system perforated by nuclear pore complexes (NPCs), a perinuclear space between the two membranes called the lumen, and the nuclear lamina[40-45]. The outer nuclear membrane is continuous with the ER, thereby making the nuclear lumen

continuous with the EDR lumen. Nevertheless, it has been proposed that the NE is structurally and functionally distinct from the EDR[41,42].

The inner membrane of the NE is lined by a polymeric network of lamina, which remains associated with it following extraction with non-ionic detergents and salt[41,46-47]. It is composed of a network of intermediate filament-type proteins called lamins. Two classes of lamin proteins have been identified in most eukaryotic cells, lamin A and lamin B. The two classes have been defined by structural characteristics, and by biochemical properties such as differences in their primary sequence, and by their behavior at mitosis[48-50]. As is characteristic of all intermediate filament proteins, lamins display a central α-helical rod domain flanked by NH_2- and COOH-terminal non α-helical end domain. In addition, lamins contain nuclear localization signals and a CaaX box (C=cysteine, a= aliphatic amino acid, X= any amino acid). The CaaX box is subjected to three successive posttranslational modifications (farnesylation, proteolytic trimming and carboxyl methylation) which are required for association of newly synthesized lamins with the nuclear membrane. This nuclear lamina acts to support nuclear shape and has been suggested to serve as a rigid framework for chromatin attachment and organization. It has been proposed that the lamins play important roles by stabilizing the NE and serving as sites for attachment of chromatin to the NE. Of the two lamin species, B-type lamins are expressed constitutively in most embryos[49,52,53] and somatic cells[51,52], while expression of A-type lamins is highly regulated during development, suggesting that lamin status may be important for cell differentiation. Recently, interest in the lamins has increased due to their identification as a major cellular substrate of the universal cell cycle

protein kinase complex, $p34^{cdc2}$-kinase[55-57]. It has also been reported that guanine nucleotide binding proteins (G-proteins), a family of heteromeric GTP-binding membrane bound proteins, known to be involved in cell signaling are ~~an~~ integral components of the nuclear lamina. Further, there is evidence for a nuclear signaling system of the nuclear lamina involving phosphoinositol-4,5-bisphosphate (PIP2) metabolism[58]; see Section IV, 6a.

Embedded in the nuclear membranes, where the inner and the outer membranes merge are elaborate supramolecular structural assemblies called nuclear pore complexes (NPCs; Figure 2). The NPCs are large proteinaceous structures with mass of 1.25×10^8 daltons, and a roughly cylindrical shape of approximately 120 nm in diameter and 70 nm high[59-67]. Recent electronmicroscopy (EM) studies have revealed more about the basic architecture of the NPC[65-73]. The NPC has been described as a tripartite assembly built from a framework of symmetrically arranged proteins in views perpendicular to the NE. When nuclear envelopes are positioned in a 3-dimensional (3-D) map, part of the NPC assembly lies within the lumen of the NE. The 3D map shows that each of the 8-spokes seen in face views of the NPC is built from 4 morphological features termed annular, column, rings and lumenal subunits (Figures 2b and 2d)[65]. There are two copies of each subunit in a spoke. Within a single spoke there is an intricate network of connections between the subunits, giving an overall underlying structure conforming to 822 symmetry. The structural elements of the pore complex are arranged to form a large central channel with a functional diameter of 26 nm and eight peripheral channels, each with a diameter of 9-10 nm (Figure 2B). At the NPC periphery are radial arms involved in

anchoring of the NPC in the membrane[68]. It has recently been shown by high resolution scanning electronmicroscopy (HRSEM)[69-72] and transmission electron microscopy (TEM) preparations that there are basket-like structures protruding into the nucleoplasmic face of the NPC (Figure 2c)[73]. The filaments of the basket which usually number up to eight, are approximately 40-45 nm in length with a diameter of 10 nm, and are attached to the outer periphery of the column ring between two adjacent subunits. These filaments extend into the nucleoplasm, where each attaches a distal basket ring (Figure 2c). The diameter of the basket filament is consistent with that of the intermediate filament of the nuclear lamina[67]. In fact it has been shown that it is the filamentous structures of the lamina which form a regular basket weave pattern over the entire surface of interface nuclei. It has been suggested that the baskets could function as an initial excluder to prevent the NPC becoming clogged with materiai that is too large to be transported. Baskets could also prevent large ribonucleoprotein granules (e.g Balbiani ring granules) from entering the NPC until they have been transformed to rod shaped particles that can be transported through the NPC[73].

In contrast to the relatively large numbers of structural studies, less is known about the biochemical composition of the NPC. Only a small number of NPC proteins, probably accounting for less than 10 % of the NPC mass, have been identified and described to date[74,75]. The first polypeptide examined was gp210, a transmembrane glycoprotein bearing N-linked (via Asn) high mannose type oligosaccharides[76-80]. This protein consists of a large NH_2-terminal domain located in the NE lumen, a single, 21-amino acid long transmembrane segment, and a short, 58-residue-long COOH-terminal domain which associates with NPC. This

topology has led to speculation that gp210 may act as a membrane anchor for the NPC and/or play a topogenic role in membrane folding during nuclear pore formation. Other NPC proteins termed nucleoporins have also been identified[81-85]. These proteins are modified with O-linked N-acetyl glucosamine residues and appear to be involved in nucleocytoplasmic transport as it is inhibited by both mouse anti-nucleosporin monoclonal antibodies (mAbs) to these glycoproteins as well as the lectin wheat germ agglutinin (WGA). Furthermore, a complex consisting of at least three of these glycoproteins (p62, p58, and p54) is required for NPC function, as depletion of NPCs of this complex renders them unable to mediate protein import into the nucleus. Molecular cloning and sequencing of several of these NPC proteins in yeast revealed highly repeated motifs which led to the division of these proteins into two groups: a) those which contain several copies of a more or less degenerate pentapeptide motif, **XFXFG** (NSP1, NUP1 and NUP2) and those which contain a degenerate tetrapeptide motif, **GLFG** (NUP49, NUP100 and NUP146)[83-87]. These frequently observed repeated motifs in the different NPC proteins so far sequenced suggest that they may be assembled in a modular fashion which could account for their mutual interaction. Another NPC protein, named NUP153, has been identified in rat and has been shown to contain four zinc fingers (Cys_2-Cys_2) which bind DNA and contribute a terminal ring of the nuclear baskets[88].

The NPC has been suggested to act both as a regulated gate and to physically propel molecules into the nucleus, based on the fact that it mediates both passive and active transport across the NE. Recent EM studies by Richardson et al. have led to the description of a model illustrated in Figure 2, which may account for passive and active nuclear

transport[89]. Proteins as large as 60 kDa are thought to diffuse through the nuclear pore of ~ 9-10 nm passively into the nucleus and be retained as a result of association with DNA or other nuclear components[90]. The diffusion of small molecules which occurs through the peripheral channel of the pore complex, is at a rate inversely proportional to their mass[90]. Dextrans with a molecular weight of less than 20 kDa diffuse very rapidly through the nuclear pores, those of 40 kDa more slowly, and those of 70 kDa, not at all. Thus, proteins and ribonucleoprotein (RNP) particles that are larger than 60 kDa must be actively transported into the nucleus. This has been shown to be at least a two-step process: i) an energy independent accumulation of an import substrate at the nuclear periphery followed by ii) an energy and temperature dependent translocation across the central channel of the NPC. The energy independent step which is as yet poorly characterized requires functional targeting sequences within the protein that allow recognition by the nuclear pore transport complex, or if not the protein must be bound to another protein that does[91,92]. Such signals have been identified for a number of proteins; (see next section). The requirement of energy has been demonstrated in cell free systems following depletion of ATP with apyrase or with deoxyglucose and sodium azide in cultured cells[89]. In the absence of ATP, or in the cold, import substrates accumulate at the nuclear periphery without being transacted into the nucleus. The addition of wheat germ agglutinin (WGA) has a similar effect, blocking translocation through the pore but not the binding of an import substrate to the cytoplasmic surface of the NPC[93-95].

It has recently been reported that introduction of a monoclonal anti-gp210 antibody into the perinuclear space, interfered with both

active and passive transport processes. These results raised the possibility that pore function might be sensitive to regulatory mechanisms (e.g. changes in Ca^{2+} concentrations) operating via the lumen of the EDR[96], since that is where gp210 is located.

The NE has been shown to be a dynamic structure that undergoes dramatic changes during mitosis when it is reversibly disassembled (i.e. disassembles at the onset of mitosis and reassembles at the end of mitosis)[57,97-102]. A number of experimental systems have been developed to investigate the mechanisms and to identify the components involved in the reversible NE disassembly. Assembly involves the attachment of vesicles to the chromatin followed by the fusion of these vesicles to produce the double membrane system. With the use of demembranated *Xenopus* sperm, chromatin and an extract from *Xenopus* eggs, it is possible to reconstruct these events *in vitro*.[57,97-102-113]. The general distribution of proteins and enzyme activities characteristic of the EDR[107,108] indicates that the vesicle subpopulations that contribute to NE assembly are distinct from the majority of EDR derived vesicles. Therefore, although the mechanism of vesicle fusion in the two systems may be similar, the NE is assembled from the subset of ER derived vesicles.

Studies on the mechanisms of nuclear envelope assembly and disassembly, as indicated earlier, have provided evidence for the requirement of GTP and ATP, the identification of G-proteins in NEs, and the requirement of their GTPase activity in nuclear membranes. Low molecular weight G-proteins have been implicated in regulating processes of membrane vesicle fusion. Other G-proteins of higher molecular weight have also been identified which may be involved in

nucleocytoplasmic transport by playing a direct role in processes of nuclear protein association with the NE and/or they may be involved in nuclear membrane signaling system, see Section IV.6b.

b) Structural features of nuclear localization signal sequences

Nuclear localization signal sequences (NLSs) have been identified for a number of nuclear destined proteins[116]. A protein may have a single NLS (as in SV40 large T)[116-121], dual signals (as in MATα2)[122], multiple signals in a single polypeptide (as in *Fos* protein)[123], or multiple signals in a multimeric protein (as in nucleoplasmin)[91,116,124-126]. Dual and multiple signals can be distinct and interdependent or they can be functionally equivalent. No strict consensus sequence for NLS has been determined and it is apparent that strict maintenance of an exact primary structure is not required for NLS function. However, many of them share common features that include clustering of basic residues and the presence of an α-helix breaker[116]. NLS containing proteins are suggested to interact with an ATP dependent transporter assembly situated in the central channel of the pore complex prior to nuclear entry (Figure 2b)[116].

Nuclear import and tight nuclear association of ligand bound steroid receptors have been intensively studied. Picard and Yamamoto have examined the subcellular distribution of deletion mutants of rat GR in order to identify the nuclear localization determinants of the protein[128]. The authors showed that the rat GR contains two NLSs that are involved in the nuclear targeting. One is located in the hinge region (NL-1), which has been mapped between residues 497-525, corresponding to residues 485-513 in mouse. The other signal (NL-2) is

within the hormone binding domain, residues 540-795 corresponding to 538-783 in mouse. In the WT GR both signals depend on hormone for function. However, NL-1 alone can direct nuclear accumulation in the absence of hormone when attached to a non nuclear protein. GR therefore constitutes an example of a protein with masked NLS that is unmasked or made accessible upon hormone binding. Within NL-1 there is a specific basic residue rich targeting sequence (TKKKIKG) that is homologous to the minimal sequence (PKK^{128}KKRKVE) of the extensively studied NLS of SV40 large T-antigen[117-119]. This sequence has been shown to be sufficient to target *E. coli* β-galactosidase, an otherwise cytoplasmic protein to the nucleus. A single missense mutation of Lys^{128}-Thr or Asn in the SV40 large T-antigen targeting sequence completely abolishes the ability of the encoded protein to direct nuclear accumulation[118,120]. The NL-1 sequence resembles a bipartite signal, i.e., it consists of two clusters of basic residues separated by ten amino acids, YRKCLQAGMNLEARKTKKKIKGIQQATA. Sequences flanking the core NLS have been shown to impair but do not abolish the ability of the large T-antigen to accumulate in the nucleus[117].

Nuclear localization signals have also been mapped for the other SHR/THR. Guiochon-Mantel et al. have studied the signals responsible for the localization of rabbit progesterone receptor (PR), using a series of deletion mutants[129]. Its main NLS is a stretch of amino acids located in the hinge region around position 638-642 and bearing similarities to the NLS present in the SV40 large T-antigen, (RKFKKFNK). This sequence is completely conserved between different species of PR. If other steroid hormone receptors, including GR, androgen receptor (AR) and mineralocorticoid receptor (MR,) are aligned through their DNA binding

regions, homologous sequences are found at exactly the same positions (10 amino acids after the last conserved cysteine, except in the case of the ER, where a homologous sequence is found eleven amino acids after the last conserved cysteine[129-139]. This hinge NLS in PR has been shown to be constitutively active, when deleted it causes the receptor to become cytoplasmic but PR could be shifted into the nucleus in the presence of hormone with a retention of biological activity. This implies that in the PR, the initial nuclear localization is not a prerequisite of biological activity. It also demonstrates that there is a second NLS in PR. This second NLS has now been shown to be located in the second zinc finger of the DNA binding domain (residues 454-486 in cPR) and it is activated either through the binding of hormone or by the deletion of the steroid binding domain which generates a constitutively active receptor[129,130]. The mechanisms that unmask or mask this second NLS are identical to those regulating the activity of the DNA binding function[129]. A much weaker and hormone inducible NLS has also been shown to be present in the HBD[130] of PR. This may function similarly to NL-2 of GR.

In the case of the human ER, a 48 amino acid fragment located in the hinge region (residues 256-303) mediates efficient nuclear localization of an ER β-galactosidase fusion protein[130]. This portion of the receptor includes three basic stretches (referred to as proto-signals): amino acids 256-260, 266-271 and 299-303. The first two basic stretches are almost perfectly conserved between the different species that have been sequenced. The third stretch is not well conserved. None of these basic stretches, could suffice on its own as a NLS. Like GR and PR, another hormone inducible NLS is also present in the HBD[130].

Cooperative effects of all three basic stetches and HBD NLS is required for efficient nuclear localization.

In the absence of ligand the AR is found predominantly in the perinuclear region of androgen containing cells[131]. However, upon androgen stimulus, the receptor is rendered nuclear. The amino acid sequence requirements for androgen dependent AR nuclear import have been determined by immunostaining transiently expressed full length WT and mutant human ARs in COS-7 cells and in transcriptional assays[133]. The sequence required for predominant nuclear localization is between residues 580-661 which comprise the second zinc finger region of the DNA binding domain, a 17-amino acid putative targeting sequence, and 28 residues of flanking carboxyl-terminal sequence[133,134]. Mutagenesis studies revealed a bipartite nuclear targeting sequence between the DNA binding and hinge regions mapped to amino acid residues 613-633. Deletion of this region resulted in loss of nuclear transport and transcriptional activity in the presence of androgen. However, the hinge region sequence contributes to complete nuclear localization.

As mentioned above, the SV40-like NLS homologies have also been found within the amino acid sequence of other members of the steroid receptor superfamily, including the MR[134], *c-erb*A/thyroid hormone receptors (THRs)[135,136], retinoic acid receptor (RAR)[138] and vitamin D receptor (VDR)[139]. However, a newly discovered member of the steroid receptor superfamily, the chicken ovalbumin upstream promoter transcription factor (COUP-TF) lacks any homology with the signal in the hinge region[140]. Unlike most steroid receptors, COUP-TF does not exceed the diffusion limit of the NPC. Therefore either COUP-TF may not require a NLS or its NLS may have an entirely different sequence.

In striking contrast to predominantly cytoplasmic localization of the unliganded GR and perinuclear localization of AR in the absence of their respective ligands, there is ample immunocytochemical and biochemical evidence that the ER and PR are nuclear, independent of hormone status[114]. This concept is also supported by some studies on the PR[115,129] suggesting that in these receptors probably the constitutive NLSs are not masked in the context of the receptors.

c) Nuclear localization signal binding proteins and cytosolic factors involved in NPC-mediated transport

It has been presumed that NLSs are necessary for nuclear import because they interact with nuclear localization signal binding proteins (NBPs). NBPs have been suggested to be adaptor molecules that bind individual signals and bring them to a common receptor at the nuclear pore complex[142]. It is known that colloidal gold particles coated with different nuclear proteins such as SV40 large T and nucleoplasmin, are imported through the same pores, suggesting that the same NBPs may mediate interaction of a group of NLS-containing proteins with the nuclear pore complex (NPC). However, the lack of a consensus NLS may suggest that there are structurally and functionally distinct NBPs and that each type of NBP recognizes a different group of ligands thereby functioning analogously to amino acid transporters[144].

Several groups have identified NBPs by a variety of approaches such as chemical cross-linking or affinity labeling of a synthetic NLS peptide to cell subfractions[141-153]. For example, LaCasse and Lefebvre have identified nuclear and cytosolic proteins of 66 kDa and 70 kDa from rat liver and mouse S49 lymphoma cells which bind NLS in the hinge

region of the GR (NL-1) and THR[145]. Other investigators have also identified NBPs from yeast and viral sources, as nuclear associated polypeptides that recognize NLS. Among these proteins two of 56 kDa and 66 kDa have been found consistently[143]. The NBPs exhibit functional properties of NLS receptors, i.e. they show saturation kinetics, and are competitively inhibited with an excess of homologous synthetic unlabelled signal peptide but not by mutant peptide, thus indicating that the binding is specific. This has led to a suggestion that NBPs may themselves serve as receptors for NLS-containing proteins[92]. Surprisingly, rather than being NPC-associated these proteins are found predominantly in the cytoplasm and less on the NE and nucleoplasm, suggesting that they may function as 'shuttling carriers' and be recycled for several rounds of transport[143,152].

Recent reports have also implicated other cytosolic factors in nuclear protein import. Cell free or digitonin permeabilized cell systems that reproduce transport *in vitro* have directly demonstrated the requirement for cytoplasmic factors in nuclear protein transport[153-157]. These include a component that interacts with O-linked glycoproteins of the NPC[152,155], the ubiquitous cellular hsp 70 cognate factor (hsc70)[155]. The process of nuclear import can be inhibited when hsc70 is depleted from cytosol used in permeabilised cell assay with ATP-agarose, or with bacterially expressed hsp70 and hsc70 detected after fractionation of cytosol. In another study, Imamoto et al. isolated a 69 kDa protein by nucleoplasmin NLS affinity chromatography, which was identified by protein sequence analysis to be hsc70[158,159]. Two hsp70 cognate proteins have been shown to shuttle between the nucleus and cytoplasm of *Xenopus* oocytes[160,162], and hsp70/hcp70 bind to NLS of

karyophilic proteins and NLS-peptides and subsequently co-localize to the nucleus with these karyophilic proteins, suggesting a possible transport function for these proteins.

Studies with the *in vitro* digitonin cell permeabilised system supplemented with exogenous cytosol have been used to investigate the possible involvement of GTPases in nuclear protein import. This system was coupled to an ELISA based assay to allow rapid quantitative analysis[163]. These studies demonstrated that non-hydrolyzable analogues of GTP such as guanosine-5'-O-3-thiotriphosphate (GTPγS) rapidly inhibit the rate of nuclear import. This inhibition was found to be dependent on the concentration in permeabilised cells and cytosol, and was strongly enhanced by cytosolic factors[164]. Since non-hydrolyzable analogues of GTP are often potent inhibitors of GTPases, this suggested that GTPases may play an important role in nuclear protein import. Furthermore, two groups have reported the involvement of Ran/TC4, a member of the *Ras* superfamily of GTP-binding proteins with GTPase activity in protein transport[144,163]. This protein together with its guanine nucleotide release protein, RCCI, which catalyses the release of GDP were found to be highly concentrated on the nuclear envelope using photocross-linking approaches[158]. These two proteins have been previously implicated in a diverse range of cellular functions, including DNA replication, cell cycle feedback control, and RNA processing and export. Thus these recent studies suggest that Ran/TC4 may serve to integrate nucleocytoplasmic trafficking with these other nuclear functions.

These advances on NLS, NLS-receptors and identification of other cytosolic factors have led to a suggestion that the NPC-mediated

transport pathway of proteins into the nucleus occurs by several distinct steps, some of them still being hypothetical[166-168]; a) In the cytoplasm the protein to be imported is complexed to a cytosolic receptor (the NLS-binding protein) via a specific NLS[142,147,155,169]; b) depending on additional cytosolic factors, this protein receptor complex then docks at the NPC by specific binding to some 'peripheral' NPC structure such as the cytoplasmic ring or the cytoplasmic filaments[89,171,172]; c) from this peripheral docking site, the protein receptor complex is next delivered to a central channel complex which harbors the actual transport machine; d) active translocation of the protein-receptor complex through the central channel complex occurs after channel gating to accommodate a particular size and shape of the protein receptor-complex; e) after release into the nucleus, the protein-receptor complex dissociates, and the receptor may be recycled for a further round of transport[147,155]. In this multi-step model of protein import several issues such as the site(s) and mechanism(s) of ATP utilization remain elusive[89,167,170].

2. *Export and Shuttling of Nuclear Targeted Proteins*

Unlike the mechanisms of nuclear protein import that have been studied extensively, very little is known about the export of macromolecules from the nucleus. Transport of RNAs and RNPs from the nucleus is poorly understood and very little information is available on signals that control export and recycling of steroid hormone receptors and other nuclear proteins. However, RNA efflux is mediated by NPC[173]. In certain ways, RNA export shares characteristics of the protein import pathway. WGA blocks export of many of the RNA species, thereby suggesting a role of O-linked N-acetyl nucleoporins in this process. It

has been possible to observe the movement of large mRNP complexes such as the Balbiani ring granules of *Chirorimous* exiting from the nuclear pores[173]. In addition, RNA coupled to colloidal gold particles and injected into *Xenopus* oocyte germinal vesicles was found associated with the NPC during its efflux[148]. Electron microscopy studies have indicated that some RNAs are transported with associated proteins: specifically, 5S rRNA first associates with the La antigen during its export and requires either interaction with transcription factor IIIA or ribosomal protein L5[174]. The influenza virus protein, M1, has been reported to be necessary for nuclear export of viral RNA-protein complexes as well as preventing nuclear re-import of the RNPs. The export of tRNAs is saturable and possibly mediated by a common carrier and is dependent on the structure of the D and T stem loop structures of tRNAs[175]. Ribosomal subunits as well as mRNA are transported to the cytoplasm by a facilitated and energy-dependent mechanism[176,177]. Until recently the only signal that has been identified as being positively required for nuclear export is the 5'monomethyl cap structure[178], present at the 5'end of polymerase II transcribed RNAs (mRNAs and snRNAs). The U1 snRNA molecule has an m^7GpppG cap at its 5' end that is also required for its export[178]. These results imply the existence of a nuclear cap-binding activity that facilitates mRNA export, and candidate proteins (cap-binding proteins) for such a function have recently been identified[179,180].

It was earlier demonstrated in nuclear transplantation experiments in amoeba that some proteins could migrate constantly (shuttle) back and forth between the nucleus and cytoplasm[181]. These experiments could not be extended to higher organisms until recent studies based on

micro-injection into *Xenopus* oocyte nuclei or the establishment of the heterokaryon assay which led to the identification of shuttling proteins in vertebrates such as the major nucleolar proteins (nucleolin and NO38)[182,183] and several other proteins (e.g. members of the 70 kDa family of hsps[162], the heterogeneous nuclear RNA-packaging protein, A1[184], and the progesterone and glucocorticoid receptors[185,186]). Studies on nucleocytoplasmic transport of shuttling proteins have become of considerable interest, because it has been postulated that these proteins might contribute to coordinating nuclear and cytoplasmic activities, or some might function as carriers in nucleocytoplasmic transport processes.

At present, investigators are seeking to determine the mechanisms by which shuttling proteins are exported from the nucleus. As very little information is currently available on this subject, two fundamentally distinct mechanisms have been proposed as models for nuclear protein export[187]. According to one model, shuttling proteins might contain export signals acting positively to direct such proteins to the cytoplasm. The second model proposes nuclear export to be a default process, with non-shuttling proteins prevented from leaving the nucleus by nuclear retention as a result of tight binding to intranuclear structures or the formation of large complexes[187]. In order to distinguish between these alternatives, one recent study based on assays of protein transfer between interspecies nuclei of heterokaryons and microinjection into *Xenopus* oocytes sought to determine the nature of sequence elements required for the export of shuttling proteins. HeLa cells were transiently transfected with cDNAs encoding the WT or deletion mutant forms of chicken nucleolin and then fused to NIH 3T3 mouse fibroblasts to study

their export rate, as they migrate from the HeLa cells into the mouse fibroblasts. This study yielded interesting results[187]; it demonstrated that none of the domains of nucleolin was essential for export and shuttling, and reached the conclusion that there are no specific export signals, instead, it was suggested that export is the default pathway. However, a recent report by Guiochon-Mantel et al. based on studies involving the role of NLSs of the PR and SV40 large T-antigen fusion β-galactosidase protein on nuclear export demonstrated that the addition of the NLSs imparted to the β-galactosidase protein the ability to shuttle between the nucleus and the cytoplasm[189]. Since micro-injected protein devoid of a NLS were unable to exit from the nucleus, it was concluded that the same NLSs are involved in both the inward and outward movement of proteins through the nuclear membrane. Thus these observations which have been the subject of a recent controversy suggested that a protein needs a specific signal to be able to exit from the nucleus. The exact portion of the NLS existing as the nuclear export signal, operating during nucleocytoplasmic shuttling, has not directly been characterized. However, these investigators also indicated that preliminary data from their laboratory have shown that by using deletion mutants of the PR-NLS a close correlation existed between the nuclear localization of the various mutants and their ability to shuttle in micro-injection experiments and heterokaryon assays. One could argue from these findings that the different systems used in these experiments may account for the differences in the results obtained, or export requirements may be different for different nuclear proteins.

An important question is whether the export of proteins from the nucleus is due to passive diffusion through nuclear pores or whether it is

an energy requiring process. Guiochon-Mantel et al. earlier reported that the shuttling progesterone and estrogen receptors still escaped from the nucleus in the presence of metabolic inhibitors, suggesting a diffusion mechanism[186]. In contrast Schmidt-Zacchmann et al.[187], reported the ability of cytoplasmic pyruvate kinase with or without tagged NLS injected into *Xenopus* oocyte nuclei to be retained at 4 ^{o}C, suggesting that it does not escape by diffusion. Until recently, the role of energy in the mechanism of export has been difficult to resolve. Attempts to look at the effect of temperature on U1A export were reported by Kambach and Mattaj to yield inconsistent results[188]. Using energy synthesis inhibitors the recent report by Guiochon-Mantel et al. also demonstrated that there was a rapid efflux of these fusion proteins from the nucleus when cells were depleted of energy[189]. Thus this observation supports a previous report from the same laboratory that protein efflux from the nucleus is not energy dependent[186].

C. Phosphorylation and Nucleocytoplasmic Trafficking of Nuclear Targeted Proteins

1. *Phosphorylation and Nucleocytoplasmic Distribution of Transcription Factors*

Nucleocytoplasmic transport is an active process that serves as a major control point for the action of many proteins that require entry and maintenance in the nucleus to exert their biological function. Many transcription factors accumulate in the cytoplasm in an inactive state in a complex with their anchoring proteins but are poised to rapidly translocate to the nucleus in response to external stimuli or cell cycle

signals[190-209]. For example, the NLS of *rel*-like[199,204,205] and myogenic[207] factors are masked when complexed with their anchoring proteins. Activation is mediated through a phosphorylation cascade that promotes complex dissociation and leads to a rapid relocation of the transcription factor component to the nucleus. Subsequently, the action of phosphatases may be required to promote re-incorporation of the factors into the inactive cytoplasmic complex to await a subsequent stimulus.

The rate of nuclear translocation of fluorescently labeled WT and mutated SV40 large T antigen proteins, as measured by Laser microfluorometry has been shown to be dependent on the phosphorylation of two serines near the NLS phosphorylated by casein kinase II (CK-II) and cell cycle regulated $p34^{CDC2}$ kinase[191,192]. Similar adjacent NLS and CK-II phosphorylation site motifs have been found in other nuclear proteins and although the molecular basis of the enhanced translocation is obscure these observations raise the possibility that nuclear translocation might be regulated by variations in specific kinase activities[193-199]. It is notable that other factors which play roles in growth regulation (e.g. p53) have NLS adjacent to $p34^{CDC-2}$ kinase consensus sites. However, no physiological relevance has been assigned to some of these observations.

Phosphorylation has also been proposed to stimulate nuclear translocation of the *c-rel* protein[193]. A consensus site for protein kinase A (PKA) enzyme, **RRPS***, is found near the NLS in this protein and several members of the *rel* family. Insertion of two amino acids into the PKA site led to the shift of *c-Rel* from the cytoplasm to the nucleus. Changing the serine residue to an alanine (a non phosphorylatable serine

analog), had no effect on localization properties, i.e., the protein remained cytoplasmic. However, mutating the serine residue to aspartic acid or glutamic acid (both negatively charged amino acids, thought to mimic a phosphorylated serine) resulted in a diffused distribution of the protein in both nuclear and cytoplasmic compartments, suggesting that nuclear transport requires activation of this site[193]. Therefore it appears the subcellular localization of the *rel* family of transcription factors is controlled by phosphorylation of subunits in the protein and/or in some cases of their cytoplasmic anchorage proteins.

The nuclear localization of at least two other proteins may be regulated by PKA. The transcription factor NFIL-6 has been shown to bind to the serum response element of the *c-fos* gene in response to forskolin treatment; this inducible binding correlates with an increase in phosphorylation and nuclear localization[194]. In addition, nuclear accumulation of c-*fos* requires continual stimulation by growth factors in a cAMP-dependent manner. In neither case however, has it been shown that the transcription factors are directly phosphorylated by PKA[195]. Finally, it has been recently shown that while *c-Jun* is transported to the nucleus in a cell-cycle dependent manner, translocation of the viral *v-Jun* protein is most rapid during G_2[196]. This difference has been mapped to a cysteine to serine mutation in the *v-Jun* NLS. A small sequence which includes these amino acids is sufficient to confer cell-cycle-dependent nuclear entry upon a heterologous protein, although no evidence for differential phosphorylation is yet available.

By contrast to the stimulatory effect that phosphorylation has on the nuclear uptake of the above proteins, certain protein factors such as SWI5 and Rb are rendered nuclear in their underphosphorylated forms.

Phosphorylation by $p34^{CDC-2}$ kinase has been shown to play a role in the regulation of subcellular localization of *Saccharomyces cerevisiae* SWI5 gene product, which is required for G_1 specific transcriptional regulation of the HO site-specific endonuclease [197,198]. The SWI5 protein is produced throughout the cell-cycle, but remains in the cytoplasm until anaphase, whereupon it is translocated into the nucleus in an underphosphorylated form, as cells enter G_1 [198]. The SWI5 NLS was identified by deletion analysis to be a fifty amino acid region located between residues 624-659 [198]. Within and near the NLS are three serine residues (Ser552, Ser646 and Ser664) that are phosphorylated *in vivo* in a cell cycle dependent manner, with the phosphorylation state correlating with cytoplasmic localization. All three serine residues are phosphorylated *in vivo* by $p34^{CDC-2}$ kinase homologue of S. *cerevisiae*, CDC-28 dependent H1 kinase. Therefore, unlike the stimulatory effect of casein kinase II on SV40 T antigen nuclear translocation, phosphorylation of SWI5 by CDC-28 inhibits uptake.

Another example of a protein whose subcellular distribution is regulated by phosphorylation is the retinoblastoma tumor suppressor gene product (Rb protein). This is a nuclear phosphoprotein involved in the regulation of the cell cycle by restricting cell progression from G_1 to S phase [199-203]. In the G_1 phase, where control of cell progression often occurs, the underphosphorylated form of Rb protein is tightly associated with a particular nuclear locale. However, as cells enter S-phase the protein becomes hyperphosphorylated and cytoplasmic [200]. This has led to a suggestion that the binding of Rb protein to nuclei is determined by a cell cycle regulated phosphorylation [197-199].

Whereas the subcellular localization of many of the transcription factors discussed above is influenced by direct modification, the same result may be achieved by modulating the activities of associated factors. A clear example is offered by the NF-κB transcription factor, first identified as a DNA-binding protein expressed constitutively in mature B-cells[204,205]. The activity of this protein was subsequently discovered to be inducible in many cell types by a large variety of stimuli (e.g. phorbol esters, interleukin-1, double stranded RNA and cAMP). The subcellular distribution studies of the transcription factor, NF-κB, have indicated that this nuclear targeting protein shows graded and regulated distribution between the nucleus and cytoplasm that is determined by phosphorylation[204]. The cytoplasmic form of NF-κB is associated with an inhibitory protein, IκB, that results in the formation of a complex that is unable to bind DNA. Phosphorylation disrupts the IκB-NF-κB complex with the release and entry of NF-κB into the nucleus.

The regulatory subunit of cAMP-dependent protein kinase type II (PKA) is thought to anchor the catalytic subunit to intracellular membranes. Binding of cAMP to the regulatory subunit causes release of the catalytic subunit, which is then transported into the nucleus[206]. This mechanism appears to be similar to that of NF-κB, although whether protein phosphorylation is involved or not remains to be elucidated.

MyoD is a nuclear phosphoprotein that belongs to a family of myogenic regulatory factors and acts in the transcription activation of muscle-specific genes. It has been demonstrated that its localization to the nucleus is regulated by PKA[207]. Purified MyoD protein microinjected into the cytoplasm of rat embryo fibroblasts is rapidly translocated into

the nucleus. Inhibition of PKA activity by injection of specific inhibitory peptide, PKI, prevents the nuclear localization. Site-directed mutagenesis studies of all the serine residues of the putative PKA sites to alanine had no effect on nuclear import of the protein. This excludes the possibility of direct involvement of PKA in nuclear translocation of MyoD, which leads to a suggestion that the nuclear import of MyoD may be mediated by phosphorylation of other components involved in nuclear import by PKA.

Cytoplasmic localization of a transcription factor by association with a cytoplasmic anchor has been well documented for GR[17,208-210]. The dissociation of hsps from unliganded GR during hormone induced activation leads to exposure of NLS of the receptor and increases the phosphorylation state of the receptor. Although a direct link has never been made between nuclear import and phosphorylation of GR, dissociation of hsps from the receptor is associated with a subsequent nuclear localization.

2. *Phosphorylation of Steroid Receptors and Functional Implications*

Protein phosphorylation is an important regulatory mechanism in a variety of physiological processes. The coupling of phosphorylation cascades with other signaling pathways can coordinate processes that occur within distinct cellular compartments, in combination increase regulatory capacity or provide a mechanism for cell- and tissue- specific response without the need to generate entirely new signaling pathways. Recent evidence has begun to link steroid action with other signal transduction pathways.

Results of several investigations suggest that the steroid/thyroid hormone receptors (SHR/THR) are hypophosphorylated in the absence of hormone and become hyperphosphorylated in the presence of hormone[211-218]. However, the functional significance of increased receptor phosphorylation has not been defined. Possible roles that have been suggested for receptor phosphorylation /dephosphorylation include increase in steroid binding capacity (SBC)[213,214] nuclear uptake[215], transactivation[216], desensitization[217] and receptor recycling[219]. For example, studies with intact mouse thymoma cells and fibroblasts have demonstrated that ATP depletion by exposure to 2,4- dinitrophenol (2,4-DNP) resulted in the loss of glucocorticoid binding capacity (GBC) of GR that is reversed by restoration of ATP levels, even in the presence of protein synthesis inhibitors[213,214]. In addition, a transition metal oxyanion such as molybdate represses both the temperature dependent inactivation of steroid bound receptors and the ligand mediated receptor activation[222,224]. The repression of activation may possibly occur by the prevention of hyperphosphorylation of the receptor molecules by impairing their dissociation from the inactive hetero-oligomeric complex. In the steroid free state, GR has on average 2-3 moles of phosphate per mole of receptor. The phosphate to receptor molar ratio increases to 5 upon glucocorticoid mediated GR activation but not with the glucocorticoid antagonist RU486[214,217]. The main sites of GR phosphorylation, as determined by proteolytic cleavage studies using ^{32}P-orthophosphate are in the N-terminal domain[222]. Phosphorylation sites in the steroid binding domain have also been identified[224,225]. Phosphoamino acid analysis revealed that serine and threonine are the phosphorylation targets and that there are about seven potential Ser/Thr

phosphorylation sites in the mouse GR, six of which are serine and one threonine[222,223]. Although tyrosine phosphorylation was not observed by these authors there have been other reports that tyrosine residues in the GR of human breast epithelial cells and rat hepatocytes are also phosphorylated[226]. Studies by Rao and Fox suggest that the epidermal growth factor (EGF) stimulates phosphorylation on both serine and tyrosine residues of human GR in cultured cells, which increases the phosphorylation state from a Ser/Tyr ratio of 3:0 to 8:1, in the presence of hormone. This suggests that GR may serve as a substrate for a protein tyrosine kinase[227].

Recently, a number of studies have examined the effect of phosphorylation mainly on the transcriptional activity of steroid receptors[228-237]. Several groups have shown that stimulation of phosphorylation pathways by modulators of PKA and PKC often has a striking positive influence on the steroid hormone response[228-242]. However, studies which have sought to identify receptor phosphorylation sites whose modification correlated with increased transcriptional activity have produced mixed results, as often no direct correlation could be observed. In some studies, these modulators produced very minor changes in the phosphorylation state of the receptor as revealed by phosphopeptide mapping[231,232]. Thus it has been suggested that these modulators may have no direct effect on modification of receptor despite the fact that they are able to effect changes in transcriptional activity[231,232]. One possible reason is that even if phosphorylation changes occur directly on the receptor, they may be transient and difficult to measure effectively by the current mapping procedures.

3. Control of Nucleocytoplasmic Shuttling and Cycling of GR and Other SHRs by Phosphorylation/ Dephosphorylation Cycles

The subcellular distribution of GR is directly controlled by effector molecules. Following hormonal stimulus, activated GR becomes tightly associated with the nuclear compartment and affects transcription through specific cis-acting DNA sequence elements. However, as mentioned earlier, even in the presence of steroid, receptors appear to return transiently to the cytoplasm and have been shown to transfer between nuclei in heterokaryons[186,189]. Studies by Guiochon-Mantel et al.[186], using inhibitors of energy synthesis showed that the nuclear occupancy of PR reflects a dynamic situation resulting from continuous diffusion of receptor from the nucleus into the cytoplasm, counterbalanced by an active transport back into the nucleus. Another observation made in this study was that when a constitutively karyophilic signal located between amino acid residues 638-642 was deleted, the mutant PR became cytoplasmic. However, this mutant was rendered nuclear in the presence of hormone, and was returned to the cytoplasm when the cells were depleted of ATP. This same mutant was deprived of nuclear entry in ATP depleted cells, even in the presence of hormone, suggesting that the second karyophilic signal which was hormone dependent had been utilized to direct an ATP-dependent nuclear entry of the receptor. Thus in the shuttling process, at least the import mechanism is ATP-dependent.

Studies by Munck and co-workers, using a different approach also demonstrate an ATP-dependent nucleocytoplasmic cycling of GR[218,219,244-247]. Their studies were based on the hypothesis that if the loss of glucocorticoid binding capacity (GBC) of GR in ATP-depleted cells

is reversed by restoration of ATP levels, and if in cell-free systems phosphatase activity was associated with reduced hormone binding capacity, then GR could undergo an ATP-driven cycle, i.e. the recycling of the receptor may occur possibly through phosphorylation /dephosphorylation mechanisms upon hormonal stimulus. To test this hypothesis, subcellular fractionation studies followed by phosphate content measurements of the different forms of GR were carried out. Subsequently, phosphopeptide mapping and sequence analysis of the receptor intermediates were determined. This showed that the phosphorylation status of GR changes from three (unliganded) to five (transformed). Nuclear unextractable, non-steroid binding form of receptors, referred to as the 'null receptors' have also been shown to exist[219,245]. The 'null receptor' is commonly found in ATP-depleted cells tightly bound to the nucleus, even without steroid. The phosphate content of this receptor is 2/3 that of cytosolic receptors from normal cells, i.e., it has only two phosphates as compared to the cytosolic untransformed form which contained 3 phosphates. Thus these studies substantiated the hypothesis that GR may undergo cycling involving its phosphorylation and dephosphorylation.

The involvement of phosphorylation in different aspects of the signal transduction pathway of GR have also been investigated recently. DeFranco and co-workers have demonstrated that GRs that are capable of modulating gene expression can subsequently reaccumulate in the cytoplasm. However, RU486 bound receptors are efficiently retained in the nucleus[248]. Since RU486 treatment has been shown to maintain the receptor in an underphosphorylated state, this observation may

provide evidence for the role of protein phosphorylation in the cytoplasmic reaccumulation of nuclear GRs.

Studies on heterocomplex assembly of the rat GR by Pratt and co-workers, using an *in vitro* transcription/translation rabbit reticulocyte lysate system indicated that the association of glucocorticoid receptors into the inactive heterocomplex, consisting of the receptor itself, a dimer of hsp90, a monomer each of hsp70 and hsp56, and other factors, is ATP/Mg^{2+} and monovalent cation dependent. Hsp70 has been suggested to have a protein unfoldase activity which is also both ATP and monovalent cation dependent[249].

As has been suggested by Munck et al. the unoccupied receptor must be phosphorylated to maintain hormone binding capacity[245-248]. Therefore these studies suggest a cyclic model involving phosphorylation of the receptor at various steps in the cycle.

Thus regulation of nucleocytoplasmic trafficking of SHRs and other nuclear destined proteins by phosphorylation may serve as an important control mechanism for many nuclear targeted proteins.

D. Proposed Experimental Model

The model of glucocorticoid action at the cellular level which has led to the explanation of some of the pathophysiology of glucocorticoid resistance is still evolving and many crucial questions still remain. For example: a) while determinants of nuclear uptake of the receptor are understood, little is known about how receptors leave the nucleus and why they shuttle i.e. migrate rapidly back and forth between nucleus and cytoplasm, or cycle i.e. nucleocytoplasmic turnover of the receptor; b) the participation of phosphorylation and dephosphorylation in the activation

or inactivation, nuclear import, export, and shuttling as well as turnover of the receptor is not well understood; c) the mechanisms by which glucocorticoids alter translatability and stability of specific mRNAs still remain an enigma.

Since nuclear translocation phenotypes are observed in 20-30 % of GR resistant cell lines, to broaden our knowledge of the molecular basis of steroid resistance, we have focused on the molecular mechanism of nucleocytoplasmic transport of the GR. We initially chose to further characterize the molecular defect of the mutant GR isolated from the S49 lymphoma nuclear transfer deficient (nt^-) cell line.

Earlier investigators demonstrated that mouse lymphoma tissue culture cells (S49.1A) were normally killed by dexamethasone (dex), a synthetic glucocorticoid hormone[250]. Dex-resistant clones were isolated from this line, some of which retain the ability to specifically bind dex but have altered receptor proteins. Addition of [^{3}H]-dex to cultures, followed by cell fractionation, revealed that the nuclear transfer of hormone-receptor complexes in some of these variant clones is deficient (nt^-), while others show increased nuclear transfer (nt^i) relative to the parental line[250,251]. The detailed characterization of these receptor defects carried out to date is as follows: a) Yamamoto et al. initially demonstrated from mixing experiments that the defects in S49 cell lines are a result of faulty receptors and not defects at the level of the subcellular organelle with which the receptor interacts[250]; b) when selected members of each class were studied in an effort to elucidate the molecular determinants involved in the receptor-nucleus interaction *in vivo*, it was observed that the apparent DNA-binding affinity of the nt^- receptors was lower relative to the WT, whereas the nt^i receptors bind

DNA with an affinity higher than the parental molecules[250]; c) sucrose gradient sedimentation analysis also showed that the nt^i receptor variants have sedimentation properties different from receptors from the WT cells[250]; d) photoaffinity labeling studies with radioactive steroids followed by analysis by gel electrophoresis under reducing and denaturing conditions showed that the molecular weight of the receptors in nt^- S49 cells was the same as in WT (94 kDa), but was less than normal in nt^i S49 cells (40 kDa)[252-256]; e) anti-receptor antibodies have been used to characterize receptors in WT and mutant S49 cells[257,258]. Both WT and nt^- receptors, but not nt^i receptors, react with antibodies directed toward the immunoreactive domain of the receptor molecule, indicating that this domain is missing from nt^i receptors; f) when GR RNA in WT S49 cells was analysed by Northern hybridization using a cDNA for the GR, the nt^i cells were found to contain receptor mRNA which was reduced in size compared to receptor RNA in WT and nt^- S49 cells, consistent with the hypothesis that receptor in nt^i mutants is synthesized as a shortened molecule[260]; g) Nuclear association studies also showed that the nt^- receptor associates with nuclei with lower affinity[14]; h) affinity labeling studies carried out in our laboratory, using nuclear envelope preparations and dexamethasone mesylate reagent have shown that the nt^- mutant GR-complex does not specifically associate with the NE in a glucocorticoid regulated manner whereas the WT GR does[259].

From these earlier studies it was revealed that the nt^- receptor mutant selected for resistance to glucocorticoid induced cytolysis contains mutated GR that although it retains its hormone binding affinity and immunogenic properties, is unable to translocate efficiently

to the nucleus in the presence of steroid. Sequence analysis of this mutant by Ringold and co-workers identified three amino acid substitution mutations relative to the WT, Val437-Gly, Arg484-His and Tyr770-Asn. Hormone binding, nuclear association and transcriptional assays have demonstrated that whereas the Val437-Gly does not affect receptor function, Arg484-His change which alters a residue in the second zinc finger of GR and adjacent to NL-1 in the hinge region, impairs nuclear association and abolishes transcriptional activity of the receptor[14], while Tyr770-Asn lowers the affinity of the GR for steroid binding[14].

This information led us to reason that the defect at positions 484 and 770 could be directly altering the mechanisms of nucleocytoplasmic trafficking, interpreted in the following way: that Arg 484 altered nuclear occupancy because of its decreased ability to bind DNA and that the Tyr770-Asn substitution could affect nuclear occupancy because of its decreased ability to bind hormone. Mouse Arg484 in the corresponding rat sequence (Arg496) is at the beginning of NL-1, i.e., exactly one amino acid away from NL-1. Substitution of a linear side chain with an imidazole ring may alter in addition to DNA binding, the conformation of NL-1. Therefore the altered flanking region sequence could be altering nuclear translocation by reducing the efficiency of nuclear import. Tyr770 is in the hormone binding domain; its substitution with an Asn residue may also in addition to altering hormone binding affect NL-2 function. Thirdly, I have identified a putative PKC site in the hormone binding domain (residues769-771), with the sequence **KYS***, using the phosphorylation site sequences and consensus specific motifs assigned for protein kinases[261]. This sequence is in agreement with the

consensus motif **K/RXS*/T***, present in proteins which serve as substrates for PKC. Phosphorylation of the Ser771 could modify this residue to mimic an aspartate residue in which case the Tyr770 could also serve as a potential tyrosine phosphorylation site. I therefore postulated that by a synergistic action involving Ser/Thr and Tyr kinases, both Ser771 and Tyr770 may be phosphorylated to affect nucleocytoplasmic transport of the receptor.

1. *Specific Aims*

The specific aims of my Ph.D. research project were:

1) To determine the amino acid substitution responsible for the nuclear transfer deficient phenotype of the nt$^-$ S49 lymphoma cell line.
2) To investigate the effects of modulators of phosphorylation on nucleocytoplasmic trafficking of GR.

2. *Experimental Plan*

I chose to further investigate the nt$^-$ mutant GR clone, using Tyr770-Asn (which may be a potential phosphorylation site) and Arg484-His (with site specific DNA defect) singly amino acid substituted mutants to: a) identify the amino acid substitution(s) in the S49 nt$^-$ GR that is (are) responsible for aberrant subcellular localization and b) investigate the role of protein phosphorylation on the kinetics of nucleocytoplasmic trafficking of GR.

The experimental plan was to transiently express the mouse WT, the nt$^-$ and the Tyr770-Asn and Arg484-His single amino acid substituted mutants in COS-7 cells and then after hormonal stimulus

monitor the kinetics by a quantitative indirect immunofluorescence of a) nuclear uptake of the various GR derivatives; b) of cytoplasmic accumulation of nuclear receptors after hormone withdrawal; and c) to repeat the procedures in a) and b) but after treatment with various modulators of protein phosphorylation prior to kinetic measurements.

We chose to use COS-7 cells as the transfection recipient because they lack endogenous GR. Secondly, COS-7 cells have been infected with the SV40 large T-antigen vaccine virus and so they could produce SV40 large T-antigen. Thus transfection of a plasmid recombinant with SV40 replication origin in COS-7 cells results in their replication to a high copy number, thereby maximizing the expression of the cloned gene.

Transiently expressed GR has been successfully used for immunofluorescence studies because of the advantages this procedure has over stably transfected cells; a) it is rapid; b) it ensures a large amount of receptor molecules/cell with a subsequent strong immunofluorescence signal; and c) because the cloned gene is not integrated into the genome after transfection, receptor mutations are not introduced[262].

II. MATERIALS AND METHODS

A. Glucocorticoid Receptor Expression Plasmids and DNA Preparation

1. *GR Expression Plasmid Constructs*

The mouse GR plasmid constructs had been prepared previously from cDNA libraries in the pCD vector system, in mammalian cells using polyadenylated RNA from wild type, (W7.2), nt^- (pNB1) mouse lymphoma derived cell lines[263-265] and a hormone binding mutant (Glu547-Gly substituted) clone (pN10)[266,267]. Subsequently, a WT mammalian expression pSV2 expression vector described in Figure 1B, was constructed from the W7.2 clone (pSV2Wrec) in mammalian cells, as described[267]. All other mouse receptor plasmid constructs used in this report had been constructed by restriction fragment swapping of regions of the mutant constructs with homologous regions in the WT construct[14].

Preparation of the human GR construct, pRShGRα, containing the full length hGR cDNA, and plasmids containing deletion receptor mutants, has been described previously[270,271]. This expression vector consists of an ~ 3.0 kb human GR cDNA driven by the Rous sarcoma virus long terminal repeat promoter (RSV-LTR) and contains the SV40 origin of replication and polyadenylation sites. Each plasmid was checked by restriction endonuclease mapping before it was used in experiments.

2. *Preparation of Large Scale Purified Plasmid DNA for Transfection Experiments*

a) Preparation and Storage of Competent Bacterial Cells[272]

Bacterial cells were grown to log phase as measured at OD_{600} of approximately 0.3-0.6 in 2 X LB medium [10 g bacto yeast extract, 16 g bacto tryptone; and 5 g NaCl, made up to 1 L] (100 ml in a conical flask). The cells were pelleted by centrifugation (3000 rpm for 10 min at 4 ^{o}C, using Beckman Model J2-21 centrifuge). Cells were resuspended in 1/10 volume of transformation and storage buffer (TSB): [LB broth, pH 6.1, containing 10% polyethylene glycol (PEG, MW=3350), 5 % DMSO and 20 mM Mg^{2+} (10 mM $MgCl_2$ + 10 mM $MgSO_4$)] at 4^{o}C and incubated on ice for approximately 10 min. For transformation, 0.1 ml aliquots of the cells were pipetted into cold polypropylene tubes and mixed with 100 pg of plasmid DNA. Cells were returned to ice for 15-30 min. Cells were grown in 0.9 ml of TSB supplemented with 20 mM glucose and incubated at 37 ^{o}C with shaking (225 rpm) for 60 min, to permit expression of the ampicillin resistance gene. Cells were plated on ampicillin-containing agar plates overnight at 37 ^{o}C for selection of transformants. Cells in TSB were frozen and stored at -70 ^{o}C for use at a later date (usually more than 12 months) without a significant loss of cell viability or transformation efficiency.

b) Large Scale Preparation of Plasmid DNA

The alkali lysis method by Maniatis et al.[272] was modified, as described below:

One colony of bacterial strain carrying the GR construct was inoculated in 2 X LB medium [20 ml of 2 X LB medium supplemented

with ampicillin to a final concentration of 150 µg/ml]. Cells were allowed to grow overnight in a shaker at 37 °C (150 rpm; Lab-line Incubator shaker). Each 500 ml lot of 2 X LB medium in 2 L conical flask, also supplemented with ampicillin at the same concentration was inoculated with 2 ml of the overnight culture. The culture was incubated at 37 °C with vigorous shaking (300 cycles/min. on a rotary shaker) to obtain an OD_{600} of approximately 0.6. A solution (2 ml) of chloramphenicol (42.5 mg/ml in EtOH) was added to a final concentration of 170 µg/ml. The culture was incubated for a further 12-16 h at 37 °C with vigorous shaking (150 rpm on a Lab-line incubator shaker)[272].

The bacterial cells were harvested from a 500 ml culture by centrifugation at 7 K rpm for 10 min at 4 °C in GSA rotor, using a Beckman Model J2-21 centrifuge. The cell pellet was resuspended in 20 ml of pre-chilled TE buffer [25 mM Tris pH 8 and 10 mM EDTA pH 8]. Cell pellet was collected by centrifugation and the washed pellet was re-suspended in 40 ml of ice-cold TEG buffer [25 mM Tris-HCl, 10 mM EDTA and 50 mM glucose pH 8] containing 12 mg/ml added lysozyme, for 30 min at 4 °C. A mixture of 10 M NaOH and 20 % SDS solution (40 ml; 200:1) was added to the lysozyme-treated DNA solution, mixed gently and kept on ice for 5 min. Sodium or potassium acetate at half volume was added (30 ml). The contents were mixed by hand shaking in a centrifuge tube several times and then centrifuged at 9 K rpm for 20 min, using a Beckman Model J2-21 centrifuge. The supernatant was filtered through 4 layers of cheese cloth and two volumes of 95 % EtOH was added, mixed and kept at -20 °C for 30 min. The nucleic acid suspension was centrifuged (9 K rpm for 20 min, using Beckman Model J2-21 centrifuge), the pellet was washed with 70 % EtOH, dried and re-

suspended in 15 ml TN [50 mM Tris-HCl pH 8 and 100 mM NaCl]. 5 M LiCl was added to a final concentration of 2 M, mixed and kept on ice for 10 min to precipitate mainly rRNA. 95 % ETOH was added to the supernatant after centrifugation (9 K rpm for 20 min, using Beckman Model J2-21 centrifuge). The nucleic acids were precipitated in the cold for 30 min at -20 ^{o}C, then dried and re-suspended in TEN buffer [50 mM Tris-HCl, 10 mM EDTA and 100 mM NaCl pH 8]. RNAse A and T were added to a final concentration of 100 and 50 µg/ml respectively, for 1 h at 37 ^{o}C to degrade mRNA and tRNA. Subsequently proteinase K was added to a final concentration of 100 µg/ml and incubated at 60 ^{o}C for 30 min, to degrade proteins. The supernatant was extracted with equal volumes of: a) neutralized phenol [Water saturated phenol and 1 M Tris-HCl pH 8, at 4:1], shaken and centrifuged at 5 K rpm for 10 min to separate phenol phase from aqueous phase; b) chloroform and c) chloroform and isoamyl alcohol (24:1), repeating the shaking and centrifugation procedures. 95 % EtOH was added at 2.5 volume and the plasmid DNA was precipitated at -20 ^{o}C overnight, centrifuged (9 K rpm, using a Model J2-21 centrifuge), washed with 70 % EtOH, dried and re-suspended in TE [10 mM Tris-HCl and 0.1 mM EDTA]. The plasmid was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

c) Banding Plasmid DNA by Centrifugation to Equilibrium in CsCl Gradients[272]

To 5.5 ml of plasmid DNA solution, 20 ml of saturated CsCl solution [200 g CsCl (technical grade), dissolved in 170 ml 0.1 M Tris-HCl pH 8 and 0.1 mM EDTA, at 37 ^{o}C, and filtered through 0.2 µM filter] and

1.3 ml of 10 mg/ml ethidium bromide solution were added, checking the refractive index to be 1.389. After mixing, using a disposable syringe fitted with a large needle (18 gauge 1/2 ") the solution was transferred to Beckman Quick Seal centrifuge tubes (Beckman), and were sealed, as described[272]. Centrifugation was carried out in a Beckman vertical VTi 50 rotor, using Beckman L7-55 ultracentrifuge for 18 h at 15 ^{o}C.

Three bands of DNA were visible with UV light, (UV-12- 360 mn output). The upper band was host DNA, two other bands located in the center of the gradient were nicked circular DNA (upper) and covalent closed circular plasmid DNA (lower). The deep-red pellet at the bottom of the tube consist of ethidium bromide/RNA complexes and the red precipitate, usually at the side of the quick seal tube was traces of protein precipitate. The lowest DNA band was collected, as described[272]. The banding procedure was repeated, using 4 ml Quick Seal tubes and centrifuging at 60 K rpm for 16 h, using a Beckman TL-100 ultracentrifuge. Two DNA bands were observed, the upper thin band was nicked circular and the lower thicker band was covalent closed circular. The lower band was removed with syringe and 18 gauge 1/2 " needle and the ethidium bromide was extracted from the banding solution by four extractions with isopropanol saturated with distilled-sterile water, as described[272]. Two volumes of sterile distilled water was added to DNA solution and 3 volumes of 95 % EtOH was also added to precipitate DNA at -20 ^{o}C. The DNA concentration and purity were determined by $OD_{260/280}$ ratio and by 1 % agarose gel electrophoresis which indicated that plasmid DNA was free from contaminating RNA and protein. The $OD_{260/280}$ ratio of the purified plasmid recombinant DNA

was 1.82. and contained 95 % supercoiled and 5 % nicked circular, as estimated from the agarose gel electrophoresis.

B. Cell Culture and Transfection Experiments

1. *Cell line and Cell culture*

We used simian COS-7 cells that lack endogenous GR as transfection recipients[14]. This cell line is derived from African green monkey kidney cells that have been infected with an SV40 vaccine virus so they can express the SV40 large T antigen. When utilized as recipients in transfection experiments, using vectors with SV40 origin of replication, the recombinants replicate to a high copy number thereby maximizing the expression of the cloned gene.

COS-7 cells (ATCC CRL 1651) were grown as monolayers in Dulbecco's Modified Eagles Medium (DMEM; GIBCO BRL) supplemented with 10 % fetal calf serum and maintained at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2 , as described[275].

2. *Preliminary Transfection Experiments*

a) Principles

The lipofection method involves spontaneous interaction of the DNA with solutions of lipofectin, a cationic bilayer-forming lipid, to form lipid-DNA complexes in serum free medium. This complex formation presumably is due to ionic interactions between the positively charged group on the lipofectin molecule and the negatively charged phosphate groups on the DNA. The DNA/liposome complex is added directly to cells grown in tissue culture plates and after approximately 5 h incubation period, fresh medium containing 20 % serum is added. The cells are incubated to allow the expression of the transfected gene.

Electroporation involves exposing cell suspension in an electroporation cuvette containing DNA to a pulse electric field of defined magnitude and length by a conventional power supply that reversibly permeabilises the cell membrane, facilitating introduction of plasmid DNA. Dead cell debris is removed by centrifugation and cells are then grown in normal growth medium to allow the expression of the transfected gene.

b) Transfection Assays

During the establishment of optimum transfection efficiency by lipofection, cells ($7x10^5$ cells/6 cm plate) were cotransfected using a β-galactosidase expression vector and pBR322 plasmid as a carrier, by lipofection[276,277]. The lipofectin (Life Technologies Inc.) to DNA ratio was varied as indicated in Table 1. After 5 h incubation time, DMEM-20 % FCS was added. Forty two hours posttransfection, cells were washed 3 times in PBS and the monolayers were scraped off in PBS (1 ml) using a rubber policeman and resuspended in 50 µl of lysis buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7), 10 mM EDTA]. Cells were lysed by 3-cycles of 5 min of repetitive freezing (dry ice/ethanol) and thawing (37 ^{0}C). Cells were centrifuged at 12 K for 10 min, using Fisher Scientific Microcentrifuge Model 235C. Cell extract was transferred into a new Microcentrifuge tube. Cell protein content was determined by the method of Bradford[278]. β-gal assay was determined by mixing 20 µg of cell extract (50 µl) with 500 µl Z-buffer [60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7] and 100 µl of o-nitrophenyl-β-D-galactopyranoside [(ONPG; 4 mg/ml) in 100 mM phosphate buffer, pH 7] to a volume of 650 µl. The reaction

mixture was incubated at 37 ^{o}C. Reaction time was recorded at 2 h when a faint yellow color developed, immediately stopping the reaction by addition of 300 µl of 1 M Na_2CO_3, thus making the final assay volume to 950 µl. The absorbance was recorded at 420 nm . The β-gal activity was expressed as unit (U) of enzyme that hydrolyzed 1 mmole of ONPG per minute per µg of protein at 37 ^{o}C. Data is an average of 2 experiments. β-galactosidase activity was estimated after A_{420} measurement, as Units/min/µg of protein.

When transfection was accomplished by electroporation, COS-7 cells were subjected to 2 min trypsin digestion and 5 ml of DMEM supplemented with 10 % FCS was added and centrifuged at 5 K rpm for 10 min, using Beckman J2-21 centrifuge. Cells were mixed with fresh medium and resuspended in an electroporation cuvette (1 cm x 1 cm x 4 cm high) at a density of 1.6 X 10^7 cells per 250 µl medium. The cell suspension was mixed with plasmid DNA (20 µg) and denatured salmon sperm DNA (50 µg) as a carrier. Electroporation was carried out by discharging pulses through the cell suspension (4 ^{o}C, 1 cm-wide aluminum electrodes, and 1 cm gap), by varying voltages (V), capacitances (µF), or cell density, in duplicates, using a Bio-Rad Gene Pulser equipped with a capacitance extender[277,279-281].

3. *Cytochemical Staining of Expressed β-gal*

The transfected monolayer cells in 35 mm^2 tissue culture plates were rinsed with PBS and fixed for 5 min in 3 % paraformaldehyde in PBS. The cells were rinsed twice with PBS and stained 2 h to overnight with 0.1 % X-gal (GIBCO BRL), 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS. Transfected cells (which stained blue) and non-transfected cells

(which did not stain) were counted using a light microscope (Olympus CK 2) to roughly estimate transfection efficiency.

4. Modification of Transfection Method for Indirect Immuno-fluorescence assays

Cells were transfected with WT or mutant GR using 10:3 μg of lipofectin:DNA, as described above. The following day, cells were washed 2 X with PBS and DMEM supplemented with 10 % charcoal stripped fetal calf serum (DMEM-10 % stripped) was added for 5 h. Cells were trypsinized and replated onto poly L-lysine coated (500 μg/ml) glass cover slips in 35 mm tissue culture plates containing DMEM supplemented with 10 % stripped serum for 22 h. Cover slip monolayers were washed 2 X in PBS and cells were then changed to serum free medium (DMEM-SF) for a further 21 h.

C. Western Immunoblotting

a) Principle

One-dimensional gel electrophoresis performed under denaturing conditions (i.e., in the presence of 0.1 % SDS) separates proteins based on molecular size, as they move through a polyacrylamide gel matrix toward the anode. After solubilising all the proteins by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. β-mercaptoethanol or dithiothreitol is added during solubilization to reduce proteins to their subunits, by reducing disulfide bonds. The electrophoresed protein is transferred onto an immobilon or nitrocellulose membrane and detected with an antibody raised against the protein of interest. After probing with the primary antibody, the

membrane is subsequently washed and antibody-antigen complexes are identified with Horseradish Peroxidase (HRPO) or alkaline phosphatase enzymes coupled to the secondary anti-IgG antibody. Chromogenic or luminescent substrates are then used to visualize the activity.

b) Preparation of Cell Extract

Cells were transfected by lipofection (using 10:6 µg Lipofectin:DNA) or electroporation (using 20 µg plasmid DNA and 50 µg of carrier DNA). Forty-two hours after transfection, cells were harvested by addition of 1ml TEN buffer [40 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl] and scraping with a rubber policeman. After centrifugation (5 K for 10 min), cell pellets (1.5 X 10^6 cells) were re-suspended in 50 µl lysis buffer, disrupted by sonication for 15 one second pulses, using a Fisher Ultrasonic Cell Disrupter Model 300, set at 30 % output power. Cell lysis was confirmed by examining a drop of the cell homogenate by phase microscopy. Cell debris was sedimented by centrifugation at 12 K for 15 min, using a Microcentrifuge Model 235C.

c) Gel Electrophoresis

After determination of the protein concentration of each sample by the method of Lowry[282], the cell extract (100 µg) was added to equal volume of 2 X SDS-PAGE loading solution, also called a solubilization-reduction mix [2.5 ml of 0.5 M Tris-HCl pH 6.8, 4 ml 10 % SDS, 1 ml mercaptoethanol, 2 ml glycerol, 1 ml of 1M $MgCl_2$, 0.4 ml EDTA 0.2 ml of 0.05 % w/v bromophenol blue] , boiled for 3-5 min, and cell debris was pelleted in a microfuge (12 K for 5 min). Samples were loaded onto a denaturing gel consisting of a 3.8 % of stacking gel and a 7.5 %

separating gel, according to Laemli[283], and Matsudaria and Burgess[284], and the gel was run at a constant current of 30 mA and 100 V. Biotinylated protein molecular weight standards were loaded in one gel lane to allow subsequent estimation of the molecular weight of protein bands. To prevent 'smiling' (curvature in the migratory band), the temperature was controlled with a circulating constant temperature water bath.

d) Immunoblotting and Immunodetection

The polyacrylamide gel was transferred to an immunoblotting cassette immersed in a solution of transfer buffer [25 mM Tris-HCl pH 8.3, 192 mM glycine, 20 % methanol, 0.1 % SDS made up to 4 L with 800 ml methanol]. Proteins were transferred onto nitrocellulose after electrophoresis (50 V for 4 h), to provide access for reaction with immunodetection reagents[287]. All remaining binding sites were blocked by immersing the membrane in a washing solution [20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 ml Tween, made up to 4 L with distilled water] containing 10 % skimmed milk, for 1 h. Immunoblotting was performed using mouse BuGR-2 monoclonal antibody to rat liver GR and horse radish peroxidase (HRP)-sheep antimouse IgG as primary and secondary antibodies respectively. The signal was visualized by utilization of enhanced chemiluminescent substrate, ECL (Amersham) [288,289]and after exposure to Kodak XAR-5 film at room temperature for 7 min. The principle of the ECL detection is based on the ability of HRP to catalyze the oxidation of luminol in the presence of hydrogen peroxide. Immediately following oxidation, the luminol is in an excited state which may decay to the ground state via a light emitting pathway.

D. Whole cell Glucocorticoid Receptor Binding Assay

a) Principle

The biological response to a steroid hormone is a saturable phenomenon. If the formation of hormone-receptor complexes which is obligatory for the production of a biological response is specific, and the quantity of steroid receptor is limited, there will be a finite number of binding sites. This criterion is met by the demonstration that the binding activity of interest can be saturated by a specific ligand for the receptor. This is usually accomplished by exposing the receptor to various concentrations of ^{3}H-steroid under equilibrium conditions and subsequently examining the amount of bound ^{3}H-steroid.

b) Method

COS-7 cells were transfected by lipofection, using 10:6 µg lipofectin : DNA. The cell extract was prepared by sonication [in 10 mM Tris-HCl pH 7.4], and cell debris was sedimented by centrifugation at 12 K rpm for 15 min at 4 ^{o}C. To tubes containing 250 µl aliquots of the cell extract preparation (280 µg of protein). An increasing amount of the radioactively labeled hormone (New England Nuclear) ranging between 0-50 nM was added to each tube and competition experiments were performed with a parallel set of tubes containing 280 µg (200 µl) of protein and 200-fold excess of cold dex (50 µl of 10 µM) was added, together with the radiolabeled dexamethasone (dex) to each tube to obtain a final volume of approximately 250 µl, for the determination of non-specific binding[290-291]. The whole cell extract preparation and hormone mixture in all tubes were incubated for 18 h at 4 ^{o}C, with

rotation of the tubes, to allow equilibrium to be established between hormone and receptors in forming hormone-receptor complexes. An equal volume (250 µl) of 1 % charcoal and 0.05 % dextran (Sigma) suspension was added to each tube and left at 4 ^{o}C for 15 min with vortexing every 5 min to adsorb free ligand. Each tube was centrifuged for 5 min at 12 K to separate free from bound steroid. Radioactivity was determined by scintillation counting in 0.5 ml samples and by addition of 4 ml of Hydrofluor scintillation fluid (Beckman) in plastic minivials. Counting efficiency for ^{3}H was 33 % for tritium in a Beckmann LS-3801 scintillation spectrophotometer. Binding was analysed by the method of Scatchard[292,293,294].

E. Methods for Kinetic Experiments

For induction experiments, cells containing the expressed GR were incubated in serum free medium for 21 h. Cells were stimulated with dexamethasone (dex) at a concentration of 10^{-6} M, unless otherwise stated. At various time periods cells were fixed and processed for indirect immunofluorescence.

To monitor export following hormone withdrawal, cells were pretreated with 10^{-6} M cortisol for 1 h. Withdrawal was initiated by multiple washes (using serum free medium warmed at 35 ^{o}C) followed by incubation in serum free DMEM supplemented with bovine serum albumin (BSA) to 10 % (withdrawal medium). In some cases 50 µg/ml cycloheximide was added to the withdrawal medium to determine whether there was appreciable synthesis of *de novo* GR during the 24 h time course.

With nuclear re-uptake experiments, cells were re-stimulated with 10^{-6} M cortisol, after loss of nuclear receptor molecules to the cytoplasm at the various points on the withdrawal curve as follows: 3 h, 6 h, 12 h, and 24 h. The kinetics of nuclear re-import was monitored for 1 h, using 10^{-6} M cortisol.

The import kinetic studies with modulators of protein phosphorylation was performed by pretreatment of cells with expressed WT or mutant receptors for 4 h with the modulator under investigation prior to hormone addition. The modulators used at final concentrations indicated were as follows: TPA (100 nM), Forskolin (25 μM), H-7 (100 μM), okadaic acid (OA; 100 nM), vanadate (0.5 mM). In some experiments I used 4α-phorbol ester (100 nM), an inactive analog of TPA as a negative control for TPA. The kinetics of combined effects of forskolin and TPA or forskolin and OA were monitored by simultaneous pretreatment of cells with the modulators at their above stated concentrations. In one of the combined forskolin and TPA experiments, I substituted TPA with 4α-phorbol ester (100 nM) to see whether I would observe the effect of forskolin alone.

The kinetics of the effects of modulators on the loss of nuclear receptors to the cytoplasm was studied by addition of the modulator at the above mentioned concentrations (except 50 nM TPA was used instead of 100 nM) in the withdrawal medium.

The kinetics of the effects of RU486 on nucleocytoplasmic transport of GR were performed using 10^{-6} M RU486 in place of dex or cortisol for the uptake and withdrawal experiments respectively.

For the kinetic studies involving energy synthesis inhibitors, forty-two hours postransfection cells containing WT or mutant receptors were

stimulated with 1 μM cortisol or RU486 for 1 h. Either after removal of hormone/antihormone or in the continuous presence of hormone /antihormone, cells were incubated with withdrawal medium supplemented with 6 mM 2-deoxyglucose and 50 mM oligomycin (energy depletion medium). In another set of experiments, 100 μM H-7 was added to the energy depletion medium, after cortisol withdrawal or in the continuous presence of the hormone. The kinetics of cytoplasmic return of nuclear receptor was monitored for 6 h.

All phosphorylating agents and oligomycin were dissolved in ethanol, of which the final concentration was less than 0.1 %. In all control experiments EtOH (vehicle) was added to the culture medium at a final concentration of 0.1 % .

F. Indirect Immunofluorescence Procedures

a) Principle

Adherent culture cells, usually grown on coverslips are fixed to immobilize cellular proteins, including the antigen of interest, within a specific compartment and to preserve cellular morphology. Cell membranes are then permeabilised to allow antibodies into the cell. A specific antibody is used to localize the protein (antigen) of interest within cells. Subsequently, a secondary antibody to which a fluorophore is conjugated is applied. This recognizes and binds to the primary antibody, hence the detection of the cellular antigen is by an "indirect method", which offers excellent sensitivity for antigen detection. The conjugated fluorophore emits visible fluorescent light on excitation at an appropriate wavelength under an immunoflourescent microscope.

b) Preliminary procedures

Growing cells on cover slips, fixation, permeabilization, primary and secondary antibody treatments were the major considerations [295,296].

Coating slides with Poly-L-lysine: Prior to use, the cover slips were placed in 70 % EtOH and sterilized by flaming. A solution of 500 µg/ml of poly-L-lysine (MW 35,000-70,000; Sigma) was prepared in sterile distilled H_2O and stored in aliquots at 4 °C. A liberal amount of the poly-L-lysine was applied onto each coverslip by holding the coverslip with jewelers forceps and dipping it into a 35 mm^2 tissue culture plate containing 500 µl of the poly-L-lysine. The cover slips were tilted at an angle of 90 °C to remove excess poly-L-lysine and allowed to dry for 1 h in a sterilization hood. The cover slips were then dipped in sterile distilled H_2O, tilted at an angle of 90 °C and then excess water was removed from the edges by aspiration to promote even poly-L-lysine coating. The cover slips were allowed to dry for 1 h.

Fixation: A solution of PBS was warmed to 50 °C and a paraformaldehyde powder was added to 3 %. A few drops of 1 M NaOH was added to dissolve the paraformaldehyde into solution. The solution was cooled to room temperature. The pH of the solution was adjusted to 7.4 and then filtered with a 0.2 µM sterilizing unit. Paraformaldehyde treatment fixes cells by cross-linking proteins thereby localizing the cellular antigen. Formaldehyde, glutaraldehyde or methanol could also be used. However, I noticed from my preliminary experiments that glutaraldehyde fixation resulted in a high immunofluorescence background, whilst methanol fixation sometimes altered the cell morphology.

Quenching of reactive aldehyde groups: 0.1 M glycine/PBS was added to fixed cells to quench intrinsic fluorescence of reactive aldehyde

groups produced after fixing cells with the cross-linking agent, paraformaldehyde.

Cell permeabilization: Permeabilization of cells after fixation with paraformaldehyde and other cross-linking agents is necessary for antibody penetration into the interior of the cell. If the cells have been fixed in alcohol, permeabilization is not required because in addition to having fixing properties alcohol permeabilises cells. However, detergent extraction methods appear to best preserve cellular morphology. A solution of 0.5 % Triton X-100 in PBS was prepared. Cells fixed on cover slips were immersed in the detergent solution for 30 min at room temperature and then the solution was aspirated.

Blocking: Nonspecific antibody binding can result from electrostatic and hydrophobic interactions with the substrate. Typically these interactions are best blocked by incubating the preparation in 5 % serum from the same species of origin as the secondary antibody. For example, for a goat anti-mouse secondary antibody, 5-10 % Normal Goat Serum (NGS) in PBS is applied to the fixed cells for 30-60 min at room temperature prior to antibody treatment. I also tried using 10 % BSA or 10 % skimmed milk in my preliminary experiments and observed that blocking with the NGS gave the lowest immunofluorescence background.

Primary and Secondary Antibody Treatments: The Primary antibody was diluted to low concentrations (10 µg/ml) in PBS. Cells were then incubated with the antibody (500 µl in a humidified chamber for 4 h at room temperature or overnight at 4 ^{o}C). It is important to note that primary antibodies could be recovered and used as much as 10 times. Alternatively, we tried diluting primary antibody in PBS containing the blocking agent. Often, this helped to further reduce

background, but antibody solution could get contaminated after repetitive usage. Addition of sodium azide prevented bacterial contamination but on all occasions it also decreased antibody reactivity. The ideal situation was to add blocking agent to the final rinse between antibody incubations.

Two types of immunofluoresence techniques could be employed. For direct immunofluorescence, the fluorochrome is conjugated to the primary antibody, whilst with indirect immunofluorescence, the primary antibody is unconjugated, but it is detected by a second antibody conjugated to a fluorochrome that specifically recognizes the primary antibody.

c) Quantitative Indirect Immunofluorescence Assay

Transfected cells grown on cover slips were washed 2 X with PBS and fixed for 30 min with a fresh solution of 3 % paraformaldehyde/PBS. Intrinsic fluorescence due to fixation was quenched with 0.1 M glycine/PBS for 10 min). Cells were sequentially permeabilised and blocked with 0.5 % Triton X-100/PBS and 5 % NGS for 30 min each. After aspiration of NGS, primary murine BuGR-2 antibody (Affinity Bioreagents; 10 µg/ml in PBS, for experiments performed with the mouse GR constructs), or anti-human GR polyclonal, PA1 510 (Affinity Bioreagents; 12.5 µg/ml in PBS, for experiments performed with the human GR constructs) was added to the coverslips overnight at 4 ^{o}C. Samples were washed 5 X with PBS. Finally, either FITC-conjugated goat anti-mouse or anti-human IgG secondary antibody (TAGO Inc.; 1:100) was added at room temperature for 70 min with gentle shaking. The immunoflourescent cell preparations were then mounted in glycerol/PBS (1:1 v/v). Subcellular localization was visualized on a Zeiss Axiophot

photomicroscope using standard double blind encryption. For each time point at least 400 stained cells were counted and classified into different categories according to their staining pattern. In cases where photographs were taken, Kodak T-max films (ASA 3200) were used. Each experiment was repeated 3 to 5 times over a period of several months.

G. Statistical Analysis

For the data in which small differences were observed between the control (untreated) experiments and experiments in which cells had been treated with a modulator (test), statistical analysis was performed to determine the significance of the differences. Data for a) controls, TPA, forskolin and the combined forskolin and TPA treated nuclear uptake experiments (Figure 24) or b) control, forskolin, OA and combined forskolin and OA experiments (Figure 27) were each classified into four groups and the % Nuclear Immunofluorescence obtained at the 20 min and 40 min time points were used for the analysis.

One-way analysis of variance was used to compare significant differences in the % Nuclear Immunofluorescence at the 20 min and 40 min time points, using the means of five determinations obtained for each time point. Tukey's honestly significant difference (HSD) test was used to assess which specific groups were significantly different. In addition, to specifically assess the interaction between TPA and forskolin treatment groups, two-way factorial design in which the factors were, TPA, (present or absent) and forskolin, (present or absent), were considered. The interaction in the OA experiment, b), were conducted in a similar fashion.

The analysis was repeated for 6 h and 12 h time points in experiments in which the rate of return of nuclear receptors to the cytoplasm had been measured in cells treated with forskolin, OA and combined forskolin and OA (Figure 28).

The overall *p*-value was set at a minimum of 0.01 for the analysis, using the Statistical Package for the Social Scientist (SPSS) software.

III. RESULTS

A. Preliminary Studies to Establish Conditions for a Quantitative Indirect Immunofluorescence Assay

1. *Transient Transfection of RSV β-gal*

I first established a quantitative indirect immunofluorescence assay to distinguish among different subcellular distributions in COS-7 cells which had been transfected with plasmids containing WT GR or GR mutant. Preliminary studies were carried out to determine transfection conditions that would result in the expression of high numbers of receptor molecules per cell, for subsequent generation of an adequate immunofluorescence signal at fairly low concentrations of antibody.

We initially chose to compare lipofection and electroporation methods for transfection since previously it had been demonstrated in other systems that both of these two methods produced high transfection efficiency and also high reproducibility of transfection [276,279]. COS-7 cells were transfected with the β-gal plasmid construct, a β-galactosidase expression vector with a Rous sarcoma virus promoter (RSV β-gal).

For lipofection, pBR332 plasmid was used as carrier DNA. In the first experiments we noted that if cells were plated at 70-80 % confluency they survived the lipofectin treatment better and higher transfection efficiencies were achieved than if cells were plated at greater or less than 70-80 % confluency. If fully confluent cells were used there was a two-fold decrease in signal. Previous reports also indicated that the length of the incubation period after the addition of lipofectin-DNA mixture to the cells was important both for the transfection efficiency and cell survival[276,277]. I confirmed in my experiments that incubation of cells

longer than 7-8 h with the lipofectin-DNA mixture resulted in high levels of toxicity to the cells, as indicated by increased rate of cell death. A transfection period of 5 h was chosen.

In order to determine the effect of varying the DNA concentration on lipofection, first, an experiment was carried out in which the amount of β-gal construct was kept constant (0.5 μg) but increasing amounts of carrier DNA were added. Secondly, the lipofectin amount was varied between 5-15 μg. Thirdly, the overall lipofectin to DNA ratio was also varied. After 42 h, cells were harvested and cytosolic cell extracts were prepared, and used to determine β-gal activities, as described in Materials and Methods. I chose 42 h to allow the expression of the transfected β-gal construct although there was no significant difference in the levels of β-gal expression (transfection signal) if this period was extended up to 60 h.

Tables 1a and 1b list β-gal activities obtained by varying the amount of DNA, lipofectin and lipofectin to DNA ratio and Figure 4 shows β-gal activities in histograms. Cell viability was determined 24 h post-transfection (Table 1), using the trypan blue dye exclusion assay, to ensure that a sufficient number of transfected cells survived the lipofection. The conclusions made from the lipofection experiments were as follows: a) we observed that the lipofectin to DNA ratio is the major determinant of β-gal expression, and not the amount of β-gal construct used in the transfection experiments. b) although the transfection signal, measured by β-gal activity increases with increasing DNA concentration, increasing the concentration of DNA to exceed that of lipofectin caused a marked inhibitory effect on the transfection; c) increasing the lipofectin concentration from 5 to 10 μg/μl increased the

transfection signal produced, while the signal was reduced if increased from 10 to 15 μg/μl; d) if lipofectin to DNA ratio was maintained at 5:3, irrespective of lipofectin or DNA amount used in an experiment, it constantly resulted in the highest transfection signal; e) maintaining lipofectin to DNA ratio between 10:6 and 10:9 yielded the highest transfection efficiency, with a β-gal activity of approximately 12.0 X 10^{-2} U/min/μg protein; f) cell viability was maintained at fairly high levels between lipofectin concentrations of 5 to 15 μg/μl. However, at 20 μg lipofectin with a lipofectin to DNA ratio of 5:3, cell toxicity occurred, since > 50 % of the cells died, as observed by light microscopy (Olympus CK 2). Since the survival of cells after transfection was very important to our subsequent immunofluorescence experiments, no further studies at concentrations greater than 20 μg were conducted. Therefore optimal conditions for lipofection were established as: a) plating cells at 70-80 % confluency; b) using a lipofectin to DNA ratio of 10:6 or 10:9; c) incubation of the lipofectin-DNA complex mixture for 5 h before the addition of serum-containing medium; d) allowing 42-60 h β-gal expression.

Optimal conditions for electroporation were determined by varying the following conditions of transfection; no. of cells/cuvette, voltage and capacitance. Previous reports showed that transfection efficiency could be increased by increasing the amount of transfecting DNA up to 80 μg, and that carrier DNA could be co-transfected with a smaller amount of the plasmid DNA construct to make up the DNA requirement[279]. In addition, it had been reported that the nature of the carrier nucleic acid was important, since the addition of yeast tRNA led to a decline in expressed enzyme activity[279]. I therefore added 50 μg of carrier DNA

(salmon sperm DNA) to 20 μg of plasmid construct so as to obtain a total amount of DNA concentration of 70 μg for our electroporation experiments[279]. β -gal activities obtained under the different experimental conditions are indicated in Table 2, and also represented in histograms in Figure 5. The following conclusions were drawn from the results: a) Using 0.7 X 10^7 cells gave a high transfection signal at a voltage and capacitance of 300 V and 800 μF respectively in the initial experiments. Therefore, this number of cells should have been used to further define optimal conditions. However, I did not require that many cells for the subsequent immunofluorescence experiments. Thus optimal conditions were established with a cell no. of 0.188 X 10^7. b) As indicated above, at a particular cell no. (0.188 X 10^7), setting the voltage at 260 V and a capacitance of 800 μF was required for highest transfection signal. c) Increasing the voltage between 260-340 V but maintaining the two other parameters constant decreased the transfection signal, whilst decreasing the voltage between 220-240 V decreased the signal by 2-fold; c) Changing the capacitance from 800-1180 μF also markedly lowered β-gal activity, as > 90 % of cell death occurred (Figure 4C). Therefore optimal conditions for electroporation were established at: 260 V, 800 μF and 0.188 X 10^7 cells/ cuvette, which typically yielded a β-gal activity of 48 X 10^{-2} U/min/μg protein.

2. *Transfection Conditions and Immunoblot Analysis of COS-7 Cells After Transfection with GR.*

A comparison of the two transfection methods showed that the optimum signal obtained with the β-gal construct after transfection by electroporation was 4-fold greater than that of lipofection. These

preliminary experiments suggested that the electroporation method was more effective. The WT GR cDNA was expressed in COS-7 cells and immunoblot analysis of whole cell extracts was performed to ascertain whether I could extrapolate the same optimal conditions we had established with β-gal transfection for GR, (Figure 6a). The GR from liver cytosol of adrenalectomised rat and mouse S49 lymphoma cell preparations were used as positive controls, while an extract from COS-7 cells which had been transfected with the β-gal construct was used as a negative control (mock transfection). Surprisingly, very low levels of GR expression were obtained after electroporation when the GR plasmid constructs were used. A band at 90/92 kDa which corresponded to the size observed for mouse GR isolated from S49 cells was observed in the transfected cell extracts. Two other bands with mobilities faster than 90 kDa (75-85 kDa) were also detected. These smaller forms of the GR detected in the analysis might have resulted from: a) alternate transcription start site of the GR cDNA; b) multiple translational start site of the GR mRNA; and/or c) mild proteolytic cleavage of the receptor, as these bands did not show up in my negative control in which primary antibody was omitted (Figure not shown). As shown in Figure 6a and 6b, lanes containing expressed GR cell extracts in which 5:3 (6a, Lane 1) and 10:6 (6b, Lane 2) μg lipofectin to DNA ratio had been used for transfection showed that at 5:3, the level of expression of GR is relatively low, while at 10:6 the expression of GR was very high. I performed preliminary immunofluorescence assays, using these two ratios and observed that at 5:3 the immunofluorescence signal was a bit low, I needed to obtain a good enough signal since I set out to quantitate the data by classification of the immunofluorescence stained cells. At 10:6

the rate of expression was very high, as observed with β-gal expression but unfortunately I observed that I could not use this ratio in my uptake experiments because the kinetics were so fast that it was difficult to separate the nuclear uptake component from the export component. I therefore used a lipofectin to DNA ratio that was in between the two, i.e., 10:3 . I also used a GR expression period of 42 h after transfection in all our experiments, since degradation of the receptor begins to occur slowly from 48 h and GR levels are reduced by ~ 20 % by 60 h. In all subsequent experiments transfection of GR was done by lipofection.

3. *Quantitative Characterization of Hormone binding Affinity and Receptor Number/Cell*

To estimate the numbers of expressed GR in transfected COS-7 cells, I determined the binding capacity of whole cell extracts for labeled synthetic glucocorticoid, dexamethasone ([^{3}H]-dex). I added increasing amounts of the radioactively labeled hormone (1-50 nM) to assay tubes, which contained extracts of the expressed receptor preparation. After equilibration, the bound ligand was separated from the free by the charcoal adsorption method[290-292]. This is rapid, reliable, inexpensive and lowers non-specific binding because charcoal is a strong adsorbent of free steroids. The concentration of the bound ligand was then determined. Non-specific binding was determined by adding 200-fold excess of unlabelled dex to a second series of tubes. By subtracting the non specific binding (NS) from the total binding (NS+SB), measured with the first set of tubes, I estimated specific bound hormone to receptor (SB). Figure 7A shows a saturation plot of the Bound dex vs dex concentration. The data was subsequently analysed by construction of a

Scatchard plot i.e. the plot of the ratio of Bound to Free vs. Bound ligand concentration, (abbreviated B/F vs. B; Figure 7B). As shown in Figure 7A, there is a considerable NS binding. This is due to using whole cell extracts which were obtained by sonication of COS-7 cells. Sonication results in cell membrane disruption and liberates numerous membrane phospholipids and proteins which are known to bind steroid[290,291]. Therefore the binding parameters obtained from Figure 7B are only rough approximations. The dissociation constant, Kd, estimated from the graph was 2.2 X 10^{-9} M. Finally, the number of receptor molecules/cell, which is an index of the subsequent immunofluorescence signal was estimated to be 1.2 X 10^6, based on the following: a) I used the general assumption that 1 mole of receptor = 1 mole of dex; and b) I estimated transfection efficiency by cytochemical staining of the expressed β-gal to be ~3 %.

4. *Quantitative Indirect Immunofluorescence of GR*

To examine nucleocytoplasmic trafficking of GR, I transiently transfected an expression plasmid of the WT receptor in COS-7 cells by lipofection and monitored the subcellular distribution of expressed receptors by indirect immunofluorescence using a fluorescein labeled second antibody.

It has been shown that cycling properties of steroid hormone receptors are influenced by the cell cycle[297]. As cells are competent to cycle receptors in G_0, to avoid possible complications in interpreting data from cycling cells we performed all experiments on cells maintained in serum free medium. This allowed me to eliminate the cell-cycle variable from consideration of the data. In addition, expression of GR to a level

well above that of endogenous transcription factors permitted us to eliminate specific protein-protein interactions between GR and other phorbol ester sensitive transcription factors such as AP-1 or other limiting nuclear components as possible factors in regulating the redistribution of GR[28-32].

To quantitate the assay, each experiment was performed between 3-5 times over a period of several months. Individual data points were generated from counting an average of 400 immunofluorescent cells, evaluated using a double blind encryption. I always observed a partial and not a complete distribution of staining within the cytoplasmic and nuclear compartments of cells upon immunofluorescence, either in the presence or absence of hormone. We therefore classified the immunofluorescence staining pattern into five categories[130], shown in Figure 8 as follows: N, cells with exclusively nuclear staining; N>C, cells which contain > 50-90 % nuclear staining; N=C, cells in which the intensity of nuclear staining equals cytoplasmic; N<C, cells in which the intensity of cytoplasmic staining exceeds that of nuclear; C, cytoplasmic staining. Graphs were plotted as % Nuclear Fluorescent cells, defined by a total of all nuclear staining ([N+N>C]) or exclusively nuclear staining ([N]), as a function of time. I plotted only [N] and [N>C] distributions because prior to hormonal stimulus less than 5 % staining was found in these two categories whilst distribution of fluorescent cells in the N=C, N<C and C categories were as follows: ~ 50 %, 20 % and < 30 % respectively, so I termed these latter categories as total cytoplasmic distributions. Thus upon ligand stimulus I measured a shift from the total cytoplasmic distributions of GR to total nuclear ([N+N>C]) or

exclusively nuclear ([N]) distributions as a function of time, as an index of the rate of nuclear uptake.

5. *Limitations*

The limitations of this assay system is that although transient assays are rapid and free from interference by chromosomal position effects there is an inherent low efficiency of the process. Secondly, assumptions could only be made in correlating an assay system in which cells have been overexpressed with the receptor with an endogenous receptor system. Thirdly, cell counting is very subjective, despite the fact that I employed the double blind encryption procedure.

B. Kinetic Studies on Nuclear Import, Export and Nuclear Re-Uptake of GR

1. *The Arg484-His Substitution Decreases the Equilibrium Nuclear Distribution of the nt⁻ GR*

To evaluate the influence of the Arg484-His and Tyr770-Asn substitutions on nuclear occupancy of GR, we expressed WT (as a control) and mutant forms of mouse GR in COS-7 cells from transiently transfected plasmids. The plasmid constructs employed are summarized in Figure 9A[14,270,271]. In the first experiment shown in Figure 10, several different mutations which include the Arg484-His and Tyr770-Asn single amino acid substitutions in the mouse receptor were tested for an influence on nuclear uptake of GR following treatment of the transfected cells with dex.

To enable me to make a distinction between nuclear accumulation (rate of nuclear uptake and steady state levels) of the WT receptor and the receptor mutants, (Figure 9A), I initially stimulated the expressed cells with 10^{-6} M dex and monitored the rate of nuclear uptake over 24 h. Prior to hormone treatment, less than 5 % of transfected cells contained total nuclear GR and fewer than 1 % contained exclusively nuclear GR. Addition of 10^{-6} M dex led to rapid uptake ($t_{1/2}$=4 min; Figure 10; Table 3) of WT GR into the nucleus. At equilibrium, which persisted for 24 h, greater than 90 % of cells contained receptors that were exclusively nuclear and nearly 95 % of cells contained receptors that were at least mostly nuclear (Table 3). Further, the maintenance of nuclear WT GR for 24 h following hormone treatment in the presence of cycloheximide (data not shown) which did not differ from untreated cells indicated that new synthesis was negligible, and thus it does not affect the subcellular distribution pattern in this period of time.

As mentioned in the Introduction, the Arg484-His mutation decreases DNA binding[24,250], we reasoned that it may also affect NL-1 function. This mutation markedly decreased the steady-state distribution of GR expressed in COS-7 cells, with the exclusively nuclear [N] GR component dropping by a third, i.e., close to 62 %. However, the initial rate of nuclear uptake of this mutant following dex treatment, when calculated as a percentage of steady-state was not appreciably different from that of WT GR ($t_{1/2}$=4 to 5 min; Figure 10; Table 3) i.e. the proportion of transfected cells with Arg484-His change in the nucleus also reached close to 50 % of equilibrium levels within 4-5 min.

Previous studies of ligand binding established that the Tyr770-Asn substitution results in generation of a receptor mutant with only 50 % of

hormone binding affinity[14]. This substitution is also in the span of the second nuclear localization signal (NL-2) and could affect signal function. Furthermore, I identified the Tyr770 to be a putative tyrosine phosphorylation site. Thus the effects of these three parameters could all affect the rate of nuclear import. However, by monitoring the kinetics of nuclear uptake of this mutant at 10^{-6} M dex, (Figure 10), it was observed that both the rate of import and the equilibrium nuclear distribution were identical to the WT receptor.

The GR Glu547-Gly mutant, is the other allele in all steroid resistant clones isolated from the S49 lymphoma. The Glu547-Gly substitution has been shown to completely eliminate hormone binding. I therefore used this mutant as a hormone binding negative control in the nuclear uptake experiments. This Glu547-Gly change produced a receptor that was unaffected by hormone treatment and remained almost exclusively cytoplasmic within the 24 h time course. This result indicated that if hormone binding should have an influence on Tyr770-Asn substitution of the receptor it probably would affect the rate of nuclear uptake. Further, a double mutant (nt^- GR), containing the Tyr770-Asn substitution in addition to Arg484-His showed no additional effects on distribution other than the effect demonstrated by the Arg484-His. I concluded from the data that the Arg484-His substitution is solely responsible for the reduced nuclear equilibrium levels of the nt^- mutant receptor, and that at least at saturating concentrations of dex the Tyr770-Asn has no effect on nuclear import.

Since the rate of nuclear import was not altered in the Arg484-His mutant GR, we reasoned that the reduced nuclear equilibrium levels demonstrated by this mutant could be due to lowered nuclear retention

due to impaired DNA binding. To determine whether the defect in Arg484-His is due to an effect on NL-1 or DNA binding, the experiment was repeated with a DNA binding mutant of human GR construct (shown in Figure 9B), which has a deletion in the first finger (hΔ420-451) but has intact NL-1 and NL-2. It too demonstrated a reduced nuclear equilibrium level and showed no difference in the rate of nuclear uptake, as compared with a corresponding human WT receptor (Figure 11). Thus we generalized our observation that DNA binding mutants of GR demonstrate altered steady state levels of nuclear receptor possibly by rendering nuclear unbound receptors available for export.

To further distinguish between the effects of lack of DNA binding and altered NL-1 of the Arg484-His mutant on nuclear occupancy, I also investigated the kinetics of nuclear import of two other DNA binding mutants: (hΔ450-487) which has deletions of the second zinc finger and also an N-terminal deletion of NL-1; (hΔ428-490) or both zinc fingers and N-terminal deletion of NL-1. Interestingly, these mutants displayed a drastic decrease (60-fold) in the rate of nuclear import (Figure 11; $t_{1/2}$=5 h) as well as reduced equilibrium levels of nuclear receptor, calculated as a percentage of steady state to be total nuclear, 78 %, 65 % and exclusively nuclear, 55 %, 45 % for the first and second zinc finger mutants respectively. These results are consistent with deletion of NL-1 sequence imparting a marked reduction in the rate of nuclear uptake, and DNA binding mutations resulting in reduced equilibrium of nuclear distribution of receptors. Thus these observations were consistent with the reduced nuclear equilibrium levels observed with the Arg484-His mutant being due to effects distal to the initial rate of nuclear import.

2. *Dose-Response Studies Demonstrated that Tyr770-Asn Change Does Not Affect Nuclear Import of GR*

We reasoned that since at saturating concentrations of dex the Tyr770-Asn change did not result in any observable alteration in the kinetics of nuclear uptake. Lowering the hormone concentration might slow down the rate of nuclear import if this receptor mutant has a decreased affinity for steroid. I therefore carried out time course studies of nuclear uptake with dex concentrations ranging between 10^{-10} to 10^{-6} M, (Figure 12). The following conclusions were drawn from the dose-response studies: a) in general, as dex concentration was lowered there was a marked reduction in the rate of nuclear uptake for WT and all mutants, as indicated by the calculated $t_{1/2}$ values in Table 5; b) low doses of dex retarded the rate but did not affect the equilibrium of nuclear uptake for the different GR derivatives. c) Surprisingly, the Tyr770-Asn mutant displayed import kinetics similar to WT, even at dex concentrations lower than physiological, confirming that this substitution does not affect nuclear import; d) at lower concentrations of dex, (10^{-9} M and 10^{-10} M), the DNA binding point mutants (Arg484-His and nt$^-$) displayed kinetics with an initial lag in the first 20 min, i.e., as the hormone concentration was lowered, the initial rate of uptake was slightly slowed in these mutants, as compared to WT receptor and the Tyr770-Asn mutant, suggesting that the Arg484-His substitution may have a marginal effect on NL-1 function at lowered concentrations of ligand.

3. *Mutations That Impair DNA Binding of GR Promote its Export From the Nucleus*

My data from the uptake experiments showed reduced equilibrium levels of nuclear GR with the DNA binding mutants. This might be the result of the impaired DNA binding influencing the rate of nuclear export of the mutant GRs by sequestration of the receptor away from DNA.

To determine whether mutations in the DNA binding domain of GR increase the rate of return of GR from the nucleus to the cytoplasm, I stimulated cells expressing WT and mutant GRs with the glucocorticoid hormone, cortisol, and then monitored redistribution of nuclear GR to the cytoplasm following abrupt withdrawal of hormone (Figure 13a). In order to ensure that our results would not be influenced by persistence of hormone in the cell, we substituted the natural glucocorticoid, cortisol, which is rapidly metabolized for dex. I also included the Tyr770-Asn mutant in my series, reasoning that persistence of a low concentration of glucocorticoid in the cell would result in a faster export of a receptor mutant with lower affinity for steroid than WT receptor.

For export experiments, after hormone withdrawal, I counted the nuclear immunofluorescent cells remaining in the [N+N>C] or ([N]) distributions as a measure of the rate of loss of nuclear receptors to the cytoplasm (i.e., cytoplasmic return or redistribution of nuclear receptors).

As reported previously, loss of WT receptor to the cytoplasm following withdrawal of hormone is a slow process. Indeed my results indicate that the $t_{1/2}$ for redistribution of nuclear receptor to the cytoplasm of total and exclusively nuclear mWT GR was 25 h and 12 h respectively[236,463,464]. Human WT GR was lost from the nucleus modestly faster than mouse receptor, with $t_{1/2}$ of 15 and 8 h for [N+N>C]

and [N] distributions respectively. Significantly, the Tyr770-Asn mutant returned to the cytoplasm with kinetics indistinguishable from the mouse WT receptor. This substantiated the suggestion that loss of hormone from the cell following withdrawal was not a rate limiting step in returning GR to the cytoplasm in my experiments.

GRs containing the Arg484-His and hΔ420-451 mutations were lost from the nucleus between 2-3-fold more rapidly than their respective WT receptors (Figures 13a and 14). This effect was reproduced by the addition of cycloheximide in the withdrawal medium to ensure that there was no protein synthesis within the 24 h kinetic period (Figure 13b). For the Arg484-His mutant, the half time for complete loss of GR from the nucleus was shortened from 25 to 9-11 h, while loss of the exclusively nuclear fraction dropped from a $t_{1/2}$ of 12 to 7 h. Similarly, removal of the first zinc finger from human GR decreased the $t_{1/2}$ of loss of exclusively nuclear receptors from 15 h to 4 h and loss of exclusively nuclear distribution from 8 to 3 h.

4. *DNA Binding Has no Effect on Re-utilization of Receptor in a Secondary Response*

Munck and co-workers have postulated that hyperphosphorylation of the receptor ultimately renders the receptor highly specific for binding to DNA sites (GRE), and after interaction with the transcription machinery, GR is covalently modified possibly through dephosphorylation. The modified receptor thus become competent for subsequent return to the cytoplasm in which it associates with hsps, followed by re-entry into the nucleus[236,237,247,388]. If this hypothesis were true, it would predict that the DNA binding mutant receptor would

not undergo this modification and hence would be defective in cycling. Secondly, $t_{1/2}$ for dex binding has been estimated previously to be < 10 min[211]. However, $t_{1/2}$ for export (> 12 h) of the WT receptor is relatively very long. This suggested that the on/off rate of ligand on the receptor could not be the determinant of the exit rate of nuclear receptors. We therefore wondered whether in the export experiments what was being measured was the rate of re-accumulation of the inactive GR-hsps-heterocomplex. Further, Pratt and co-workers have demonstrated that association of hsp90 with GR is required for the maintenance of a competent steroid binding state of the receptor[394]. Thus it is only the GR-hsp90-complex which is competent to bind hormone for subsequent transformation to the DNA binding state and entry into the nucleus.

I sought to determine: a) whether DNA binding has an effect on re-utilization of receptor in a secondary response; and b) whether in the kinetic measurements of cytoplasmic redistribution of nuclear receptors after hormone withdrawal, I was observing in the cytoplasm reaccumulation of the inactive 8-9S GR-heat shock protein complex (untransformed receptors) in a state competent to bind ligand, rather than free unliganded GR (unliganded 4S receptor) which is unable to re-bind steroid. I re-stimulated the cells at various points along the withdrawal curve with cortisol. At all points tested, readdition of hormone led to a rapid return of both WT receptor and the Arg484-His mutant to the nucleus in a fashion almost indistinguishable from the primary response (Figure 15). We conclude that the DNA binding does not affect the rate of nuclear re-uptake of the receptor in a secondary hormonal response. This result could only be obtained if the redistribution of immunofluorescence reflected the cytoplasmic accumulation

of GR in a state competent to bind hormone, i.e., in the inactive hsp-associated heterocomplex as opposed to the free unliganded form.

In summary, my findings suggest that DNA binding by GR in the nucleus plays a significant role in promoting nuclear occupancy of the receptor. Secondly, deletions in the N-terminal flanking region sequence of NL-1 markedly reduces the rate of nuclear import by several fold. However, a Tyr770-Asn change in the mouse GR did not affect nucleocytoplasmic transport of the mutant GR. Finally, receptor molecules that are returned to the cytoplasm upon hormone withdrawal can be quantitatively returned to the nucleus upon restimulation with hormone. We termed the cytoplasmic GR observed after hormone withdrawal "recycled GR".

C. Effects of RU486 and Modulators of Phosphorylation on Nucleocytoplasmic Transport of GR

Hormone induced hyperphosphorylation has long been implicated in many aspects of steroid receptor action[211-218]. Whereas studies on the effects of modulators of phosphorylation on steroid inducible gene transcription is slowly emerging, very little is known regarding their effect on nuclear import, export and recycling of the receptor. Another way in which phosphorylation could influence receptor function is by regulating nucleocytoplasmic trafficking of the receptor. Indeed the glucocorticoid antagonist, RU486 (known to prevent hyperphosphorylation of the receptor) has been shown to influence cycling of WT receptor in COS-1 cells[237]. OA has also been shown to affect GR nuclear re-entry in *v-mos* non-transformed cells[236]. I therefore investigated the role of RU486, (GR antagonist), TPA and Forskolin (stimulators of protein kinase C and

A respectively), H-7 (inhibitor of protein kinases), and OA and vanadate (inhibitors of PP-1/PP-2A and protein tyrosine phosphatases respectively) on nucleocytoplasmic transport of GR.

1. The Inhibition of GR Cycling by Hormone Antagonist is Independent of DNA Binding

RU486 is a powerful GR and PR antagonist and has been postulated to act by affecting DNA dependent post-translational modification of the receptor[298-301]. Further, it has been shown to generate a receptor that is unable to re-accumulate in the cytoplasm during withdrawal[237]. To test the role of DNA binding in RU486 action, I compared its effects on nuclear uptake and redistribution of the receptor into the cytoplasm upon hormone withdrawal of Arg484-His and WT mouse GR (Figure 16). First, in determining the kinetics of nuclear uptake in cells treated with RU486 (10^{-6} M), we observed $t_{1/2}$ value of 8-10 min, compared to 4-5 min of dex treated receptors. This result suggests a 2-fold decrease in the rate of nuclear distribution of GR when RU486 is the ligand.

Further, following withdrawal of RU486, the nucleocytoplasmic equilibrium of WT receptor was maintained for 24 h without any apparent decrease in the nuclear occupancy of the receptor (return of the GR to the cytoplasm). Similarly, when the kinetic studies were repeated using the Arg484-His mutant receptor, its cytoplasmic redistribution was also blocked. Previous data by DeFranco and co-workers indicated that in the liganded state, RU486-treated GR retains the ability to traffic between heterokaryon nuclei[248]. We therefore postulated that we might be observing 'apparent nuclear occupancy' of RU486-treated GR rather than a complete blockage of receptor exit from the nucleus; i.e., shuttling

was continuing but redistribution to the cytoplasm in the form of 8-9S recycled receptor was blocked.

From my results, it is clearly evident that the apparent nuclear occupancy of RU486 treated GR is independent of DNA binding as both WT GR and Arg484-His mutant were completely unable to redistribute to the cytoplasm following withdrawal of RU486 from the medium. Therefore, the effect of RU486 on both nuclear import and re-cycling must probably be exerted prior to the interaction of the receptor with DNA and be independent of any DNA dependent receptor modification.

2. *Re-cycling of GR is Dependent on a Phorbol Ester Sensitive Protein Kinase Activity*

Several groups have shown that phorbol esters potentiate the activation of transcription of GR, but some have shown that they have no detectable effect on receptor phosphorylation[232,236]. I sought to monitor the influence of TPA and a protein kinase inhibitor, H-7, on the rate of nuclear uptake as well as the rate of return of nuclear receptors to the cytoplasm following hormone withdrawal. The uptake kinetics was monitored at a lower concentration of dex (10^{-9} M) to slow down the rate of nuclear uptake so as to be able to observe changes in the rate caused by these modulators. The results of these experiments are shown in Figures 18 and 19. The $t_{1/2}$ values estimated from the graphs are also shown in Table 6 and 7. First, by monitoring the rate of nuclear uptake at 10^{-9} M dex, after 4 h pretreatment of cells with TPA, WT GR demonstrated a shift in $t_{1/2}$ values for nuclear import from 30-40 min in the absence of TPA to 15 min, i.e., there was a 2-fold increase in the rate of import. In contrast, 4 h pretreatment with H-7 resulted in ~2-fold

decrease of the rate of uptake of WT receptor. The Arg484-His mutant GR also demonstrated ~ 2-fold increase in the rate of nuclear uptake after TPA treatment and a 2-2.5-fold decrease after H-7 treatment.

TPA treatment of cells at the time of hormone withdrawal markedly increased the initial rate of redistribution of GR to the cytoplasm with $t_{1/2}$ values for return of nuclear WT receptor dropping from 24 h to 3.5 h of total nuclear and from 12 h to 3.5 h of exclusively nuclear distributions. Similarly, $t_{1/2}$ values of Arg484-His mutant GR after TPA treatment dropped from 9-11 h (controls) of total nuclear distribution to 3 h (TPA treated) and 7 h (control) of exclusively nuclear distribution to 3 h (TPA treated). Remarkably, return of both WT and Arg484-His GRs to the cytoplasm were completely blocked by H-7, resulting in > 95 % of receptors still remaining in the nucleus after 24 h of hormone withdrawal. Similar results were obtained when 10^{-6} M cortisol and H-7 were added simultaneously to the withdrawal medium (results not shown).

The effects of RU486 in blocking the return of nuclear receptors to the cytoplasm was reproduced by H-7. The fact that addition of H-7 to the medium was at the time of hormone withdrawal indicates that H-7 is able to exert its effect following hormone withdrawal. By contrast the RU486 treatment which was before hormone withdrawal indicates that the RU486 effect probably occurred prior to withdrawal of the anti-hormone. Not surprisingly, the treatment of RU486 bound WT and Arg484-His mutant receptors with TPA did not have any influence on the RU486 effect on nuclear retention (Figure 20), i.e., the RU486 bound receptor is resistant to the TPA effect on loss of nuclear receptors to the cytoplasm.

3. *cAMP-Dependent Protein Kinase Stimulator, Inhibitors of PP-1/PP-2A and Protein Tyrosine Kinase may Potentiate the Effects of Steroid Receptors on Transcription by Prolonging Nuclear Occupancy*

In most systems it has been shown that treatment of transfected cells with an activator of cAMP dependent protein kinase, such as forskolin or 8-bromo cAMP, or an inhibitor of PP-1 and PP-2A, such as OA augmented the glucocorticoid inducible GR mediated induction of transcription[228-238,240-243].

Studies with GR, PR and ER have also shown that treatment of cells with vanadate or EGF results in an enhancement of the hormone-induced receptor mediated transcription[232,241,243]. Vanadate is a protein tyrosine phosphatase inhibitor whose effect increases phosphorylation of tyrosyl residues in proteins. EGF upon interaction with its receptor results in autophosphorylation on tyrosyl residues of the receptor (EGFR). Thus both vanadate and EGF stimulate signaling pathways involving tyrosine phosphorylation.

To test whether signals originating from the cAMP-mediated cascade and in pathways involving phosphatases or requiring tyrosine phosphorylation can also influence nucleocytoplasmic trafficking of GR, the effects of forskolin, OA and vanadate on nuclear import and return of nuclear receptors to the cytoplasm after hormone withdrawal were examined. Forskolin and OA caused a moderate increase in the rate of uptake for both WT and Arg484-His (Figure 21). Statistical analysis indicated that the differences observed were significant, with a *p*-value of < 0.001. Vanadate on the other hand impaired the rate of nuclear uptake by ~2-fold for both WT and Arg484-His.

Treatment of cells with forskolin, OA or vanadate at the time of hormone withdrawal decreased loss of both WT receptor and Arg484-His mutant from the nucleus, i.e., all three agents increased 'apparent nuclear retention' of both WT receptors and Arg484-His mutant (Figure 22). However, the effect on WT GR only became apparent at 24 h. The effects of these three agents appear to be dominant over the DNA binding effect, as the Arg484-His mutant was retained in the nucleus to almost the same extent as WT receptor.

Interestingly, in hormone withdrawn COS-7 cells, re-stimulation (10^{-6} M cortisol) of both OA treated WT and DNA binding mutant receptors which had been redistributed to the cytoplasm re-entered the nucleus (Figure 23), at approximately the same rate ($t_{1/2}$=2-3 min) as the primary response. This observation is inconsistent with a previous report by DeFranco and co-workers in which they had shown that nucleocytoplasmic trafficking of GR is inhibited in *v-mos* transformed and in non transformed 6m2 cells treated with OA under normal cell proliferating conditions[236-237] due to inability of the cytoplasmic hormone withdrawn GR to re-enter the nucleus upon readdition of hormone. An explanation to this discrepancy may be that the OA effect in 6m2 non transformed cells may be a cell specific observation, or a higher concentration of OA may be required to effect receptor desensitization (inability to be re-utilized in a secondary response) in COS-7 cells.

4. *Simultaneous Stimulation of cAMP and IP3/DAG Dependent Signaling Pathways Slows Nuclear Import of GR*

In certain systems, it has been demonstrated that cross-talk exists between the cAMP-dependent pathway and inositol phospholipid signal-

ing system[302-308]. The fact that both pathways have been shown separately to synergize with steroid hormones in steroid receptor mediated transcription suggest that simultaneous activation of both pathways may generate a multi-signaling cascade to modulate receptor function.

To investigate the possible effect of simultaneous coupling of dual phosphorylation signaling pathways on glucocorticoid-inducible GR-mediated nuclear import and to be able to distinguish between effects of DNA binding and non DNA binding, we monitored the kinetics of nuclear accumulation of WT GR and Arg484-His mutant after simultaneous treatment of cells with forskolin and TPA for 4 h prior to 10^{-9} M dex stimulation (Figure 24). First, as indicated earlier, whereas forskolin and TPA alone increased nuclear import, the forskolin/TPA combination slowed the hormone inducible GR mediated nuclear import to levels obtained in the absence of either of the agents ($t_{1/2}$=40 min). A one way statistical analysis of variance (F-test) of the 20 and 40 min time points of the uptake kinetics indicated that the observed differences were significant, with a *p*-value of < 0.001, i.e., the rate of forskolin and TPA treated GRs were different from control but the rate of uptake of GRs treated with combined forskolin and TPA was identical to control. I also carried out a two-way analysis of variance to determine statistically whether interaction occurs between forskolin and TPA to bring about the down regulation effect produced by the two agents. The results indicated that interaction occurs between the two agents to produce the observed effect. Thus this data indicate that the stimulatory effect of one modulator on the dex-inducible GR mediated nuclear import may inhibit the effect of the other to bring about a reduction in the ligand binding response (trans-modulation).

5. *Down Regulation of Nuclear Accumulation of WT Receptor but not Arg484-His Mutant is Observed at Sub-Physiological Dosage of Dex after Simultaneous Treatment of Cells with Forskolin and TPA*

Down regulation of the GR action has been very well documented[387,464,465]. Although the mechanisms underlying down modulation is not very clear, it has been reported that it occurs at very low concentrations of hormone[387,464,465]. The effect has never been shown to occur at high concentrations of hormone. Recently, it has been reported that the AP-1 transcription factor (a heterodimer of *c-Jun* and *c-Fos* proteins) could interact with GR to repress the transcriptional activity of GR[463]. *c-Jun* and *c-Fos* proteins are known to be induced by TPA, whilst forskolin induces *c-Jun*. However, it has not been investigated whether the combined agents could affect nuclear accumulation of the receptor. Thus I extended the studies on nuclear accumulation of the receptor after pretreatment of cells with both Forskolin and TPA and followed the kinetics at 10^{-10} M dex concentration, i.e., at a hormone concentration one order of magnitude lower than the former concentration. I also used the WT receptor and the Arg484-His mutant to determine whether the effect was mediated by DNA binding. Secondly, I repeated the experiment in the presence of 50 µg/ml cycloheximide (CHD), to completely inhibit any *de novo* synthesis of GR that may be formed under the serum-free, cell synchronized conditions of my experiments. Thirdly, I substituted TPA with 4α-phorbol ester, an inactive analog of TPA as a control for the forskolin/TPA combination treatment to determine whether I would observe the effect of forskolin alone. In addition I included 4α-phorbol

ester alone treatment in my series of experiments to check basal levels. Finally, the time course was extended to 12 h so as to be able to determine possible effects that might occur at longer times after treatment with these two agents.

The results of these experiments are shown in Figure 25. There was no appreciable difference in the rate of nuclear uptake of both WT and Arg484-His receptors after treatment with forskolin or TPA alone compared to the untreated receptor upon 10^{-10} M dex stimulation ($t_{1/2}$ = 60 min). However, the forskolin and TPA in combination resulted in 2-fold decrease in the initial rate of import. In addition, ~ 40-50 % reduction of steady state levels of total nuclear and ~ 60 % of exclusively nuclear receptor was observed, as compared to the control untreated receptors. However, between 9-12 h of treated cells, the levels of the receptor, after treatment with both agents started increasing again. Thus the reduction which might have been brought about by down regulation of PKC was possibly overcome over a long period of time. This may indicate that either the agents were no longer effective or the down regulation effect demonstrated by WT receptor as a result of induction of a factor in the presence of these two agents is mutual, i.e., GR could also repress the levels of the factor. I also observed in my experiments with the combined forskolin and TPA treatment, the amount of fluorescent signal of the WT receptor containing cells was reduced in both cytoplasmic and nuclear compartments from the time points of 2 h to 9 h, as compared to the control samples. This may suggest a reduction of receptor molecules in the cells.

The Arg484-His mutant only showed a modest increase in the rate of uptake with the forskolin or TPA alone treatment at 10^{-10} M dex.

However, the time course of nuclear uptake of GR displayed after treatment of cells with a combination of forskolin and TPA was characterized by a prolonged initial lag with 3- to 5-fold reduction of exclusively nuclear and total nuclear distributions of receptor respectively, with maintenance of steady state levels of the mutant receptor. There was no evidence of reduction of fluorescent signal with this mutant receptor. For both WT receptor and Arg484-His mutant, experiments repeated with CHD gave identical results as those done in the absence of CHD (results not shown). The experiments performed with a combination of forskolin and 4α-phorbol ester gave identical results as those done with forskolin alone. On the other hand results obtained after treatment with 4α-phorbol ester alone were identical to the controls, i.e., those in which no agent had been administered to cells containing WT receptor or Arg484-His mutant.

These observations suggest that the simultaneous activation of the cAMP and IP3/DAG signaling pathways may serve to negate each others effect on the hormone induced GR mediated nuclear accumulation.

6. A DNA Dependent Cancellation Effect on Nuclear Retention is Produced by Simultaneous Stimulation of cAMP and IP3/DAG Signaling Pathways

I further investigated the effect of coupling of two phosphorylation signaling pathways on the kinetics of return of GR from the nucleus to the cytoplasm. As shown in Figure 26, treatment of cells with combined forskolin and TPA, after hormone withdrawal produced distinct effects on the WT receptor and DNA binding mutant. The TPA alone effect increased the rate of return of WT receptor to the cytoplasm, whilst the

forskolin alone treatment (as shown earlier in Figures 19 and 22) resulted in nuclear retention of the receptor. Thus the two kinase stimulators produce a cancellation effect on cytoplasmic return of WT nuclear receptors which resulted in approximately the same $t_{1/2}$ to equilibrium levels ($t_{1/2}$=18 h and 9 h for total nuclear and exclusively nuclear distributions respectively) as untreated control cells. I also tested the significance of the cancellation effect, using the F-test, which indicated that the differences were significant, with a *p*-value of < 0.01.

By contrast, treatment of cells with a combination of forskolin and TPA at the time of hormone withdrawal resulted in the Arg484-His mutant demonstrating the TPA alone effect ($t_{1/2}$=6 h and 3.5 h for total and exclusively nuclear distributions of receptor respectively). This observation may imply that the forskolin effect on nuclear retention of GR could be overcome by the effect of TPA but only if the receptor is defective of DNA binding. Thus DNA binding may be required for the two signaling pathways to act to bring about the forskolin alone effect.

7. *Simultaneous Inhibition of Protein Phosphatase 1/2A Activities and Stimulation of cAMP Signaling Pathway Produce an Additive Effect on Nuclear Occupancy of WT GR but not the Arg484-His Mutant*

An earlier demonstration that OA inhibition of PP-1 and PP-2A causes augmentation of cAMP levels in some cells[309] and also an increase in transcriptional activity of SHRs[231-233], prompted me to determine whether cAMP dependent protein kinase might be involved in the phosphorylation events affected by the phosphatase inhibitor. Since forskolin also augments the hormone-induced steroid receptor-mediated

transcription, I postulated that simultaneous treatment of cells with forskolin and OA could amplify the observed enhancement of the forskolin effect on the dex-inducible GR-mediated nucleocytoplasmic transport. As shown in Figure 27, pretreatment of cells with a combination of forskolin and OA prior to 10^{-9} M dex stimulation reduced the effect of the hormone-inducible GR-mediated nuclear import observed with forskolin alone. Although these effects appear small, the F-test done on the data also showed that the small differences were significant, with a *p*-value of < 0.001. Similarly, as indicated before, a two-way analysis of variance to determine statistically whether interaction occurs between forskolin and OA to bring about the reduction in the rate of nuclear uptake showed that interaction does occur between the two agents which was significant. It thus seems that the forskolin and OA in combination treatment could also decrease the hormone induced GR-mediated response, however this effect is not as pronounced as was observed with the forskolin and TPA combined treatment.

Further, simultaneous treatment of cells with 25 μM forskolin and 100 nM OA at the time of hormone withdrawal to monitor the kinetics of return of receptor molecules from the nucleus to the cytoplasm uncovered an additive effect on nuclear retention (Figure 28). After 24 h, approximately 85-90 % of WT receptors were still retained in the nucleus after this combined treatment. Interestingly, the Arg484-His mutant after forskolin and OA combined treatment displayed export kinetics which was identical to the effect with forskolin alone. Thus it appears that with the WT receptor capable of DNA binding, OA augmented the forskolin effect on nuclear retention, whilst with the Arg484-His receptor that is defective of DNA binding, no augmentation was observed and a

similar effect to that of forskolin-induced nuclear retention was observed. This together with the results obtained for treatment with the combination of forskolin and TPA suggest that different phosphorylation events may play an important role in enhancing DNA binding of the GR.

D. Effects of Energy Depletion on Nucleocytoplasmic Transport of GR

This section discusses experiments which determine whether the effects of RU486 and H-7 in blocking the cytoplasmic return of nuclear receptors is on shuttling (the rapid migration of receptors between nucleus and cytoplasm) or re-cycling (a process in which the receptor is re-incorporated into an inactive 8-9S hsp-containing complex that is competent to re-bind ligand) of the GR. Since cellular energy (ATP) has been shown to be required for nuclear import, whilst nuclear export is not energy dependent, we reasoned that if we blocked nuclear import, we should observe more cytoplasmic accumulation of nuclear receptors if the blockage was not on export but on re-cycling. If the effect was on export, RU486 and H-7 treated nuclear receptors would still be blocked from returning to the cytoplasm.

Energy synthesis inhibitors have been used previously to provide evidence of ATP requirement in some aspects of steroid receptor function [186,213,214]. Addition of high concentrations of oligomycin to cells results in inhibition of cellular protein phosphorylation and energy utilizing reactions. In contrast to uncouplers which lead to the complete discharge of all high energy compounds, oligomycin inhibits synthesis of ATP by blocking the conversion of high energy bond compounds to ATP,

i.e., it blocks Pi→ATP, but has no effect on ADP→ATP exchange reaction. Thus oligomycin inhibition would only block energy synthesis via the electron transport chain but ATP synthesis via glycolysis could still occur. I therefore added 2-deoxyglucose, an analog of glucose with hydrogen substituted for a hydroxyl group at the 2-position of the pyranose ring. This glucose analog has been shown to strongly inhibit glycolysis by > 60 %, by competitively inhibiting the hexokinase reaction (first step in glycolysis) thereby depleting the glycolytic pathway of ATP synthesis (substrate level phosphorylation).

1. *Inhibition of Nuclear Import by Energy Inhibitors Overcomes RU486 and H-7 Blockage of Redistribution of GR to the Cytoplasm Following Hormone or Anti-Hormone Withdrawal*

I supplemented the hormone withdrawal medium with 2-deoxyglucose and oligomycin and monitored export kinetics over 6 h after stimulation of cells with: a) cortisol, b) RU486, or c) cortisol, but with added 100 μM H-7 to the oligomycin supplemented medium. These series of experiments were performed either in the absence or in the continuous presence of hormone. The results are shown in Figures 29 and 30. The following observations were made from the graphs: 1) The $t_{1/2}$ of efflux of receptor molecule from the nucleus is approximately the same for both WT and Arg484-His ($t_{1/2}$=2-3 h) under all three experimental conditions in the presence of energy inhibitors; and 2) receptor release to the cytoplasm was faster in the presence of energy inhibitor, as compared with the previous untreated RU486 data in Figure 17, shown as composite graphs in Figure 31, and H-7 untreated data in

Figure 19. Treatment with an energy inhibitor provoked an efflux of the receptor from the nucleus to the cytoplasm ($t_{1/2}$=2-3 h), visible after 2 h and was complete in 4 h. This demonstrates that cellular energy depletion cause the redistribution of nuclear receptors to the cytoplasm by blocking nuclear import. This effect is independent of whether hormone is present or not (Figure 30). The rate of exit of WT is identical to Arg484-His, suggesting that in the presence of energy inhibitor, the DNA binding effect in increasing nuclear retention is lost. This may imply that tight binding to DNA requires ATP. The fact that oligomycin treatment resulted in detectable cytoplasmic GR suggest that RU486 and cortisol/H-7 treated GR continue to shuttle between the nucleus and cytoplasm and that the effects of these agents abrogate the abilities of the receptors to form cytoplasmic 8-9S GR-hsps-complex, and not in their abilities to exit.

Thus the blockage of cytoplasmic accumulation of nuclear receptors by these agents (RU486 and cortisol/H-7) is only apparent because nucleocytoplasmic shuttling is unaffected. Composite graphs for cortisol bound nuclear receptors treated with H-7 in the absence or presence of an energy inhibitor (Figures 19 and 30) were essentially the same as those shown in Figure 31 for RU486 treated and untreated cells.

Table 1. Transient Transfection of RSV β-gal by Lipofection. Shown are β-gal activities obtained after transfection with varying concentrations of lipofectin, DNA and lipofectin : DNA (μg:μg). The number of live cells was determined 24 h post-transfection by the trypan blue exclusion assay. Forty-two h post-transfection, cell extracts were prepared by freezing/thawing three times, followed by recovery of the supernatant by centrifugation at 12 K for 10 min. Protein concentration was determined by the method of Bradford[278]. β-gal activities were determined in a reaction mixture (950 μl final volume) containing the cell extract (20 μg of protein), 100 μl of 4 mg/ml ONPG and Z-buffer after incubation at 37 ^{o}C until 2 h when a faint yellow color had developed. The absorbance at 420 nm was determined after stopping the reaction with 1 M Na_2CO_3 and recording the time. β-gal activity was expressed as unit (U) of enzyme that hydrolysed 1 μmole of ONPG per minute per μg of protein at 37 ^{o}C. Data are an average of 2 experiments.

Table 1A. TRANSFECTION BY LIPOFECTION

L : DNA (μg)	CONSTRUCTS ßgal : pBR322 μg	TOTAL PROTEIN μg/ul	$OD_{420/2h}$	Av. ßgal Activity U/min/ug Protein X 10^{-2}	Cell Viability (No. of Live Cells) X 10^5/ ml.
5 : 3	0.5 : 2.5	2.16	0.269	9.55	12.0
5 : 6	0.5 : 5.5	3.00	0.175	6.20	14.2
5 : 9	0.5 : 8.5	3.36	0.187	3.40	14.1
10 : 6	0.5 : 5.5	2.44	0.223	11.72	12.0
10 : 9	0.5 : 11.5	1.20	0.384	12.00	9.0
10 : 20	0.5 : 19.5	1.98	0.187	2.20	10.5

Table 1B. TRANSFECTION BY LIPOFECTION

L : DNA (μg)	CONSTRUCTS ßgal : pBR322 μg	TOTAL PROTEIN μg/μl	$OD_{420/2h}$	Av. ßgal Activity U/min/ug Protein X 10^{-2}	Cell Viability (No. of Live Cells) X 10^5/ ml.
15 : 2	0.5 : 1.5	1.27	0.195	3.75	10.40
15 : 4	0.5 : 3.5	1.28	0.220	4.35	13.20
15 : 4	2.0 : 2.0	1.57	0.188	3.40	12.40
15 : 4	4.0 : 0.0	1.59	0.140	2.40	14.80
15 : 9	0.5 : 8.5	1.31	0.358	8.55	11.80
15 : 15	0.5 : 14.5	1.80	0.242	9.16	12.10
15 : 30	0.5 : 29.5	2.64	0.111	4.00	13.70

Table 2. Transient Transfection of RSV β-gal by Electroporation. Shown are β-gal activities obtained after transfection with varying 2a) cell density/cuvette 2b) voltage and 2c) capacitance. The number of live cells was determined immediately after electroporation by the trypan blue dye exclusion assay. Forty-two h after transfection the cell extracts were prepared by three times of freezing/thawing of cells, followed by centrifugation at 12 K for 10 min to recover the supernatant. Protein concentration was determined from cell extracts by the method of Bradford[278]. β-gal activities were determined in a reaction mixture (950 µl final volume) containing cell extract (20 µg of protein), 100µl of 4 mg/ml ONPG and Z-buffer, after incubation at 37 °C until a faint yellow color had developed. The absorbance at 420 nm was recorded after stopping the reaction with 1 M Na_2CO_3. β-gal activity was expressed as unit (U) of enzyme that hydrolysed 1 µmole of ONPG per minute per µg of protein at 37 °C. Data are an average of 2 experiments.

Table 2A. TRANSFECTION BY ELECTROPORATION

a) Varying the Amount of Cells / Cuvette

CONDITION V vs µF	Amount of cells/ cuvette (X 10^7)	TOTAL PROTEIN µg/µl	OD_{420}/2h	Av. ßgal Activity U/min/µg Protein X 10^{-2}	Cell Viability (No. of Live Cells) X 10^5/ ml.
340 , 800	0.188	2.32	0.214	13.20	4.52
340 , 800	0.375	5.20	0.328	20.00	7.82
340 , 800	0.750	3.44	0.378	23.20	20.00
340 , 800	1.500	3.56	0.208	12.80	57.00
b) Varying Voltage					
220 , 800	0.188	3.96	0.332	21.80	7.60
240 , 800	0.188	1.84	0.310	27.15	6.50

Table 2B. TRANSFECTION BY ELECTROPORATION

b) Varying Voltage

CONDITION V vs µF	Amount of cells/ cuvette (X 10^7)	TOTAL PROTEIN (µg/µl)	OD_{420}/2h	Av. ßgal Activity U/min/µg Protein X 10^{-2}	Cell Viability (No. of Live Cells) X 10^5/ ml.
260 ; 800	0.188	3.04	0.350	48.33	6.40
280 ; 800	0.188	2.60	0.105	32.30	6.00
300 ; 800	0.188	2.32	0.196	17.30	5.20
340 ; 800	0.188	2.32	0.214	13.20	4.52

c) Varying Capacitance

260 , 800	0.188	3.04	0.258	48.33 ± 2.5	6.40
260 , 1180	0.188	2.40	0.350	15.25 ± 3.2	2.90

Table 3. Subcellular Distribution at Equilibruim for Wild Type Receptor, DNA-Binding Mutants and the Tyr770-Asn Mutant, Which is Defective in Hormone Binding. Cells were prepared for indirect immunofluorecence and classified into the different categories as described in Figure 8. All GR derivatives displayed similar rates of nuclear entry but Arg484-His and nt⁻ mutants demonstrated reduced nuclear equlilibruim distributions. Results show the mean of 3-5 experiments. Figures are presented as percentages of nuclear immunofluorescent cells with indicated standard deviations.

Table 3. Subcellular distribution of WT and Mutant GRs Following 1 μM Dexamethasone Administration.

RECEPTOR	$t_{1/2}$ to equilibrium, min	% Subcellular distribution at 10 min.				
		N	N>C	N=C	N<C	C
mWT	4	91 +/- 1	3 +/- 2	5 +/- 2	0.5	0.5
Tyr770-Asn	4	93 +/- 1	3 +/- 3	4 +/- 3	0.5	0.5
Arg484-His	5	62 +/- 3	17 +/- 2	17 +/- 3	0	4 +/- 1
NBrec (nt^-)	5	64 +/- 3	17 +/- 2	16 +/- 2	0	4 +/- 1
hWT	4	89 +/- 3	4 +/- 2	5 +/- 2	0	2 +/- 2
hΔ 420-451	4	73 +/- 2	11 +/- 2	11 +/- 3	1 +/- 1	4 +/- 3

Table 4. Summary of Kinetic Data for Nuclear Export of Wild Type Receptor, DNA Binding and the Tyr770-Asn Mutants. Shown are the $t_{1/2}$ values for loss of nuclear receptors to the cytoplasm upon hormone withdrawal, as calculated from the graphs for the above GR constructs . The DNA binding mutants displayed a faster rate of export than the corresponding WT.

Table 4. Kinetics of Redistribution of GRs to the Cytoplasm Following Hormone withdrawal.

	[N+N>C]		[N]
RECEPTOR	$t_{1/2}$ for CORTISOL WD, h	RECEPTOR	$t_{1/2}$ for CORTISOL WD, h
mWT	24-25	mWT	12
Tyr770-Asn	24-25	Tyr770-Asn	12
Arg484-His	11	Arg484-His	7
NBrec (nt^-)	9	NBrec (nt^-)	7
hWT	15	hWT	8
hΔ420-451	4	hΔ420-451	3

Table 5. Summary of Kinetic Data Showing the Effect of Dex Concentration on the Rate of Nuclear Import of Wild Type Receptor, DNA Binding and the Tyr770-Asn Mutants. Shown are the $t_{1/2}$ values of rate of import at different dex concentrations, as calculated from the graphs. At higher concentrations of dex (10^{-6} to 10^{-7}) the rate of nuclear import is approximately the same for WT and other GR derivatives, however, at dex concentrations lower than the above, the DNA binding mutants display a slightly slower rate of uptake.

SUMMARY OF DOSE RESPONSE KINETIC DATA:

Table 5. Time Half ($t_{1/2}$) for the Establish ment of Equilibrium by WT or Mutant GR

A

[N+N>C]

CONSTRUCT	t1/2 to Equilibrium (min) [DEX]			
	10^{-6} M	10^{-7} M	10^{-9} M	10^{-10} M
mWT	4.0	4.5	40	60
Tyr_{770}-Asn	4.0	4.5	40	60
Arg_{484}-His	5.0	5.0	40	70
NBrec (nt-)	5.0	5.0	40	80

B

[N]

CONSTRUCT	t1/2 to Equilibrium (min) [DEX]			
	10^{-6} M	10^{-7} M	10^{-9}M	10^{-10}M
mWT	4.0	4.5	30	90
Tyr_{770}-Asn	4.0	4.5	30	90
Arg_{484}-His	5.0	5.5	40	120
NBrec (nt-)	5.0	5.5	40	140

Table 6. Summary of Kinetic Data showing the effects of modulators of protein phosphorylation on the rate of Nuclear Import of Wild Type Receptor and the Arg484-His Mutant. Shown are the $t_{1/2}$ values for import and the steady state nuclear levels of receptors, after 4 h pretreatment of cells with different modulators, as calculated from the graphs. It also describes the possible effects of the agents on nuclear import. **A**, experiments performed at 10^{-9} M dex concentrations; **B**, experiments performed at 10^{-10} M dex concentrations.

Table 6. Summary of Kinetic Data: Effect of Modulators of protein Phosphorylation on the Rate of Nuclear Import

[N]

A

10^{-9}M Dex	$t_{1/2}$ to Equilibrium after Pretreatment with modulator (min)								
CONSTRUCT	Ctl	TPA	FK	OA	H-7	Van	FK/TPA	FK/OA	**Possible Effect on N I**
mWT	35	15	12	20	85	60	30	30	TPA, FK and OA increased the rate of both WT and Arg484-His by ~ 2-fold. H-7 and Van decreased by ~2.5- and 2-fold respectively.
Arg_{484}-His	40	15	18	20	120	70	40	40	

B

10^{-10} M Dex							
CONSTRUCT	Ctl	TPA	FK	FK/TPA	FK/4PE	Steady state Levels	**Possible Effect on N I**
mWT	120	120	120	cd	120	WT Reduced by ~60 % after FK/TPA treatment	Jun/AP-1 repression of GR
Arg484-His	120	120				Arg484-His, Not affected	Trans-modulation of FK effect by TPA on GR action

N I = Nuclear import, cd = cannot be determined from the graph

Table 7. Summary of Kinetic Data for Nuclear Export of Wild Type Receptor and Arg484-His mutant. Shown are the $t_{1/2}$ values for return of nuclear receptors to the cytoplasm and % of fluorescent receptors retained in the nucleus at 24 h after hormone withdrawal, with the addition of modulator in the withdrawal medium. $t_{1/2}$ values were as calculated from the graphs for the GR constructs indicated above. It also describes the possible effects of the modulators on cytoplasmic redistribution of nuclear receptors.

Table 7. Summary of Kinetic Data: Effects of Modulators of protein Phosphorylation on the Rate of Cytoplasmic Redistribution of Nuclear Receptors After Hormone Withdrawal

[N]

A

t1/2 to Equilibrium after HWD with modulator (hr)						
CONSTRUCT	Ctl	TPA	H-7	RU486	**% Nuclear Fluorescent Cells at 24 h**	**Possible Effect on Cytoplasmic Redistribution**
mWT	11	5	cd	cd	H-7 and RU486 =95% For both WT and Arg484-His	TPA increases recycling, H-7 and RU486 completely block recycling this effect is independent of DNA binding.
Arg_{484}-His	5.5	2.5	cd	cd		

B

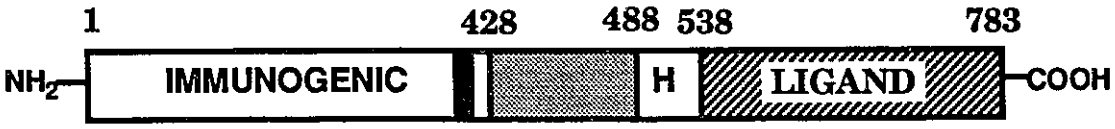
CONSTRUCT	FK	OA	Van	FK/TPA	FK/OA	**% Nuclear Fluorescent Cells at 24 h**	**Possible Effect on Cytoplasmic Redistribution**
mWT	21	cd	cd	cd	cd	Van and OA were > 70-90 % Nuclear,FK was 50 %.	All increased apparent nuclear retention, FK/TPA and FK/OA effect on WT cancelled out, and on Arg484-His, only TPA alone or FK alone effect was observed
Arg484-His	21	cd	cd	cd	cd		

HWD = Hormone Withdrawal, cd = cannot be determined from graph.

Figure 1A. Functional Domain Stuctures of Mouse GR. Shown are the immunogenic, DNA binding and ligand binding domains and the hinge region separating the latter two domains. The domains were assigned according to the primary sequence published by Danielsen et al[14].

Figure 1B. Structure of the GR Expression plasmid (pSV2Wrec). The filled-in segment and the thick black line represents coding and 3' non coding GR sequences, respectively. The unshaded areas are SV40 sequences and the thin line pBR322-derived sequences. Transcription starts at the SV40 promoter and proceeds clockwise through the GR sequences and terminates at the SV40 poly A addition site[14].

A


1
428
488
538
783
NH2
IMMUNOGENIC
H
LIGAND
COOH

B

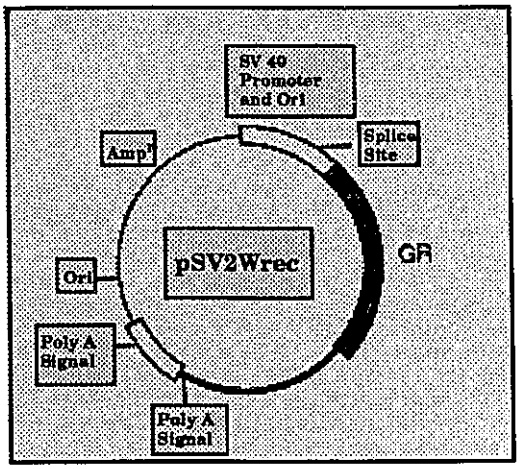
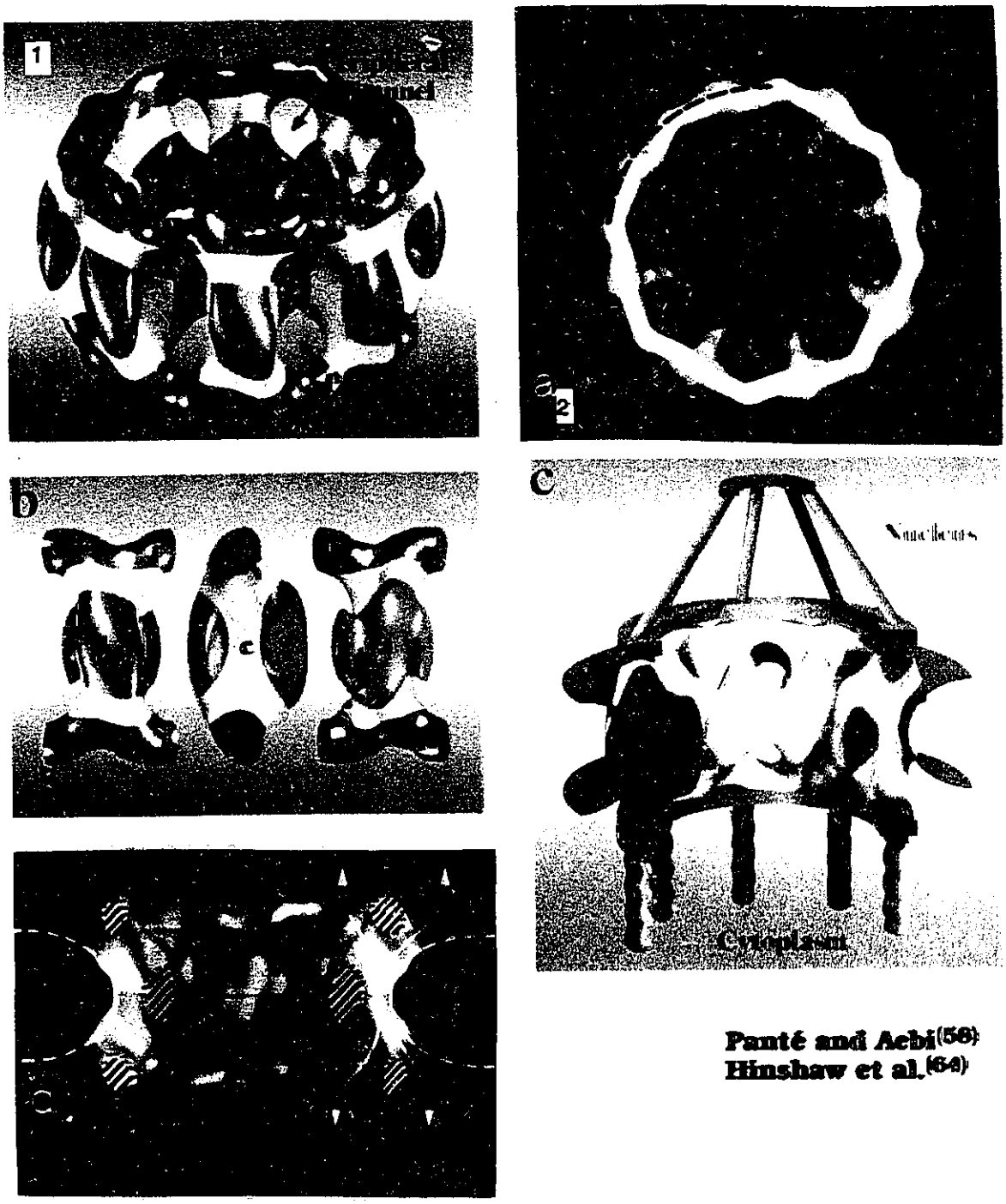

SV 40 Promoter and Ori
Amp'
Splice Site
pSV2Wrec
GR
Ori
Poly A Signal
Poly A Signal

Figure 1

Figure 2A. The Architecture and design of Nuclear Pore Complex. Shown are color coded calculated 2D and 3D maps of detergent released NPC, as obtained by EM studies coupled with image analysis. **a)** Oblique (a_1) and en face (a_2) views of the 3D map representing the basic framework, i.e. spoke complex, of the NPC which exhibits strong 822 symmetry and thus consists of 2-identical halves relative to the central plane of the NE. The map shows that each of the 8-spokes seen in en face views of the NPC is built from annular, column ring and lumenal subunits. Hinshaw et al. have proposed that the symmetric assembly revealed by the EM and image analysis plays an indirect role in active nucleocytoplasmic transport, serving as a framework that is constructed according to 822 symmetry[64]. **b)** One multi-domain spoke cut out from the basic framework of the NPC shown in **(a)** Each spoke shows the four distinct morphological elements of which they are built; i.e. annular (a), column (b), ring (r) and lumenal (l) subunits. **c)** Schematic diagram of NPC embedded in the double membrane NE (cream and brown). Its major structural component include the basic framework, showing peripheral cytoplasmic filaments (purple) and nuclear baskets (orange). **d)** The front half of an edge view has been cut away along a diagonal passing through two of the peripheral channels. Shaded areas indicate the cut ends of the rings and the inner annulus. The path of the nuclear membrane are indicated by a dashed line. Probable routes for passive nucleocytoplasmic exchange through one of the channels are indicated by double headed arrows. The subunits indicated in **(b)** are shown here in colors as follows: annular subunit (green), rings (yellow), and lumenal subunits (blue). The remaining tan colored parts of the 3D map represents the column subunits.



Panté and Aebi[58]
Hinshaw et al.[64]

Figure 2A

Figure 2B. Three dimensional surface Views of a Detergent Extracted Nuclear pore Complex. The spoke-ring assemly including cytoplasmic particles (CP) is medium blue, the lumenal ring is dark blue and the central NPC-transporter is pink. **a)** Cytoplasmic surface view of the NPC at an angle of 10^{o} Lumenal spoke (LS), Radial arm domain (RA) and cytoplasmic thin ring (CR). **b)** A view of the NPC rotated at 45^{o} about a vertical axis. The lumenal ring is highlighted in this view. The cytoplasmic particles (CP) and the nucleoplasmic thin ring (NR) are shown. **c)** A view of the NPC from the cytoplasmic surface is shown in the same orientation as **(a)** but the top portion of the cytoplasmic particles is removed to show the packing of the transporter within the inner spoke ring. **d)** A cut-away view of the spoke ring assembly is presented with the central transporter removed to show the internal surface of the spoke-ring assembly. The lumenal ring (LR) and the cytoplasmic particles (CP) are indicated. **e)** A second cut away view is shown with the transporter left intact. In this view the cutting plane is oriented along a putative radial arm two-fold axis, between adjacent spokes. **f)** A cut-away side view of the NPC is shown in the same orientation as **(d)** but with the transporter left intact. Internal channels located between the hour-glass shaped transporter and the inner spoke ring are revealed. The transporter spans the width of the NPC and the local two-fold axis of the transporter is offset from the approximate two-fold axis of the spoke ring assembly by ~ 50 Å, towards the cytoplasmic/top surface. (Akey and Rademercher)[65].

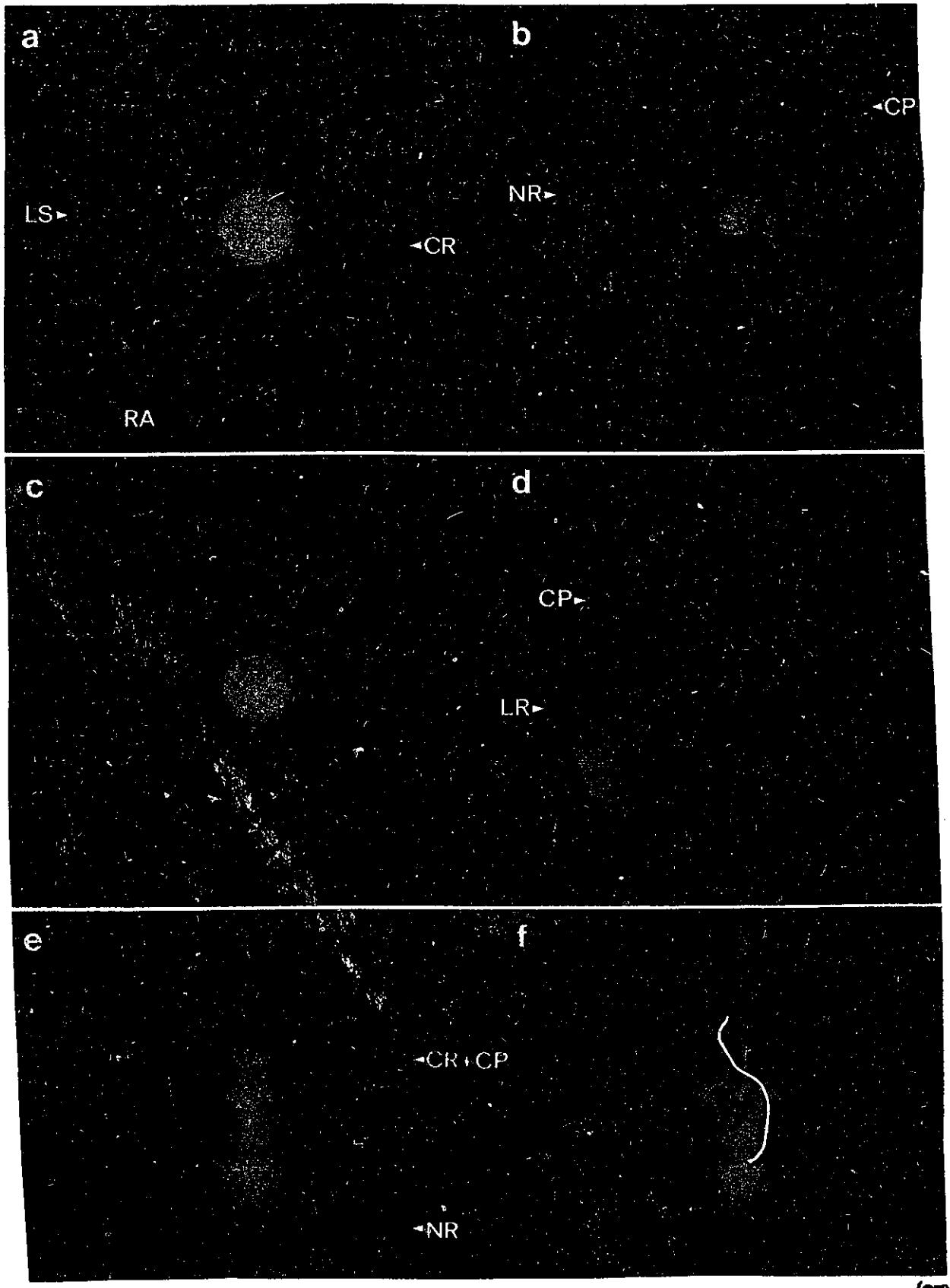
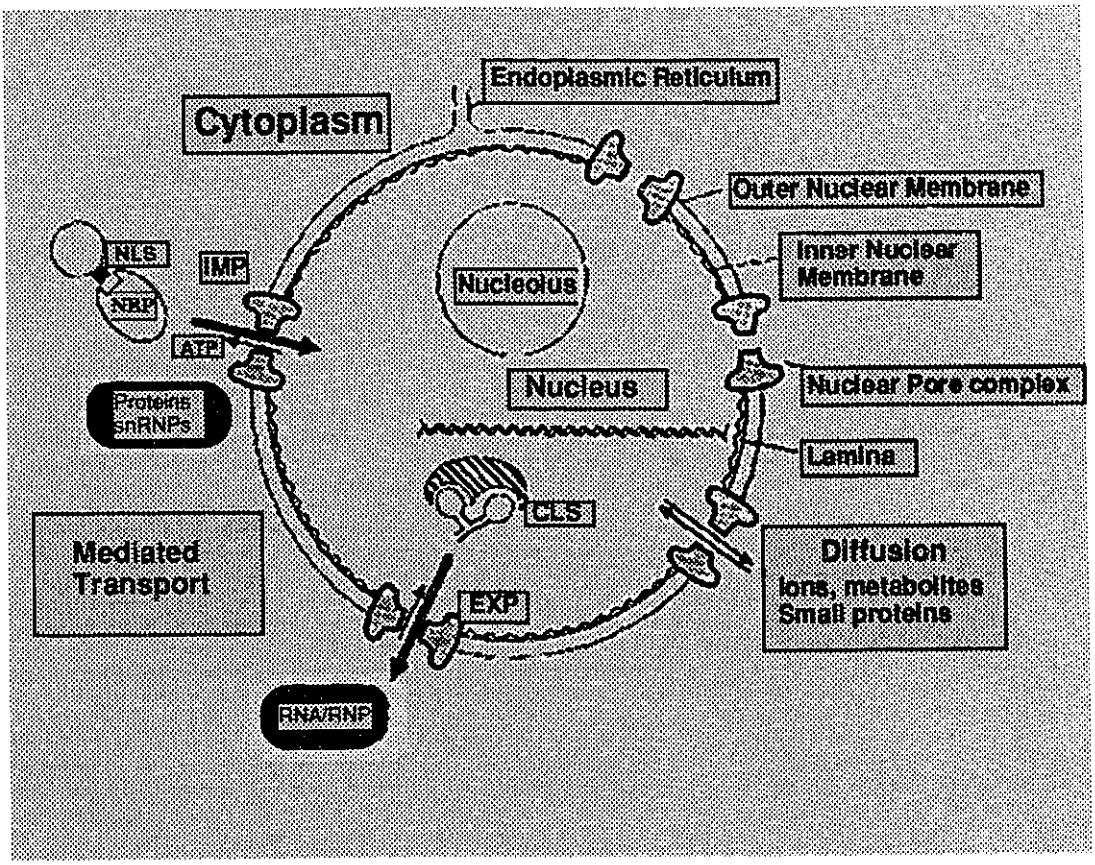


Figure 2B **Akey and Rademercher**[65]

Figure 3. Bidirectional Transport of Proteins Through Nuclear Pore Complexes. A schematic representation of the structural components of the NE include a double membrane, NPCs, and nuclear lamina. The NPCs provide aqueous channels with a functional diameter of 9-10 nm, allowing diffusion of ions, small molecules and proteins with molecular masses up to 40-60 kDa. The transport of larger karyophilic proteins through the pore is an active process: it is temperature dependent, requires ATP, and displays saturation kinetics[65]. Export of some proteins is controlled by tight binding to intranuclear structures[187] and also it may be NLS-mediated[189].
Imp, Import; Exp, Export; NE, Nuclear Envelope; NPC, Nuclear Pore Complex; NLS, Nuclear Localization Signal; NBP, NLS-Binding proteins.



Nigg et al.[167]

Figure 3

Figure 4. Optimisation of Transfection Efficiency by Varying Different Conditions of Lipofection. A bar representation of the effect of varying the lipofectin : DNA (μg/μg) on β-galactosidase expression after transient transfection in COS-7 cells. Cells were co-transfected with a β-galactosidase expression vector and pBR322 plasmid as carrier. The lipofectin:DNA was varied as indicated in Table 1. After 42 h cells were harvested and cell extracts were prepared for β-galactosidase activity, estimated after determining the absorbance at 420 nm (A_{420}) measurements as Units/μg/of protein/min as described in Materials and Methods. Data represents mean of two separate experiments.

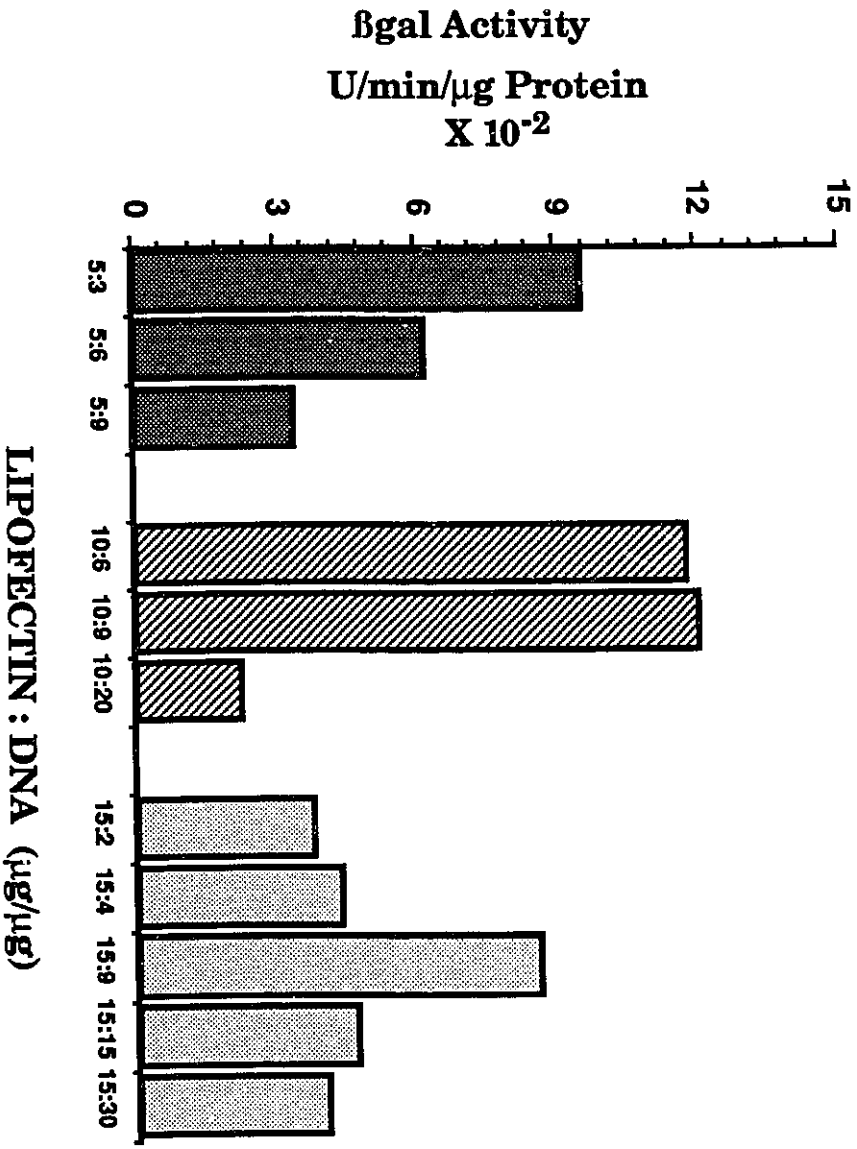

ßgal Activity
U/min/µg Protein
X 10-2
0
3
6
9
12
15
5:3
5:6
5:9
10:6
10:9
10:20
15:2
15:4
15:8
15:15
15:30
LIPOFECTIN : DNA (µg/µg)
TRANSFECTION BY LIPOFECTION

Figure 4

Figure 5a. Optimisation of Transfection Efficiency by Varying Different Conditions of Electroporation

A bar representation showing the effect of varying a) No. of cells per cuvette b) voltage for electroporation on β-gal expression after transient transfection in COS-7 cells. Cell extracts were prepared 42 h after transfection and β-galactosidase activities were determined as described. Data are means of two separate experiments.

TRANSFECTION BY ELECTROPORATION

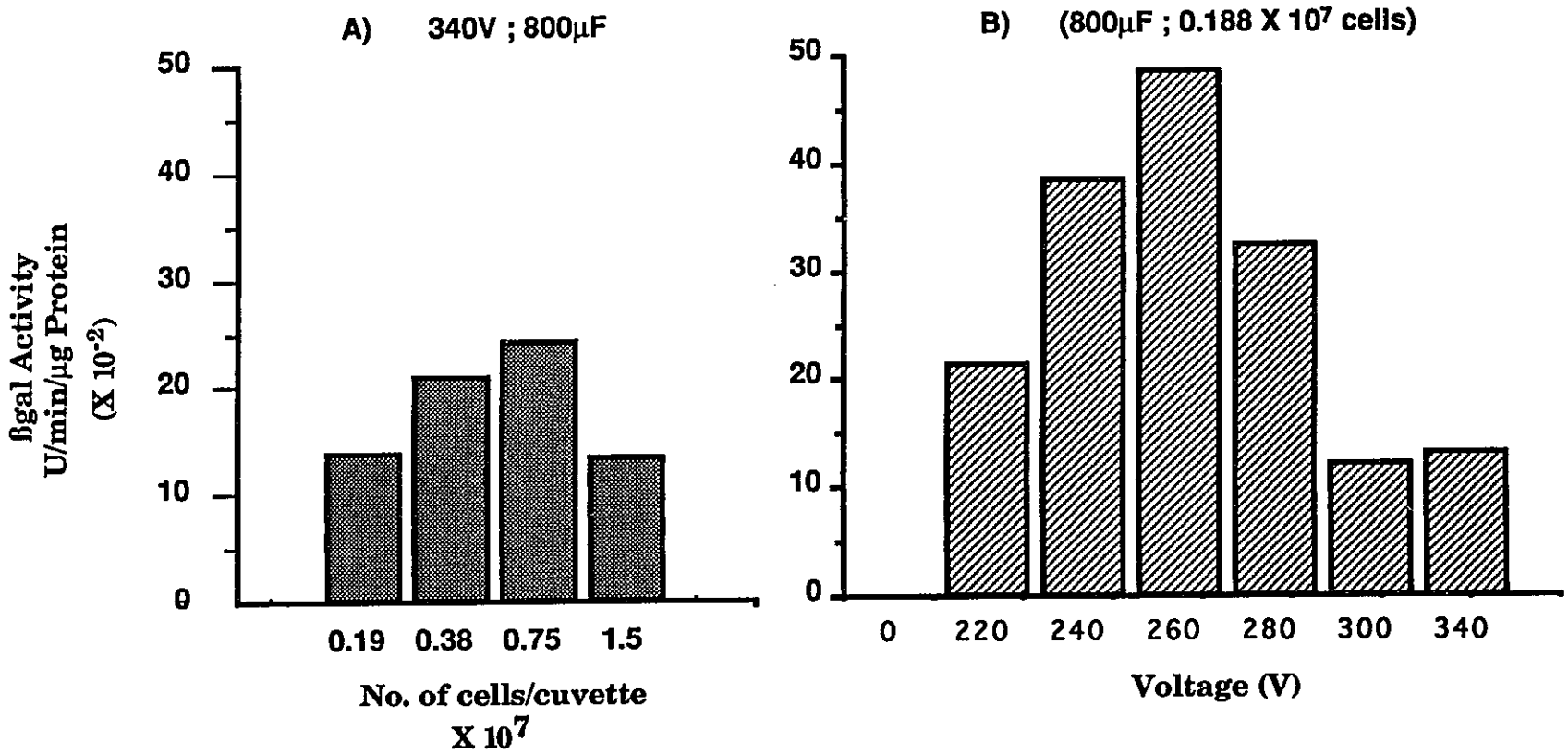


Figure 5a

Figure 5b. Optimisation of Transfection Efficiency by Varying Different Conditions of Electroporation

A bar representation showing the effect of varying: c) capacitance used for electroporation on β-gal expression after transient transfection in COS-7 cells. Cell extracts were prepared 42 h after transfection and β-galactosidase activities were determined as described. Data are means of two separate experiments.

TRANSFECTION BY ELECTROPORATION

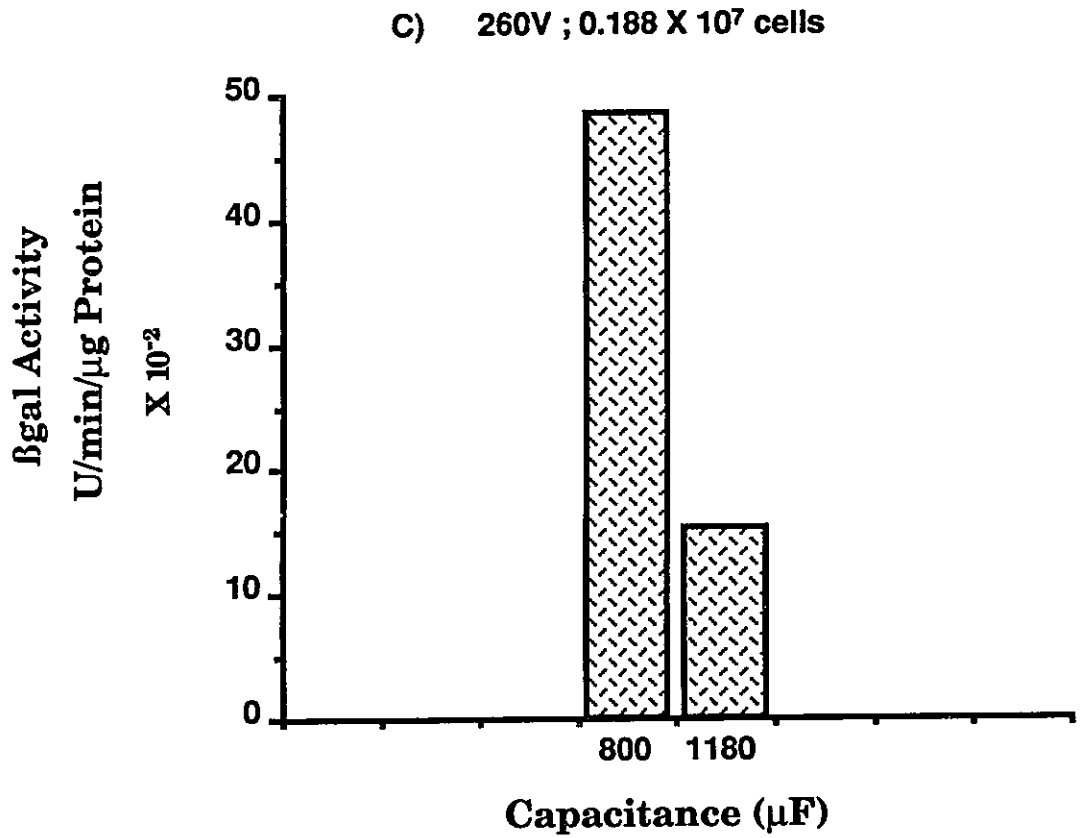


Figure 5b

Figure 6a. Immunoblot of COS-7 Cell Extract Containing Expressed Wild Type Glucocorticoid Receptor after tranfections by Lipofection and Electroporation. Wild type GR was transfected in COS-7 cells by lipofection and electroporation, varying the amount of the GR construct. Cell extracts were prepared as described in Materials and Methods, electrophoresed on 7.5 % SDS-PAGE and transferred onto nitrocellulose. BuGR-2 monoclonal and sheep anti-mouse IgG conjugated to Horse-radish peroxidase (HRP) were used as primary and secondary antibodies respectively. The amount of DNA used in the transfection experiments are shown above the lanes. All lanes were loaded with 100 µg protein. Lanes 1 to 3 were loaded with COS cell extracts obtained after transfection with 2.5, 0.5 and 0.1 µg) of the GR construct but maintaining a lipofectin to DNA ratio of 5:3, by the addition of carrier DNA to make up for the DNA requirement. Lane 4 was loaded with extract from mock transfected cells. Lanes 5 and 6 were loaded with extracts from cells transfected with 20 and 1 µg of GR construct after electroporation respectively. Lanes 7 and 8 were loaded with cell extracts from S49 whole cell (S49 WC) and cytosol (S49 C) respectively, whilst lane 9 was loaded with extracts from adrenalectomised rat liver. These lanes (7, 8 and 9) represent controls for GR. Protein molecular weight marker in one gel lane shown in kilodaltons (kDa), allowed estimation of the molecular weight of the protein bands.

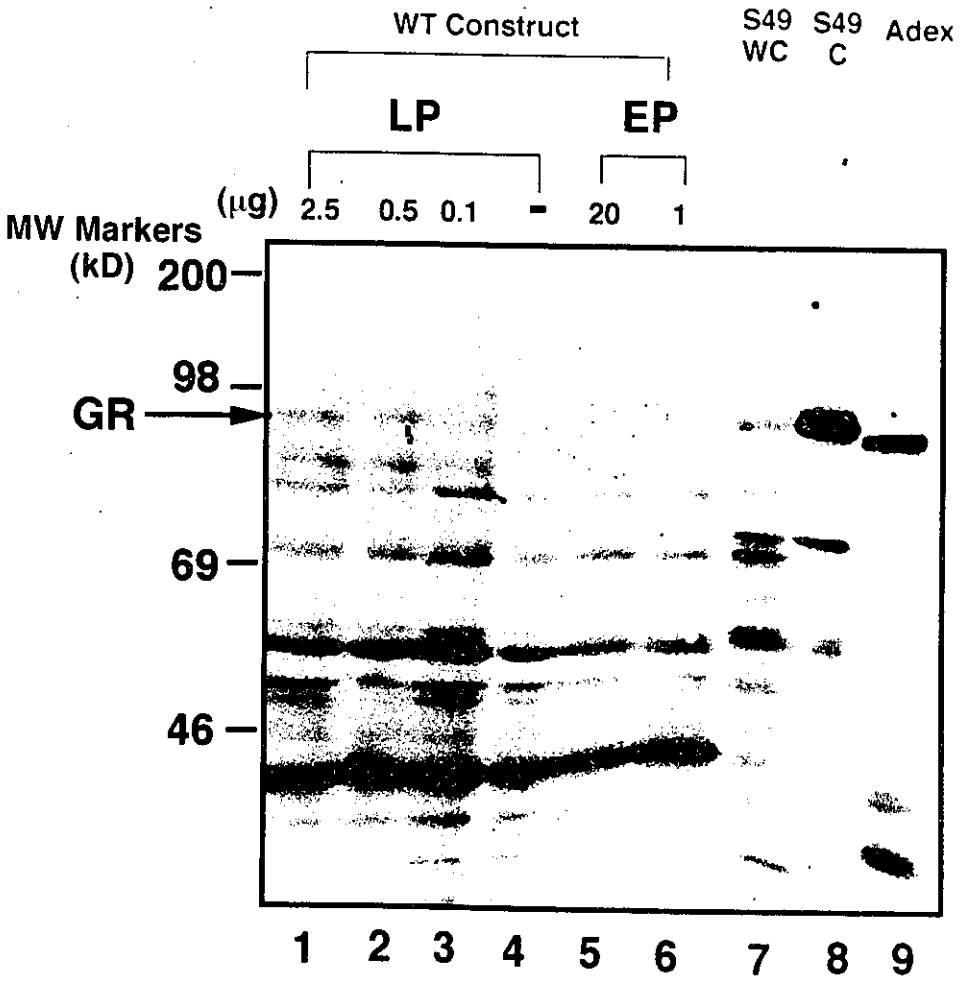

WT Construct
S49 WC
S49 C
Adex
LP
EP
(μg)
2.5
0.5
0.1
-
20
1
MW Markers (kD)
200
98
GR
69
46
1
2
3
4
5
6
7
8
9

Figure 6a

Figure 6b. Immunoblot of COS-7 Cell Extract Containing Expressed Wild Type Glucocorticoid Receptor from transfection by lipofection. Wild type GR construct was transfected in COS-7 cells by lipofection using varying amounts of the GR construct, but maintaining a lipofection to DNA ratio of 10:6 μg. Cell extracts were prepared as described in Materials and Methods, electrophoresed on 7.5 % SDS-PAGE and transferred onto nitrocellulose. BuGR-2 monoclonal and sheep anti-mouse IgG conjugated to Horseradish Peroxidase (HRP) were used as primary and secondary antibodies respectively. The amounts of DNA used in the transfection experiments shown above the lane numbers. All lanes were loaded with 100 μg protein. Lanes 1 to 3 were loaded with 2.5, 0.5 and 0.1 μg of the GR construct respectively, whilst maintaining a lipofectin to DNA ratio of 10:6. Lane 4, a negative control for GR expression, was loaded with extracts from β-gal (mock) transfected cells, using 10:6 lipofectin : DNA (μg/μg). Lanes 5 and 6 were loaded with cell extracts from S49 whole cell (S49 WC) and cytosol (S49 C) respectively, and they represent controls for GR. Protein molecular weight markers in one gel lane shown in kilodaltons (kDa), allowed estimation of the molecular weight of the protein bands.

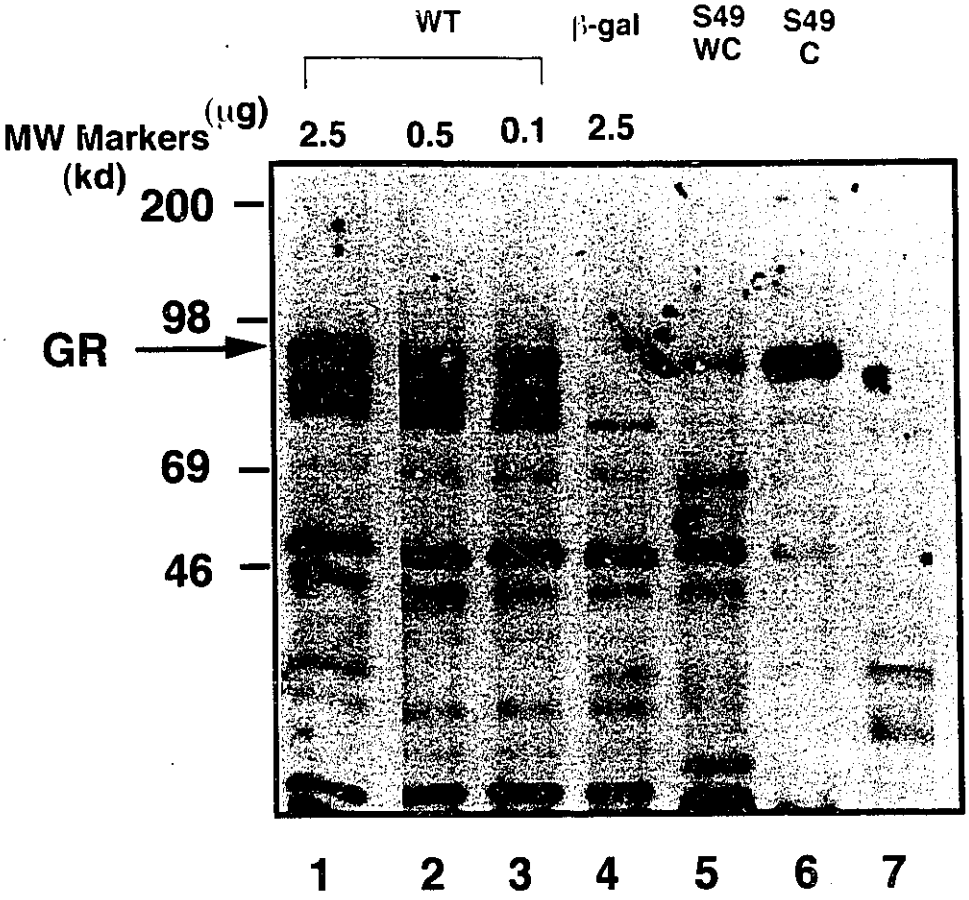
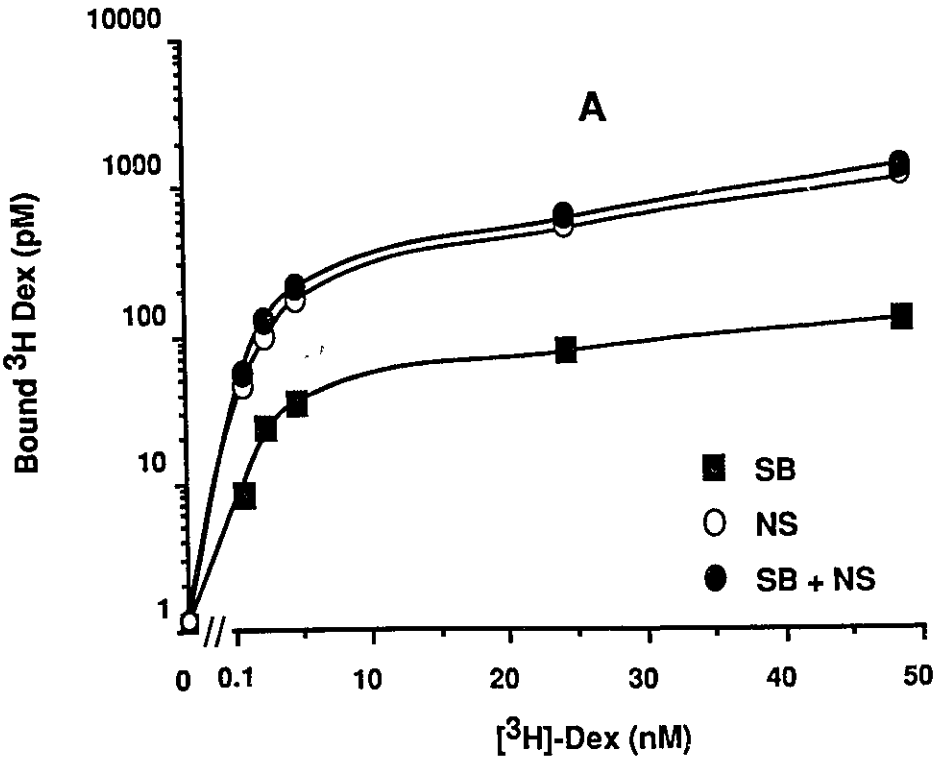

WT
β-gal
S49 WC
S49 C
(μg)
MW Markers (kd)
2.5
0.5
0.1
2.5
200
98
GR
69
46
1
2
3
4
5
6
7

Figure 6b

Figure 7. Characterization of [^{3}H]-dex Binding to Expressed Wild Type GR in COS-7 Whole Cell Extract. Cell extracts from expressed WT receptor were prepared, as described in Materials and Methods. **A)** Bound labeled ligand concentration (nM) vs Total ligand concentration, Owith and ●without the addition of 200-fold excess of unlabeled dex. ■Specific binding (SB) is the calculated difference between binding in the presence and absence of excess unlabeled ligand. **B)** Scatchard plot of specific binding data from (**A**); Plot of the ratio of Bound to Free dex vs Bound dex concentration (B/F vs B).


A
10000
1000
100
10
1
Bound ^{3}H Dex (pM)
SB
NS
SB + NS
0
0.1
10
20
30
40
50
[^{3}H]-Dex (nM)

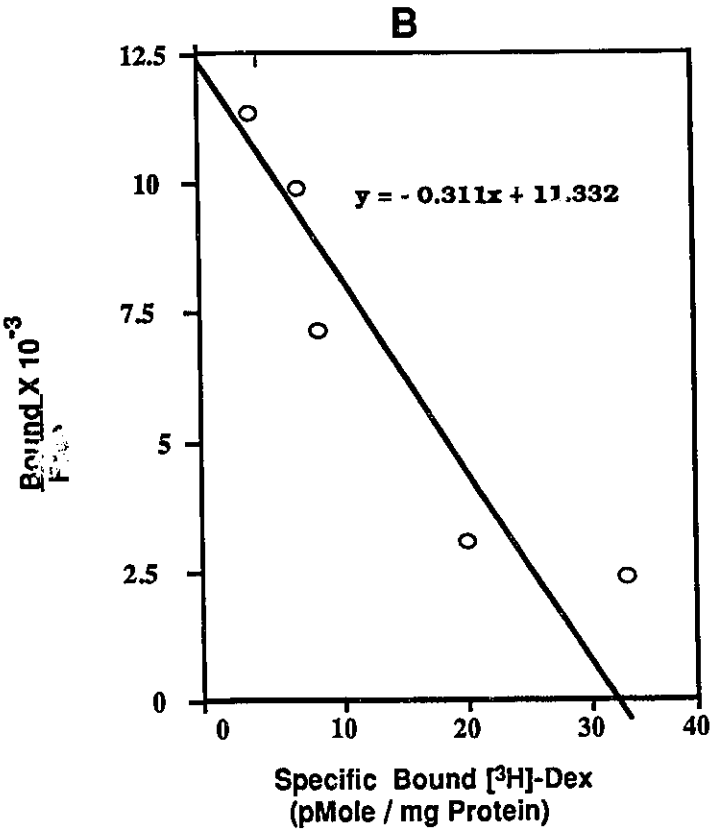

B
12.5
10
7.5
5
2.5
0
y = - 0.311x + 11.332
0
10
20
30
40
Specific Bound [^{3}H]-Dex
(pMole / mg Protein)

Figure 7

Figure 8. Classification of Immunofluorescence Staining Patterns After Transfecting COS-7 Cells with WT or Mutant Receptor Expression Vectors. COS-7 cells were transfected by lipofection, using 10:3 μg Lipofectin:DNA. Transfected cells were grown on cover slips for 42 h before hormone treatment. After hormonal stimulus, cells were fixed in 3 % paraformaldehyde, permeabilised with 0.5 % Triton X-100. Cells were stained using primary BuGR-2 monoclonal antibody (for mGR experiments) or anti human polyclonal (PA 510) antibody (for hGR experiments), followed by secondary FITC-conjugated IgG. Localization to the nucleus was assessed on a Zeiss Axiophot photomicroscope. Immunofluorescent cells were classified into 5 categories according to their staining pattern as follows: N, cells with exclusively nuclear staining; N>C, in which the intensity of nuclear staining exceeds that of cytoplasmic; cells which contain > 50 %-90 % nuclear staining; N=C, cells in which the intensity of nuclear staining equals cytoplasmic; N<C, cells in which the intensity of cytoplasmic staining exceeds that of nuclear ; C, cytoplasmic staining.

[N]

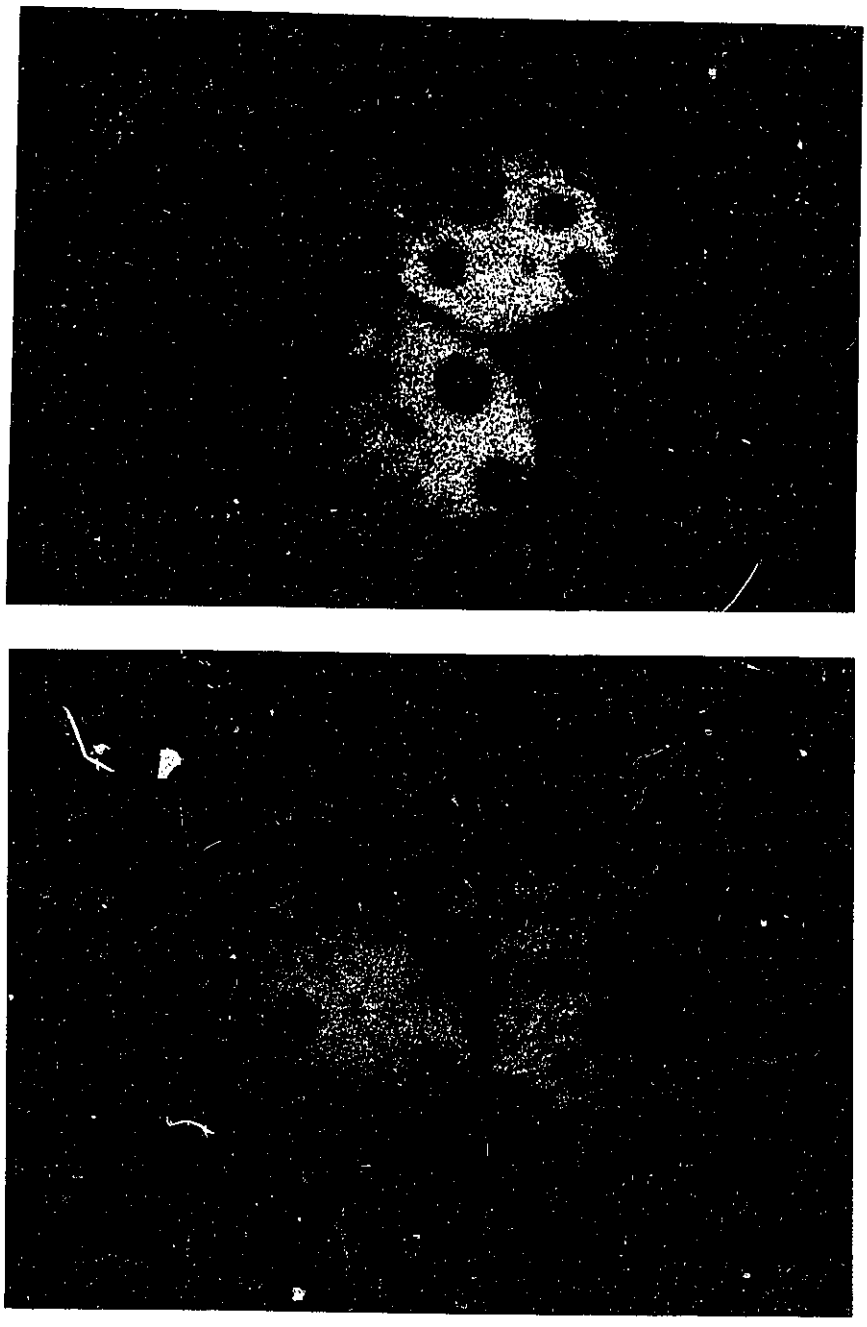


Figure 8

[N>C]

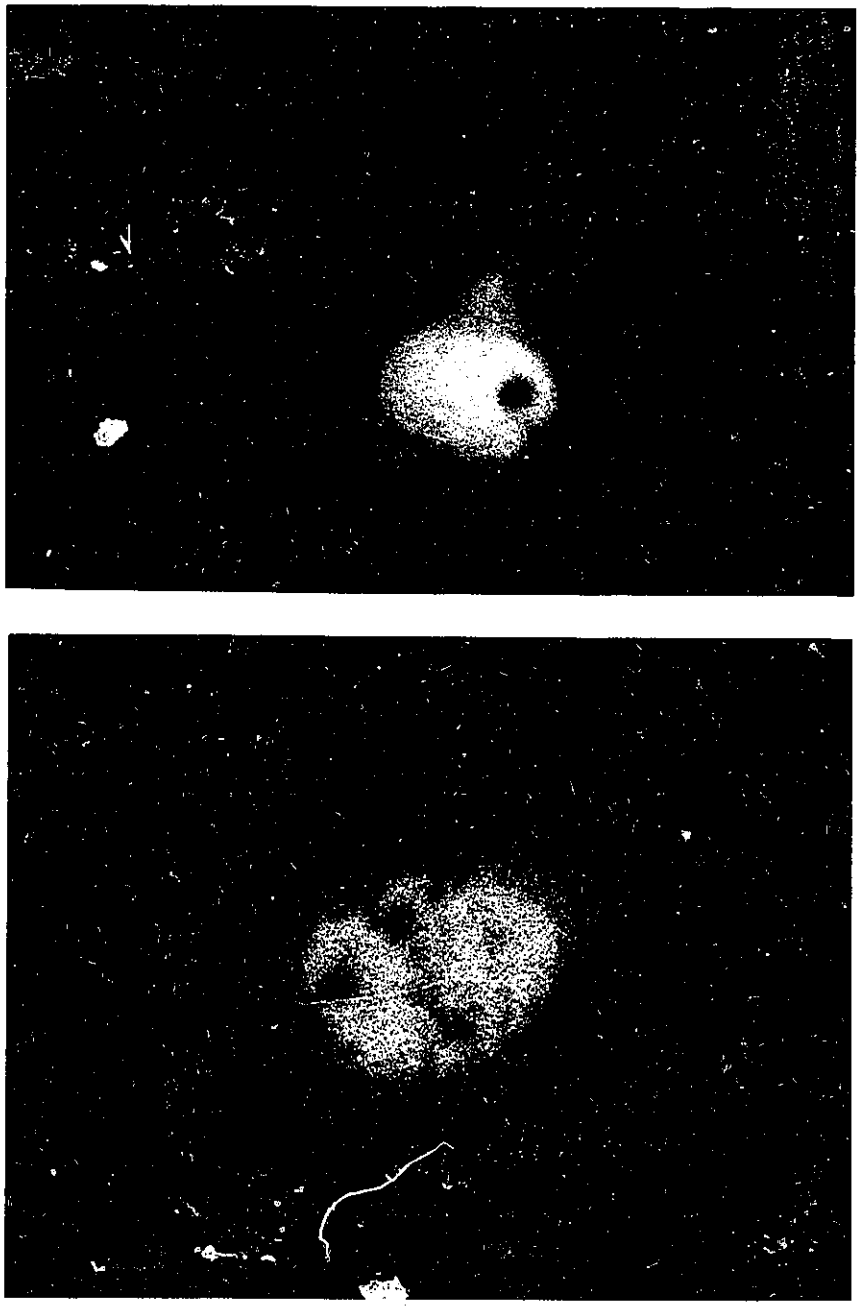


Figure 8

[N=C]

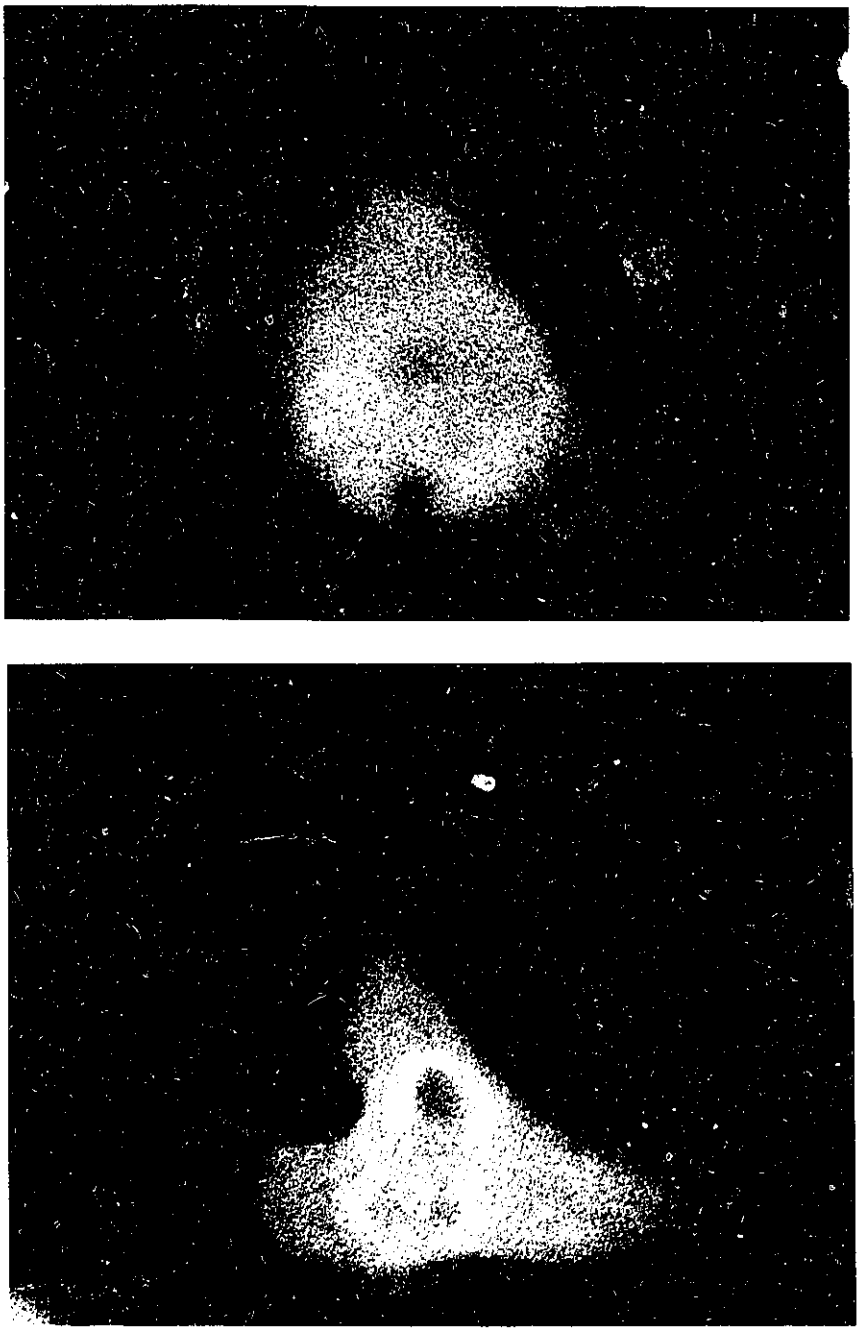


Figure 8

[N<C]

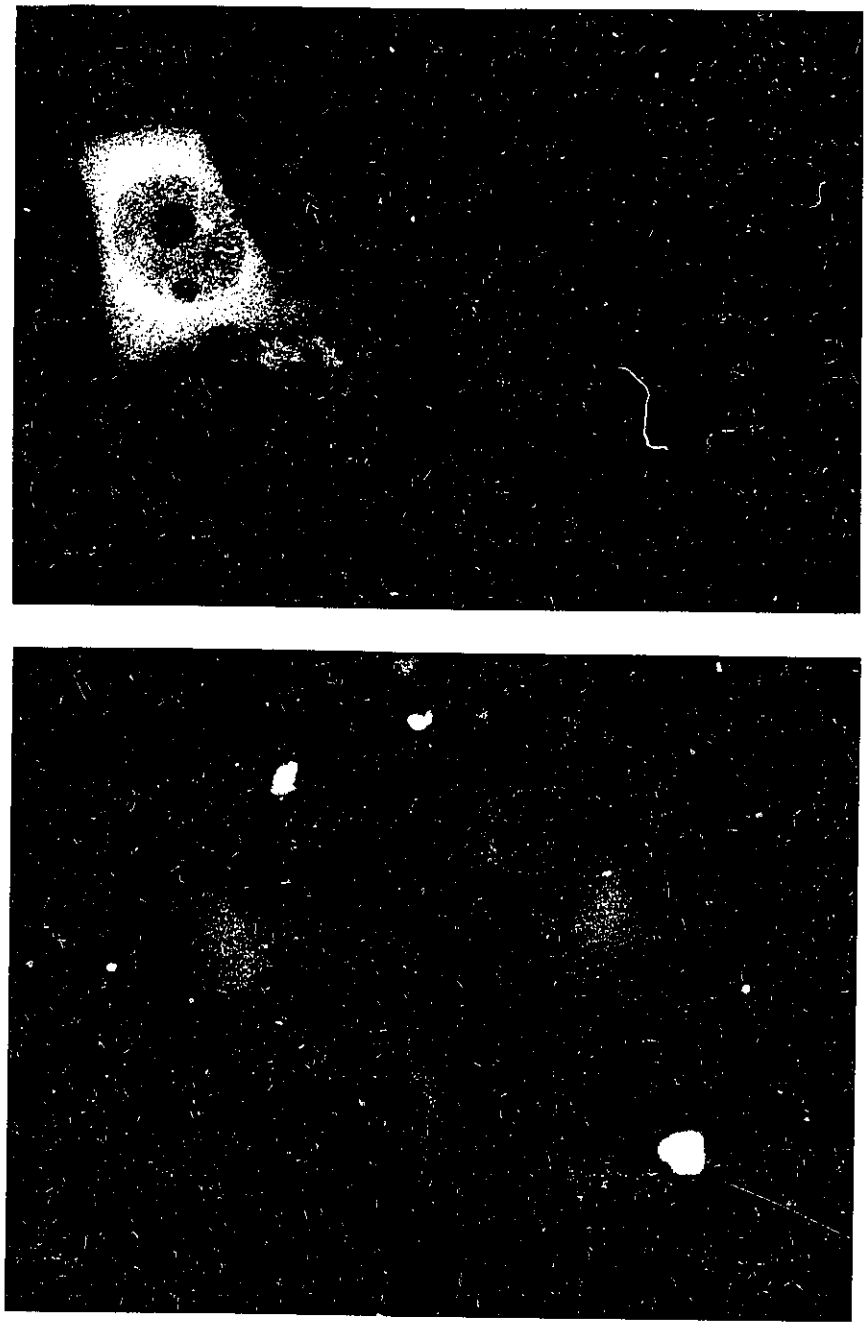


Figure 8

[C]

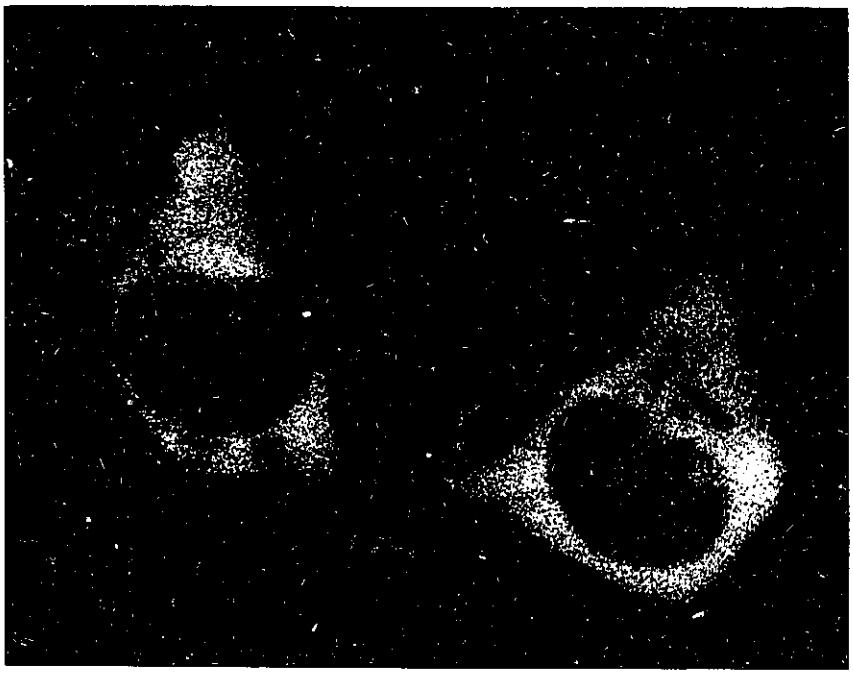


Figure 8

Figure 9. Schematic Representation of Mouse and Human GR Constructs, used in the Kinetic Studies. The numbers above each box correspond to the amino acid position in the protein sequence of the receptor. **(A)** Mouse GR; the three amino acids shown in the mouse wild type GR (mWT) are those changed in NBrec (nt^-) mutant relative to the mWT. The Val437-Gly change is a silent mutation and has been shown to be polymorphic among different cell lines[14]. Tyr770-Asn and Arg484-His are mutant GR constructs containing single amino acid substitutions present in NBrec. **(B)**, human WT GR (hWT) and a corresponding first zinc finger deletion mutant (hΔ420-451), with the deletion shown as a gap, numbers above refer to the amino acid position from -NH2+ to -COO- terminal of the deleted sequence.

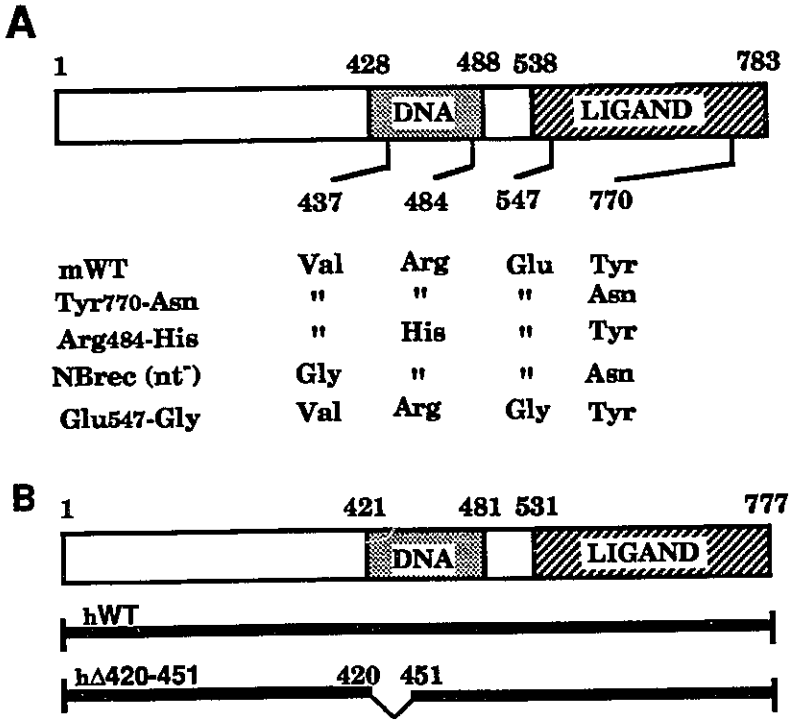

A
1
428
488
538
783
DNA
LIGAND
437
484
547
770
mWT Val Arg Glu Tyr
Tyr770-Asn " " " Asn
Arg484-His " His " Tyr
NBrec (nt⁻) Gly " " Asn
Glu547-Gly Val Arg Gly Tyr
B
1
421
481
531
777
DNA
LIGAND
hWT
hΔ420-451
420
451

Figure 9

Figure 10. Effect of Impaired DNA and Defective Hormone Binding on the Kinetics of Nuclear Uptake of Mouse Wild-Type and Mutants of GR. The kinetics of the following GR derivatives were monitored by indirect immunofluorescence: mWT as a positive control for maximal nuclear entry; a hormone binding mutant, Glu547-Gly, as a negative control; the double mutant, NBrec (nt^-); and the GR mutants containing the single amino acid substitutions in the nt- GR (Tyr770-Asn and Arg484-His). Cells were transfected by lipofection as described in Materials and Methods. After 1 μM dex treatment, at the times indicated, cells were fixed in 3 % paraformaldehyde and prepared for indirect immunofluorescence, to monitor the rate of nuclear uptake over a 24 h period. Subcellular localization was visualized on a Zeiss Axiophot photomicroscope. To quantitate the data, at least 400 fluorescent cells were counted for each time point, using standard double blind encryption and classified into one of five categories according to their staining pattern. Graphs were plotted as % Nuclear Fluorescent cells, defined by [N+N>C] or [N] as a function of time. OWT, ●Tyr770-Asn, □Arg484-His, ■NBrec and ΔGlu547-Gly. The DNA binding mutants displayed reduced steady state equilibrium levels, while the Tyr770-Asn substitution did not affect the rate of import. The error bars represent standard errors determined from 3-5 transfection experiments.

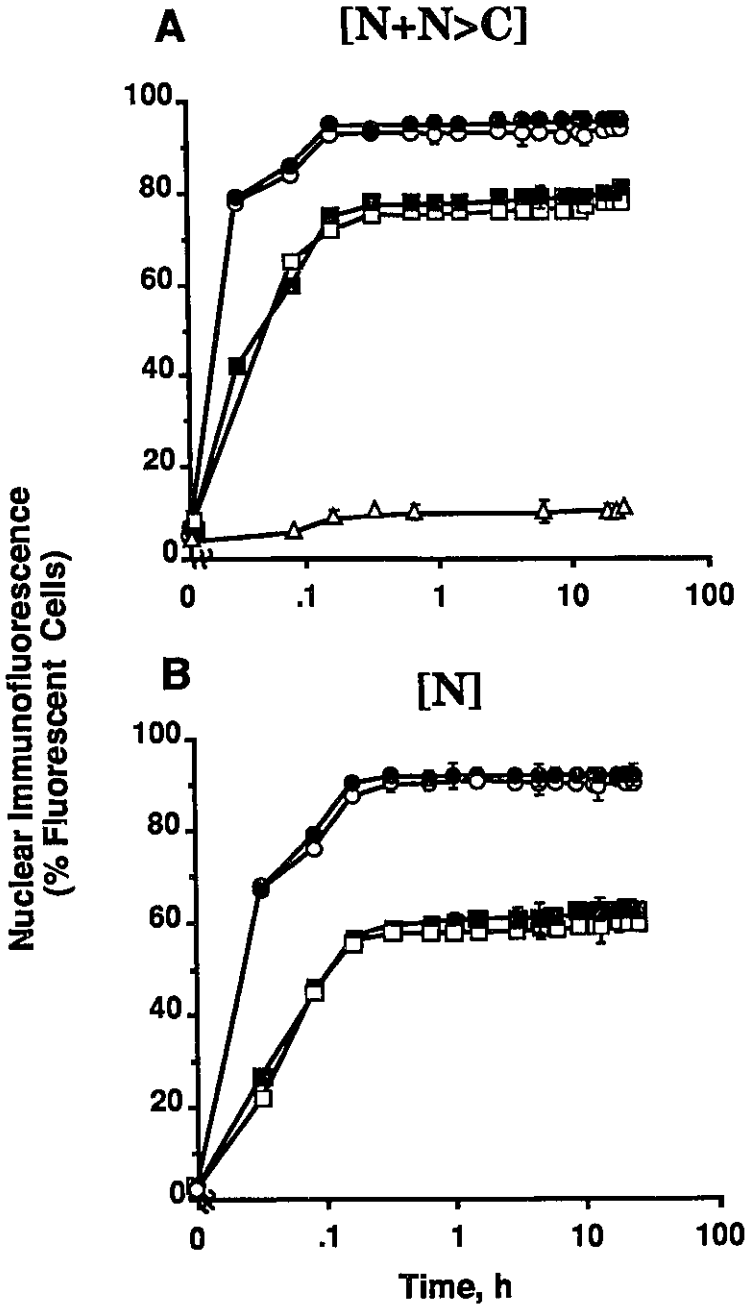

A
[N+N>C]
100
80
60
40
20
0
0
.1
1
10
100
B
[N]
100
80
60
40
20
0
0
.1
1
10
100
Time, h
Nuclear Immunofluorescence
(% Fluorescent Cells)

Figure 10

Figure 11. The Influence of NL-1 on the Kinetics of Nuclear Uptake of Human Wild Type and DNA Binding Mutants. The kinetics of nuclear uptake of the following human GR constructs were monitored after dex stimulation for 24 h: Human WT, hWT; DNA binding mutant (hΔ420-451), which has a deletion of a first zinc finger; DNA binding mutant (hΔ450-487) which has a deletion in the second zinc finger, including N-terminal NL-1; and a DNA binding mutant (hΔ428-490) which has a deletion of both zinc fingers, and also including N-terminal NL-1. COS-7 cells were transfected by lipofection and immunofluorescence, procedures for cell counting and plotting of graphs were as described in legend to Figure 10. The rate of uptake of hWT was identical to mWT (●hWT). The first zinc finger mutant with intact NL-1 (OhΔ420-451) displayed similar kinetics of uptake as Arg484-His. both DNA binding mutants ■h (Δ451-487) and (□hΔ428-490) which also had impaired NL-1 showed a marked reduction (60-fold decrease) in the rate of uptake as well as reduced nuclear equilibrium levels. The error bars represent standard errors determined from 3-5 transfection experiments.

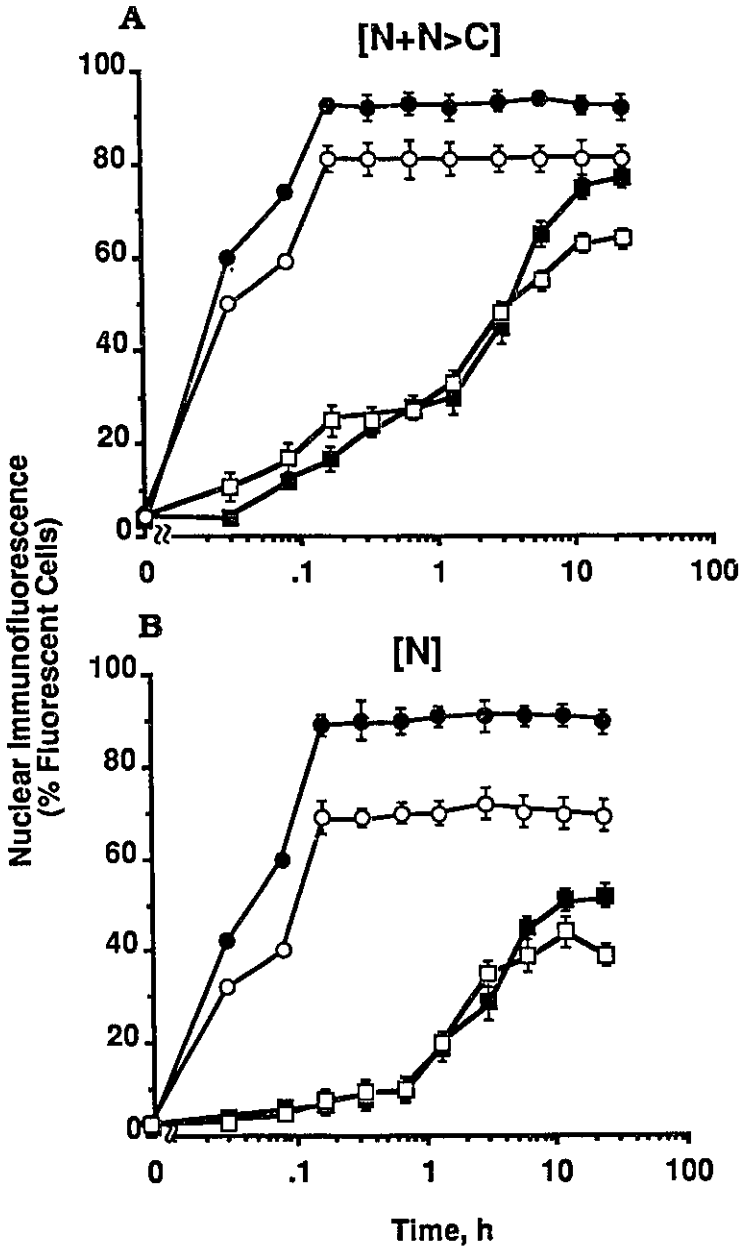

A
[N+N>C]
100
80
60
40
20
0
0
.1
1
10
100
B
[N]
100
80
60
40
20
0
0
.1
1
10
100
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h

Figure 11

Figure 12. Dose Response Kinetics of WT and Mutant Glucocorticoid Receptors. The kinetics of nuclear uptake of expressed WT and mutant receptors over a 6 h period were monitored using dex concentrations between 10^{-6} M and 10^{-10} M using the transfection, indirect immunofluorescence, and procedures for cell counting and plotting of graphs, as described in legend to Figure 10. The GR derivatives used were as follows: (**A** and **B**) mWT, (**C** and **D**) Tyr770-Asn, (**E** and **F**) Arg484-His single mutants; and (**G** and **H**) NBrec (nt^-). ●10^{-6} M, ○10^{-7} M ■10^{-9} M □10^{-10} M. The Tyr770-Asn mutant, previously reported to have only 50 % of hormone binding activity displayed identical uptake kinetics as mWT, even at sub-physiological concentrations of dex. The error bars represent standard errors determined from 3-5 transfection experiments.

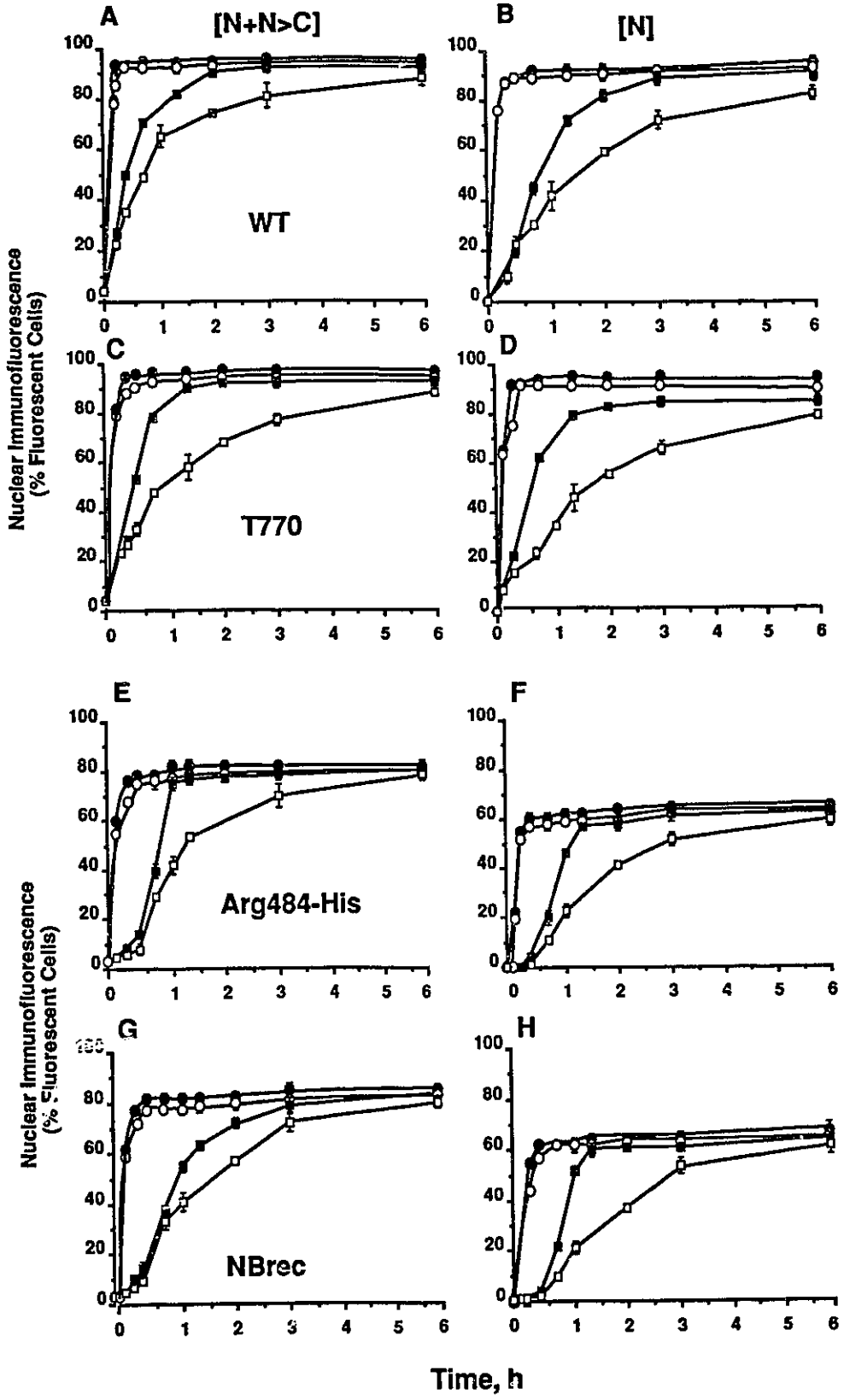

A
[N+N>C]
B
[N]
WT
C
T770
D
E
Arg484-His
F
G
NBrec
H
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h

Figure 12

Figure 13a. Kinetics of Nuclear Export of Mouse Wild Type and DNA Binding Mutants of GR Following 1μM Cortisol Withdrawal. COS-7 cells were transfected with the mWT and Arg484-His mutant by lipofection, as described in Materials and Methods. After 1 h treatment with 1 μM cortisol, 10 % BSA supplemented DMEM serum free medium (withdrawal medium) was added. At the times indicated cells were fixed with 3 % paraformaldehyde and prepared for indirect immunofluorescence, as described in the legend to Figure 10. Graphs were plotted as % Nuclear Fluorescent cells retained after hormone withdrawal, as a function of time. To enable direct comparison, the absolute [N] and [N>C] values at the time of hormone withdrawal (T_0) for WT receptor and the mutants were set to 100 %. (A), % cells showing total nuclear staining i.e. exclusively nuclear plus nuclear staining greater than cytoplasmic, [N+N>C]; (B), % cells showing exclusively nuclear fluorescent staining, [N]. ●mWT, ○Tyr770-Asn, ■Arg484-His and □NBrec. DNA binding mutants are exported 2-3 fold faster than WT. The export rate of Tyr770-Asn was identical to WT. The error bars represent standard errors determined from 3-5 transfection experiments.

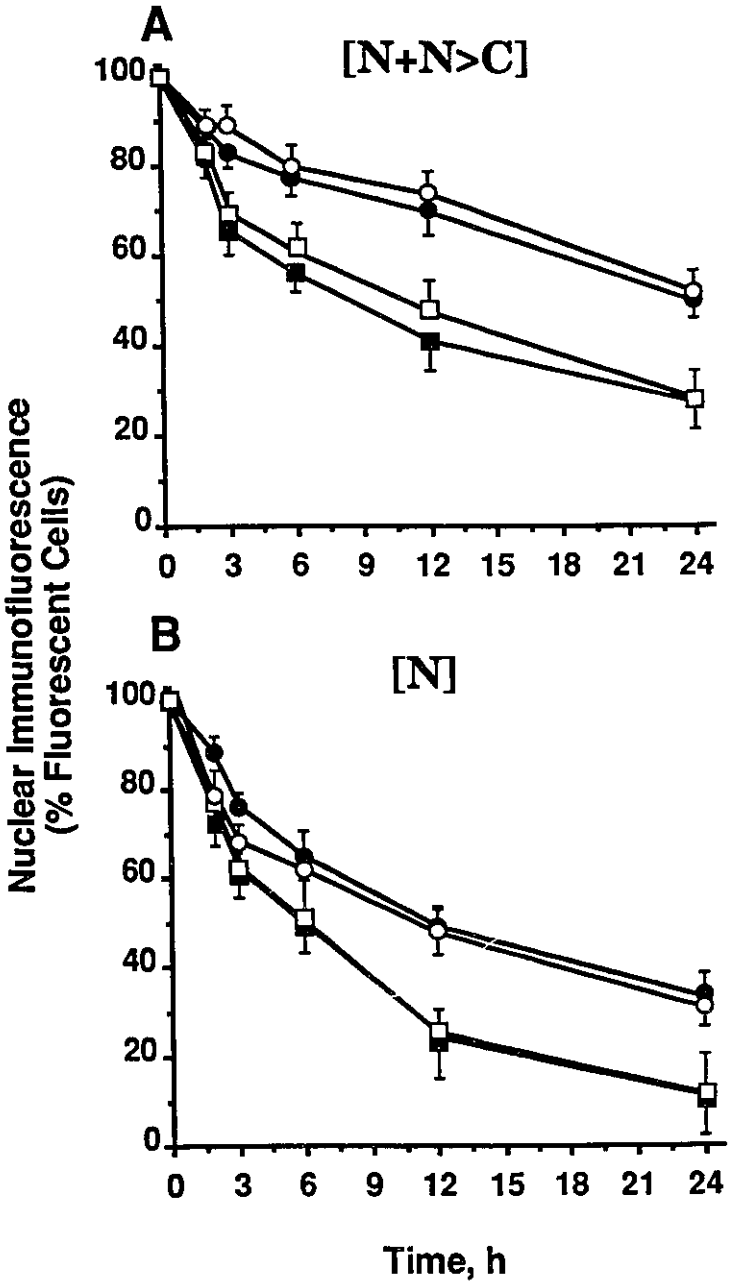

A
[N+N>C]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
B
[N]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h

Figure 13a

Figure 13b. Kinetics of Nuclear Export of Mouse Wild Type and DNA Binding Mutants of GR Following 1μM Cortisol Withdrawal With Added Cycloheximide in the Withdrawal Medium. COS-7 cells were transfected with the mWT and Arg484-His mutant by lipofection, as described in Materials and Methods. After 1 h treatment with 1 μM cortisol, 10 % BSA supplemented DMEM serum free medium (withdrawal medium) was added together with 50 μg/ml cycloheximide (CHD). At the times indicated cells were fixed with 3 % paraformaldehyde and prepared for indirect immunofluorescence, as described in the legend to Figure 10. Graphs were plotted as % Nuclear Fluorescent cells retained after hormone withdrawal, as a function of time. To enable direct comparison, the absolute [N] and [N>C] values at the time of hormone withdrawal (T_o) for WT receptor and the mutants were set to zero. (A), % cells showing total nuclear staining i.e. exclusively nuclear plus nuclear staining greater than cytoplamic, [N+N>C]; (B), % cells showing exclusively nuclear fluorescent staining, [N]. ●mWT, ○Tyr770-Asn, ■Arg484-His and □NBrec. There is no significant difference between untreated and CHD treated data shown in Figure 13 A. The error bars represent standard errors determined from 3-5 transfection experiments.

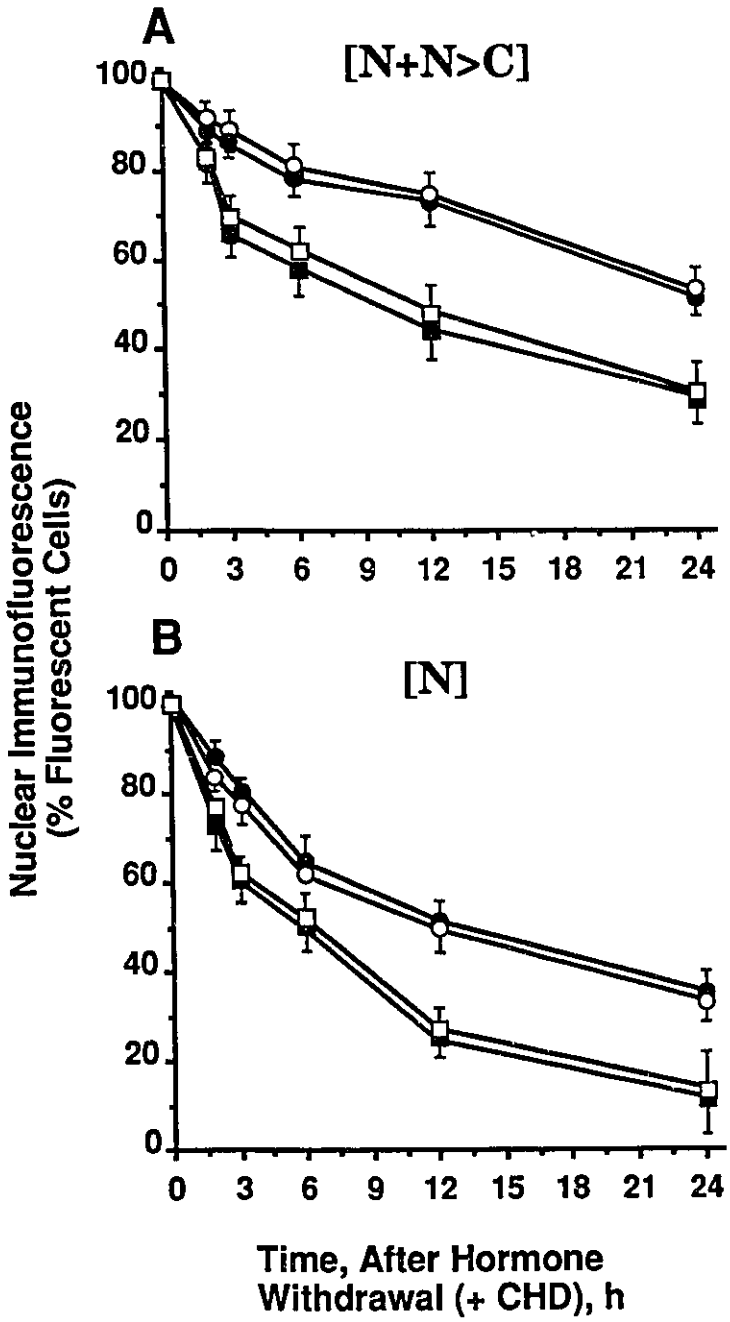

A
[N+N>C]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
B
[N]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, After Hormone
Withdrawal (+ CHD), h

Figure 13b

Figure 14. Kinetics of Nuclear Export of Human Wild Type GR and a Corresponding First Zinc Finger Mutant Following 1 μM Cortisol Withdrawal. The kinetics of loss of nuclear receptor to the cytoplasm was followed as described in the legend to Figure 13, using the ●human WT and OhΔ420-451. Cells were transfected by lipofection as described in Materials and Methods. After 1 h treatment with 1 μM cortisol, 10 % BSA supplemented serum free DMEM medium was added and at the times indicated, cells were fixed and processed for indirect immunofluorescence as described in legend to Figure 10. Shown are graphs of (**A**), percentage of cells showing total nuclear fluorescence, [N+N>C]; (**B**), Percentage of cells showing exclusively nuclear fluorescent staining [N]. As compared to the export kinetics displayed by human ●WT , the loss of OhΔ420-451 mutant GR to the cytoplasm was 3-4 fold faster than the corresponding WT. Also the rate of loss of human WT receptor is faster than mouse WT. The error bars represent standard errors determined from 3-5 transfection experiments.

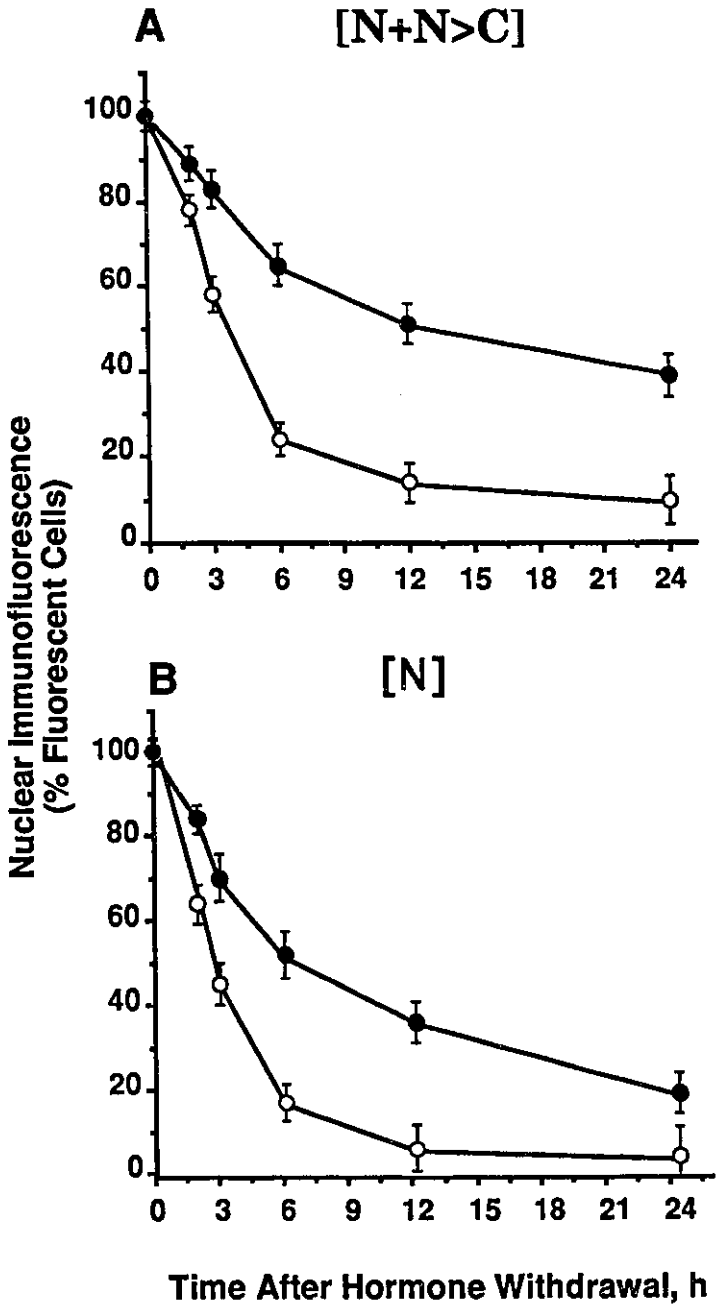

A
[N+N>C]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
B
[N]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Nuclear Immunofluorescence (% Fluorescent Cells)
Time After Hormone Withdrawal, h

Figure 14

Figure 15a. Re-uptake of Wild Type GR and Arg484-His Mutant After Return to the Cytoplasm. Transfected COS-7 cells were treated with 1 μM cortisol for 1 h. Cells were incubated in withdrawal medium, after removal of hormone. Thirty min before the end of the 3 h and 6 h withdrawal period. Withdrawal medium was replaced by serum free medium and incubated for an additional 30 min. Cells were re-stimulated with 1 μM cortisol and the kinetics of nuclear re-uptake was monitored for 1 h by the immunofluorescence. The counting of cells and procedures for plotting of graphs were as described in legend to Figure 10. Shown are graphs of percentage of total Nuclear Fluorescent cells, [N+N>C] and percentage of cells showing exclusively nuclear fluorescence [N]. At any point along the withdrawal curve readdition of hormone led to a rapid nuclear re-uptake for both ●mWT and OArg484-His mutant, thus demonstrating that DNA binding has no effect on re-utilisation of receptor in a secondary response. The error bars represent standard errors determined from 3-5 transfection experiments.

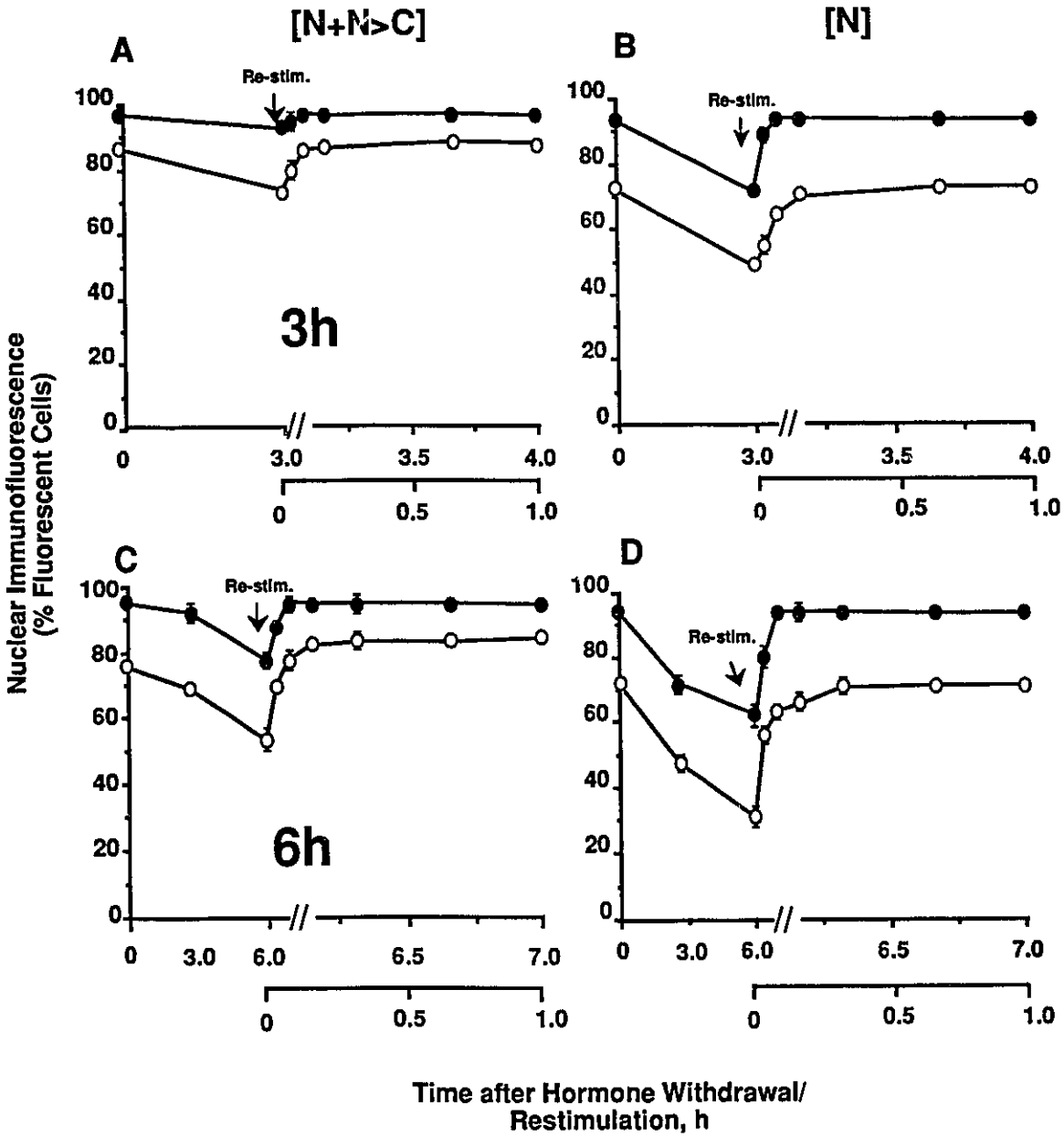

[N+N>C]
[N]
A
B
C
D
Re-stim.
3h
6h
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time after Hormone Withdrawal/
Restimulation, h

Figure 15a

Figure 15b. Re-uptake of Wild Type GR and Arg484-His Mutant After Return to the Cytoplasm. Transfected COS-7 cells were treated with 1 μM cortisol for 1 h. Cells were incubated in a withdrawal medium, after removal of hormone. At the times indicated, except that thirty minutes before the end of 12 h and 24 h, withdrawal medium was replaced by serum free medium and incubated for an additional 30 min. Cells were re-stimulated with 1 μM cortisol and the kinetics of nuclear re-uptake was monitored for 1 h by the immunofluorescence. The counting of cells and procedures for plotting of graphs were as described in legend to Figure 10. Shown are graphs of percentage of total Nuclear Fluorescent cells, [N+N>C] and percentage of cells showing exclusively nuclear fluorescence [N]. At any point along the withdrawal curve readdition of hormone led to a rapid nuclear re-uptake for both ●mWT and OArg484-His mutant, which was not different from the primary response, thus demonstrating that DNA binding has no effect on re-utilisation of receptor in a secondary response. The error bars represent standard errors determined from 3-5 transfection experiments.

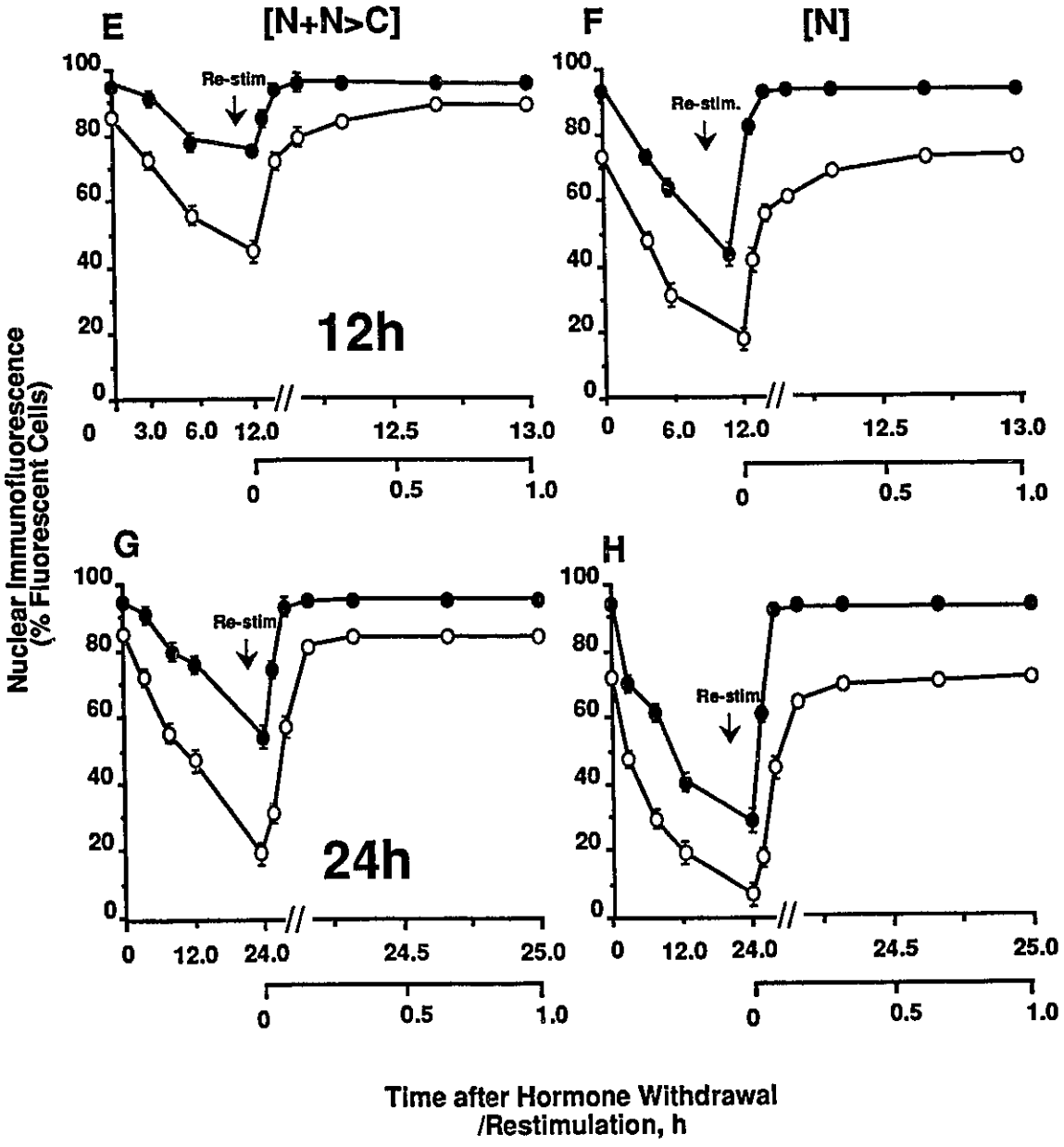

E
[N+N>C]
F
[N]
Re-stim
Re-stim.
12h
24h
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time after Hormone Withdrawal
/Restimulation, h

Figure 15b

Figure 16. Effect of RU486 on the Kinetics of Nuclear Uptake of Mouse Wild Type and a DNA Binding Mutant of GR. Mouse WT or Arg484-His GR expression vectors were transfected in COS-7 cells by lipofection, as described in Materials and Methods. Nuclear uptake was monitored over a 6 h period. After 1 μM RU486 treatment, at the times indicated, cells were fixed in 3 % paraformaldehyde and processed for indirect immunofluorescence, as described in Materials and Methods. The counting of cells and plotting of graphs were as described in legend to Figure 10. ●WT ○Arg484-His mutant. As shown from the graphs, for both WT and the Arg484-His mutant, $t_{1/2}$ to equilibruim levels for both [N+N>C] and [N], was reduced by approximately two-fold ($t_{1/2}$= 8-10 min) as compared to dex stimulated cells. The error bars represent standard errors determined from 5-8 transfection experiments.

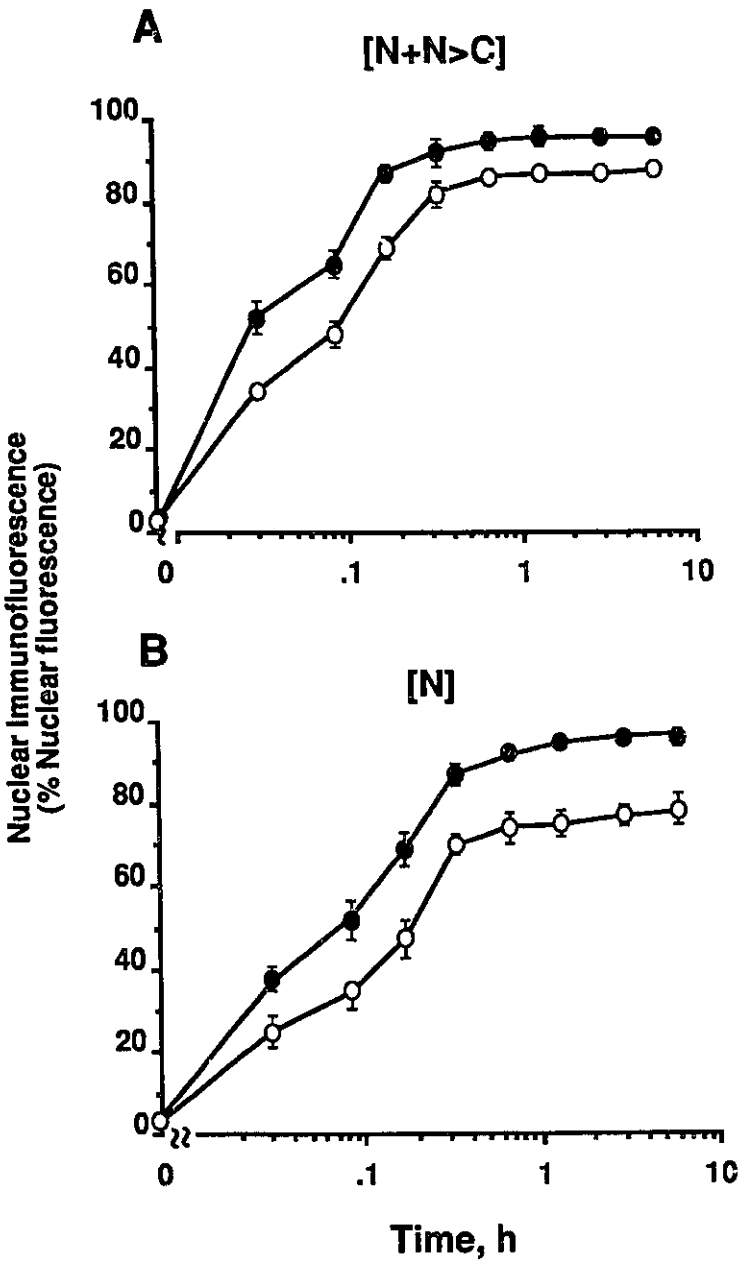

A
[N+N>C]
100
80
60
40
20
0
0
.1
1
10
B
[N]
100
80
60
40
20
0
0
.1
1
10
Nuclear Immunofluorescence
(% Nuclear fluorescence)
Time, h

Figure 16

Figure 17. Effect of RU486 on the Kinetics of Nuclear Export of Wild Type GR and Arg484-His Mutant. After transient expression of WT receptor and Arg484-His mutant in COS-7 cells, the cells were stimulated with 1 μM RU486 for 1 h. After extensive washings, withdrawal medium was added and at the times indicated cells were fixed and processed for indirect immunofluorescence, as described in legend to Figure 10. Cell counting and plotting of graphs were as described in legend to Figure 13. ●mWT ○Arg484-His mutant. Greater than 95 % nuclear retention is observed after 24 h of hormone withdrawal for both mWT and Arg484-His. Thus the effect is independent of DNA binding. The error bars represent standard errors determined from 5-8 transfection experiments.

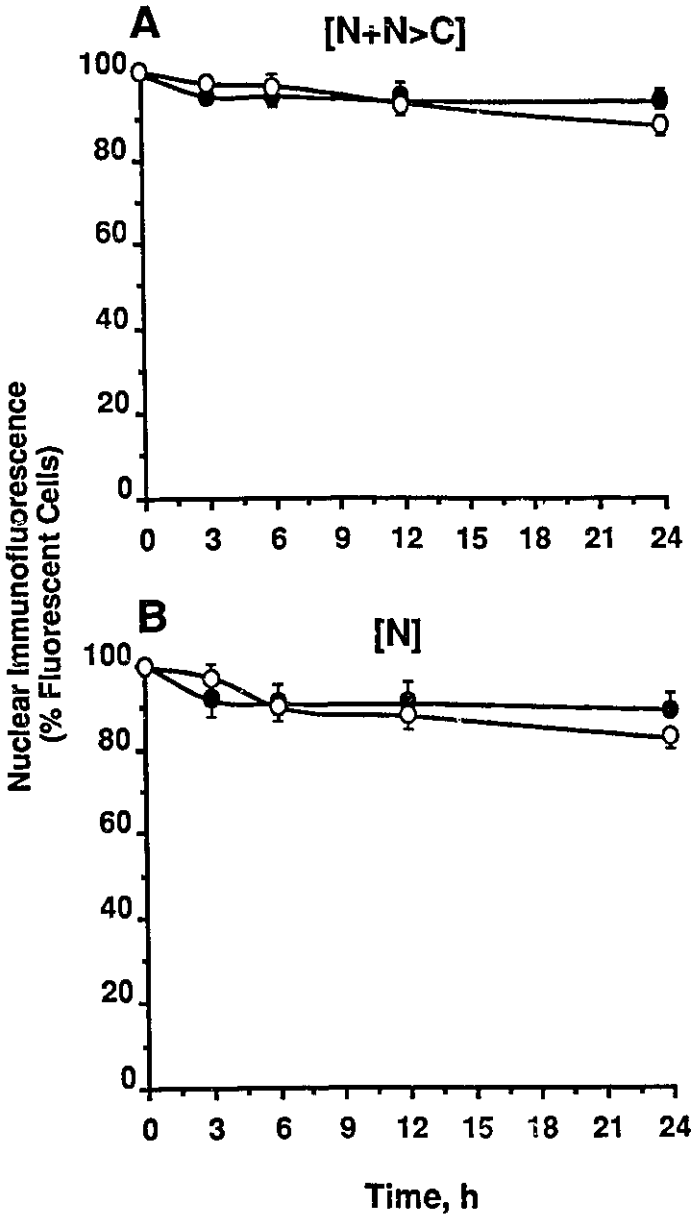

A
[N+N>C]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
B
[N]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h

Figure 17

Figure 18. Effect of TPA and H-7 on the Kinetics of Nuclear Uptake of Mouse WT Receptor and Arg484-His Mutant. COS-7 cells were transfected with mouse WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection WT and mutant receptors were pretreated for 4 h with either 100 nM TPA or 100 μM H-7, followed by stimulation with1 nM dex. Nuclear uptake was monitored for 3 h. At the times indicated cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence, as described in Materials and Methods. Cell counting and plotting of graphs were as described in legend to Figure 10. **A** and **B**, WT; **C** and **D**, Arg484-His. ●mWT or Arg484-His controls ■50 nM TPA, ○100 μM H-7. TPA treatment increased the initial rate of uptake of WT receptor by 2-fold, whilst H-7 treatment results in a 2-fold decrease in the rate of uptake. Likewise, TPA treatment increased the rate of uptake of Arg-His mutant by ~ 2-fold, whilst H-7 treatment resulted in an overall 2 to 2.5-fold decrease in the rate of uptake as compared to control. The error bars represent standard errors determined from 3-5 transfection experiments.

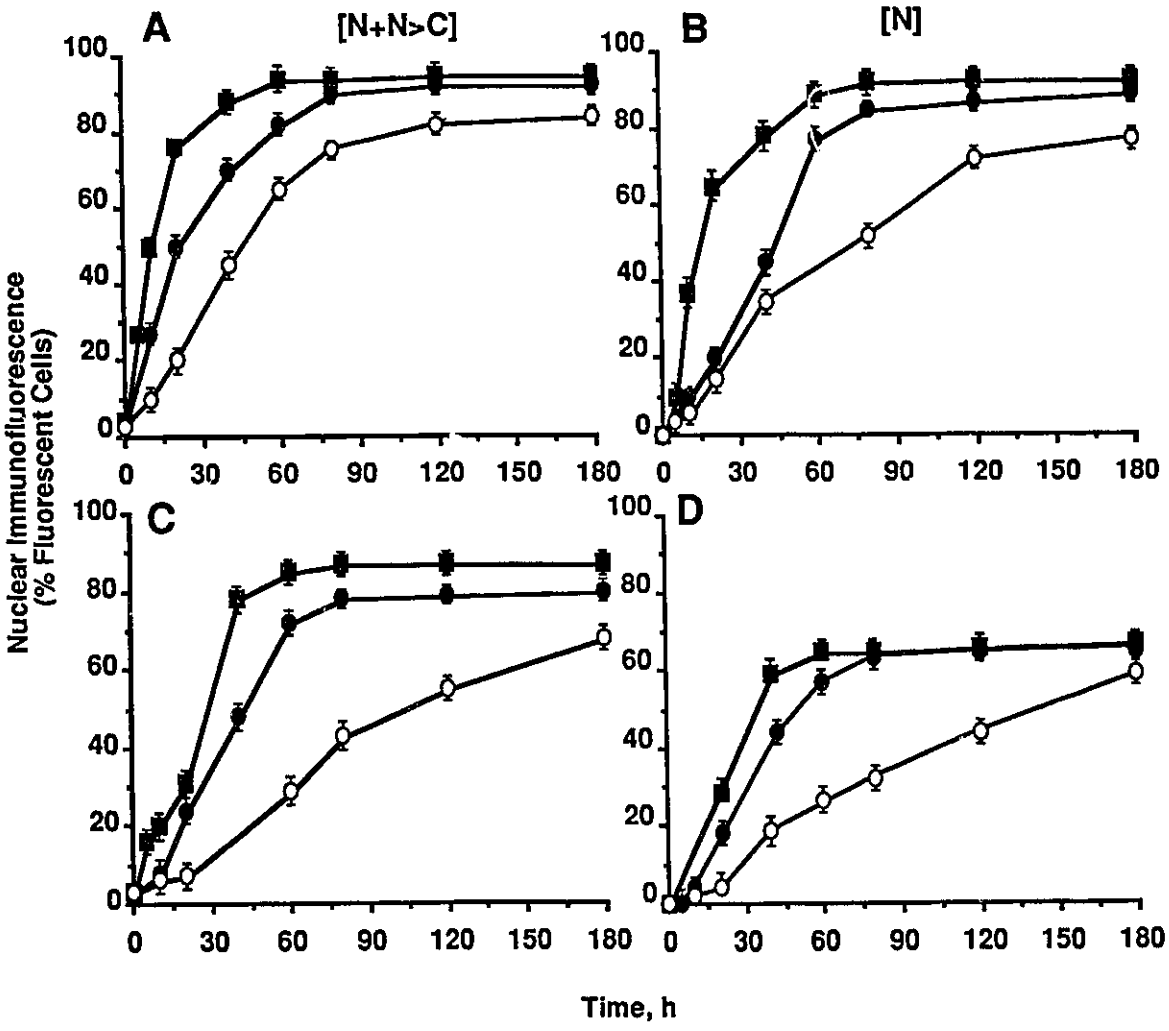

A
[N+N>C]
B
[N]
C
D
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h
0
20
40
60
80
100
0
30
60
90
120
150
180

Figure 18

Figure 19. Effect of TPA and H-7 on the Kinetics of Redistributon to the Cytoplasm of Nuclear Mouse Wild Type Receptor and Arg484-His Mutant. COS-7 cells were transfected with mouse WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection, cells were stimulated with 1 μM cortisol for 1 h. After removal of hormone, cells were incubated with withdrawal medium supplemented with 50 nM TPA or 100 μM H-7 final concentrations. The return of nuclear receptors to the cytoplasm was monitored for 24 h. At various times, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figures 10 and 13. **A** and **B**, mWT; **C** and **D**, Arg484-His. ●Controls for mWT or Arg484-His ■50 nM TPA ○100 μM H-7. TPA treatment resulted in enhanced rate of initial loss of nuclear receptor to the cytoplasm of both WT and DNA binding mutant. By contrast, H-7 treatment blocked > 95 % of cytoplasmic return of nuclear receptors. The error bars represent standard errors determined from 3-5 transfection experiments.

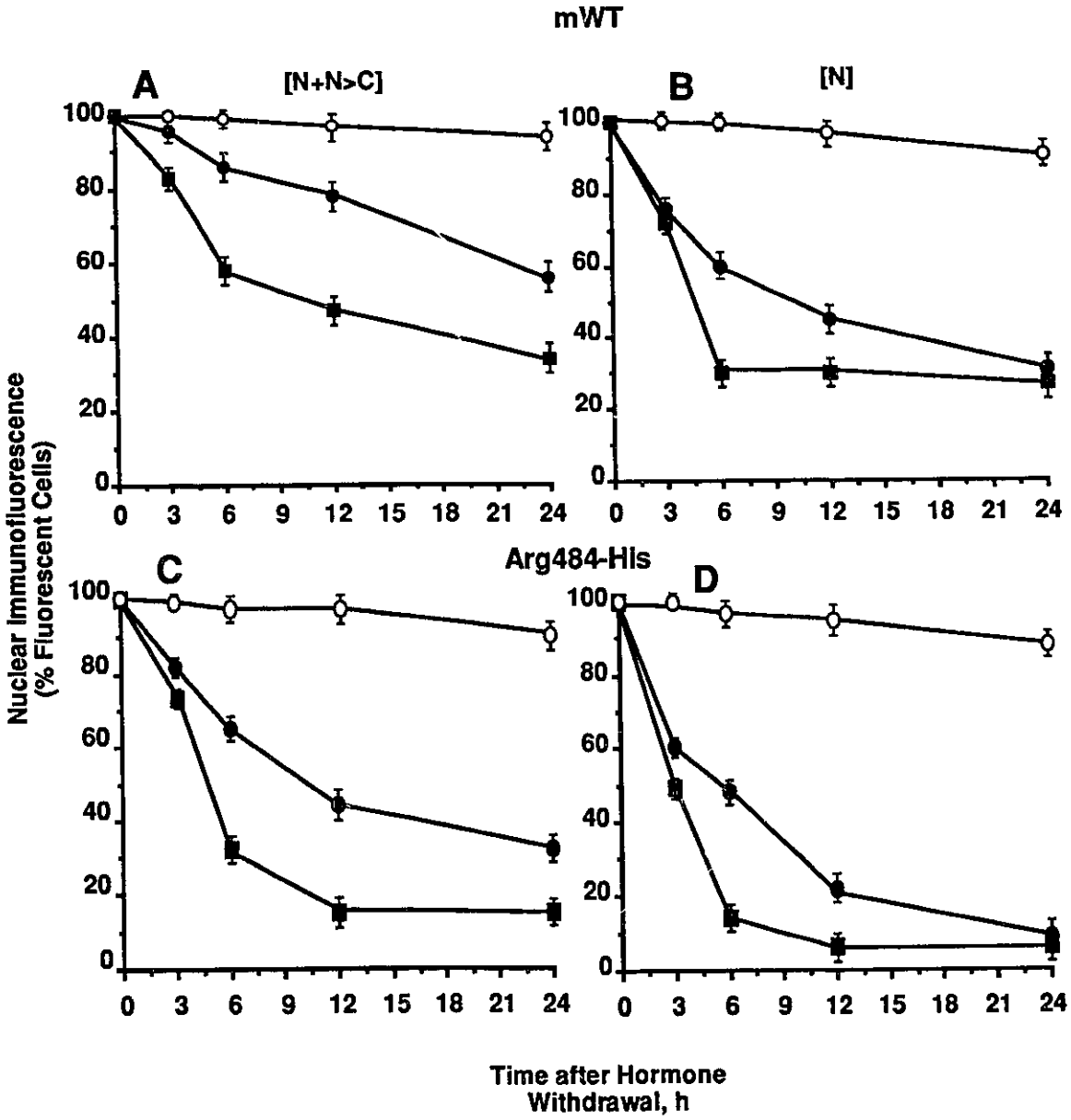

mWT
A
[N+N>C]
B
[N]
C
Arg484-His
D
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time after Hormone
Withdrawal, h
0
20
40
60
80
100
0 3 6 9 12 15 18 21 24

Figure 19

Figure 20. Nuclear RU486 bound Wild Type and Arg484-His GRs are Resistant to TPA Treatment. COS-7 cells were transfected with mWT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection cells containing WT or mutant receptors were stimulated with 1 μM RU486 for 1 h. After removal of antagonist, cells were incubated with withdrawal medium supplemented with TPA at final concentration of 50 nM. Loss of nuclear receptor to the cytoplasm was monitored for 24 h. At the times indicated, after hormone withdrawal, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. Counting procedures and graphs were plotted, as described in legend to Figure 10. ● mWT ○ Arg484-His mutant. TPA failed to stimulate redistribution of both RU486 bound WT and Arg484-His nuclear receptors to the cytoplasm. The error bars represent standard errors determined from 3-5 transfection experiments.

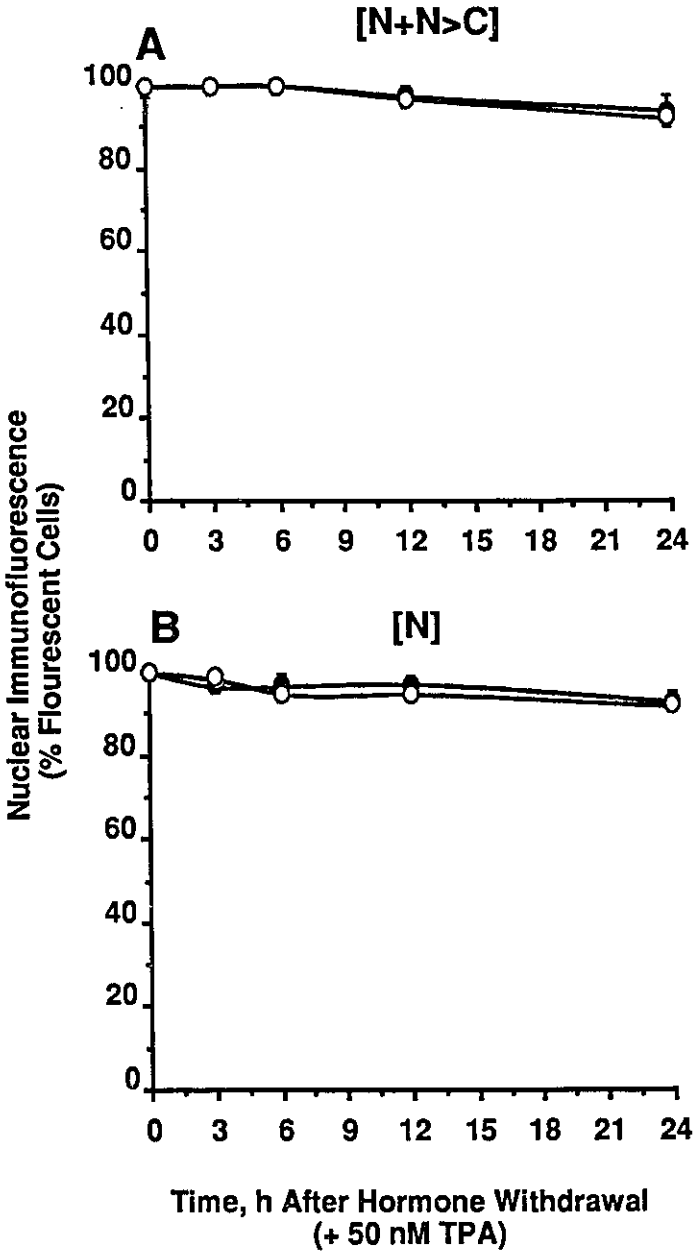

[N+N>C]
A
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
B
[N]
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Nuclear Immunofluorescence
(% Flourescent Cells)
Time, h After Hormone Withdrawal
(+ 50 nM TPA)

Figure 20

Figure 21. Effect of Forskolin, OA and Vanadate on the Kinetics of Nuclear Uptake of Mouse Wild Type and DNA Binding Mutant. COS-7 cells were transfected with mWT receptor or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours pos-transfection, WT and mutant receptor containing cells were pretreated for 4 h with one of the following effectors, at the final concentrations indicated; 25 μM Forskolin, 100 nM OA or 0.5 mM vanadate, followed by stimulation with1 nM dex. Nuclear uptake was monitored for 3 h. At the times indicated, cells were fixed with 3 % paraformaldehyde and prepared for indirect immunofluorescence. Cell counting and plotting of graphs were as described in legend to Figure 10. **A** and **B**, WT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls ■25 μM Forskolin □100 nM OA and. ○0.5 mM vanadate. Forskolin or OA treatment increased the rate of WT nuclear import by ~ 2-fold and 1.5 to 2-fold of Arg484-His. In contrast vanadate reduced the rate of uptake of both WT receptor and the Arg484-His mutant by ~ 2-fold as compared to control. The error bars represent standard errors determined from 3-5 transfection experiments.

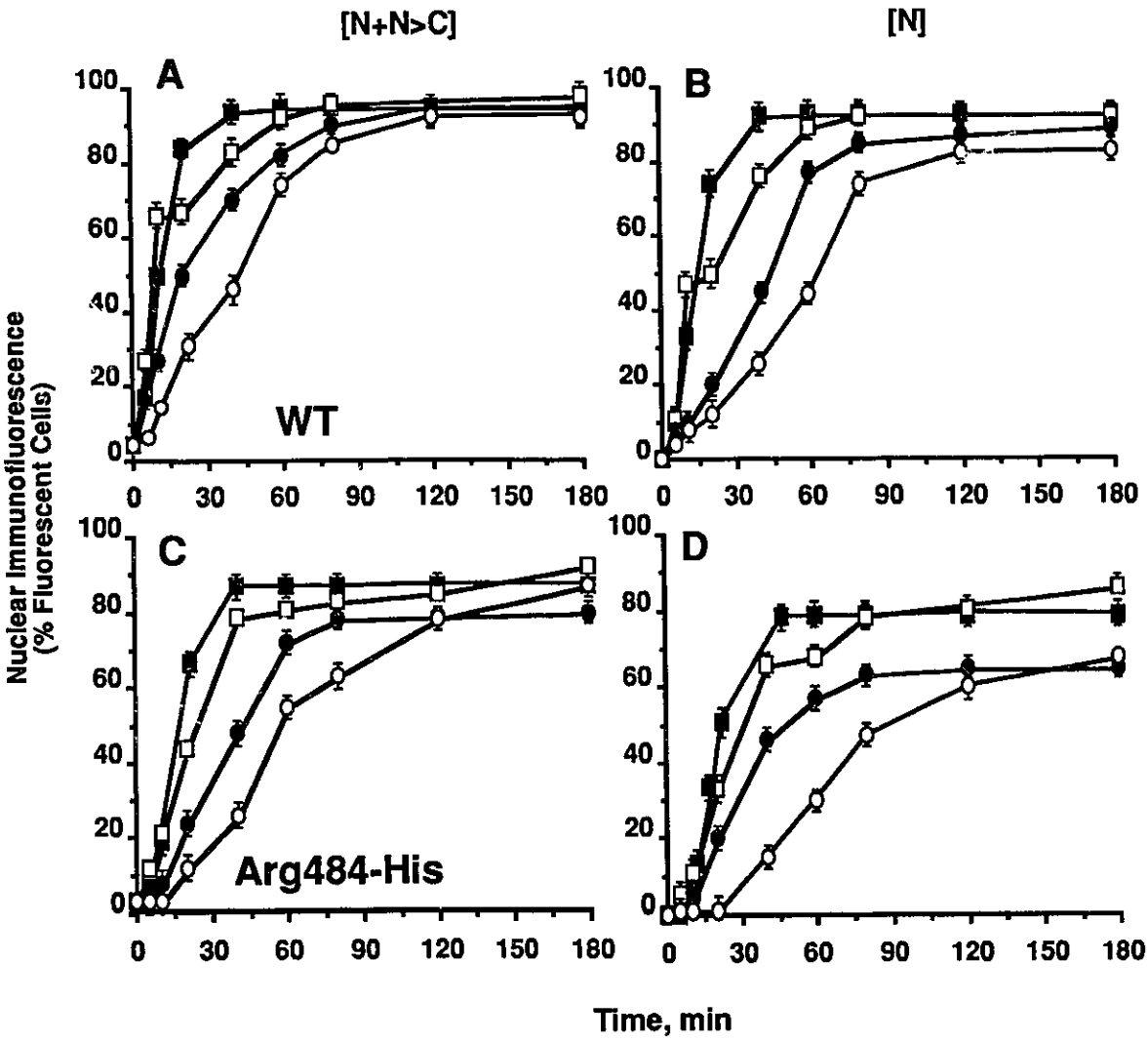

[N+N>C]
[N]
A
B
C
D
WT
Arg484-His
Nuclear Immunofluorescence (% Fluorescent Cells)
Time, min
0
20
40
60
80
100
0
30
60
90
120
150
180

Figure 21

Figure 22. Effect of Forskolin, OA and Vanadate on Cytoplasmic Redistribution After Hormone Withdrawal of Nuclear Mouse Wild Type Receptor and Arg484-His Mutant GRs. COS-7 cells were transfected with mouse WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours postransfection, cells were stimulated with 1 μM cortisol for 1 h, as described in Materials and Methods. After removal of hormone, cells were incubated with withdrawal medium supplemented with forskolin, OA or vanadate. Loss of nuclear receptors to the cytoplasm was monitored for 24 h. At the times indicated on the kinetic curve, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 13. **A** and **B**, WT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls O25 mM Forskolin □100 nM OA and ■0.5 mM vanadate. All three modulators decreased redistribution of nuclear receptors to the cytoplasm. The error bars represent standard errors determined from 3-5 transfection experiments.

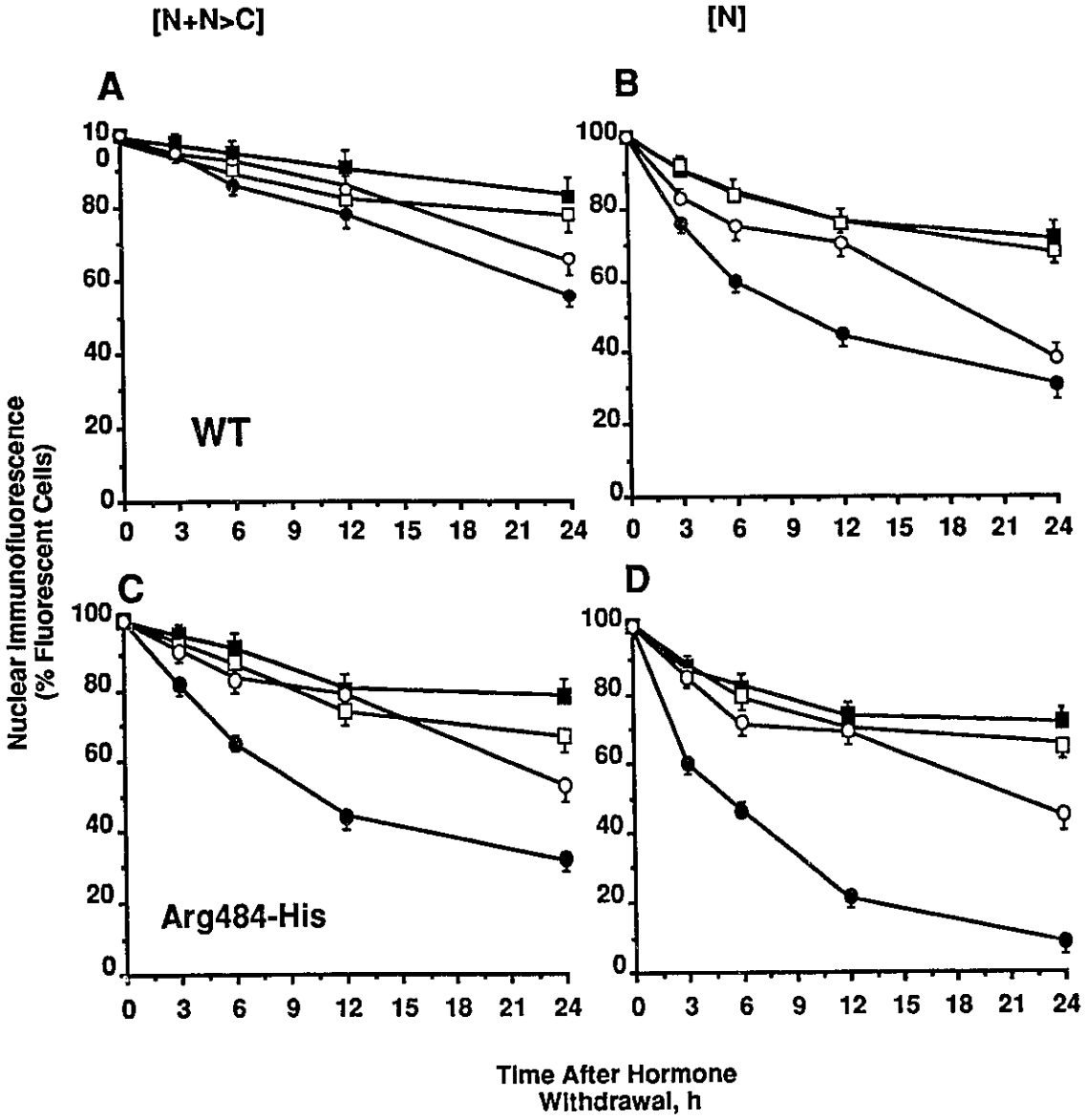

[N+N>C]
[N]
A
B
C
D
WT
Arg484-His
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time After Hormone
Withdrawal, h
0
20
40
60
80
100
0 3 6 9 12 15 18 21 24

Figure 22

Figure 23. Effect of OA on Re-utilisation of Receptor in a Secondary Response in COS-7 cells. Transfected COS-7 cells were treated with 1 μM cortisol for 1 h. Cells were incubated after hormone withdrawal at the times indicated in withdrawal medium supplemented with 100 nM OA. After 24 h, withdrawal medium was replaced by serum free medium and the cells were incubated for an additional 30 min. Cells were restimulated with 1 μM cortisol and nuclear re-uptake was monitored for 24 h. At the times indicated, cells were fixed and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 10. ●mWT and OArg484-His. Both WT and Arg484-His re-entered the nucleus, with a $t_{1/2}$ of 2-3 min. The error bars represent standard errors determined from 3-5 transfection experiments.

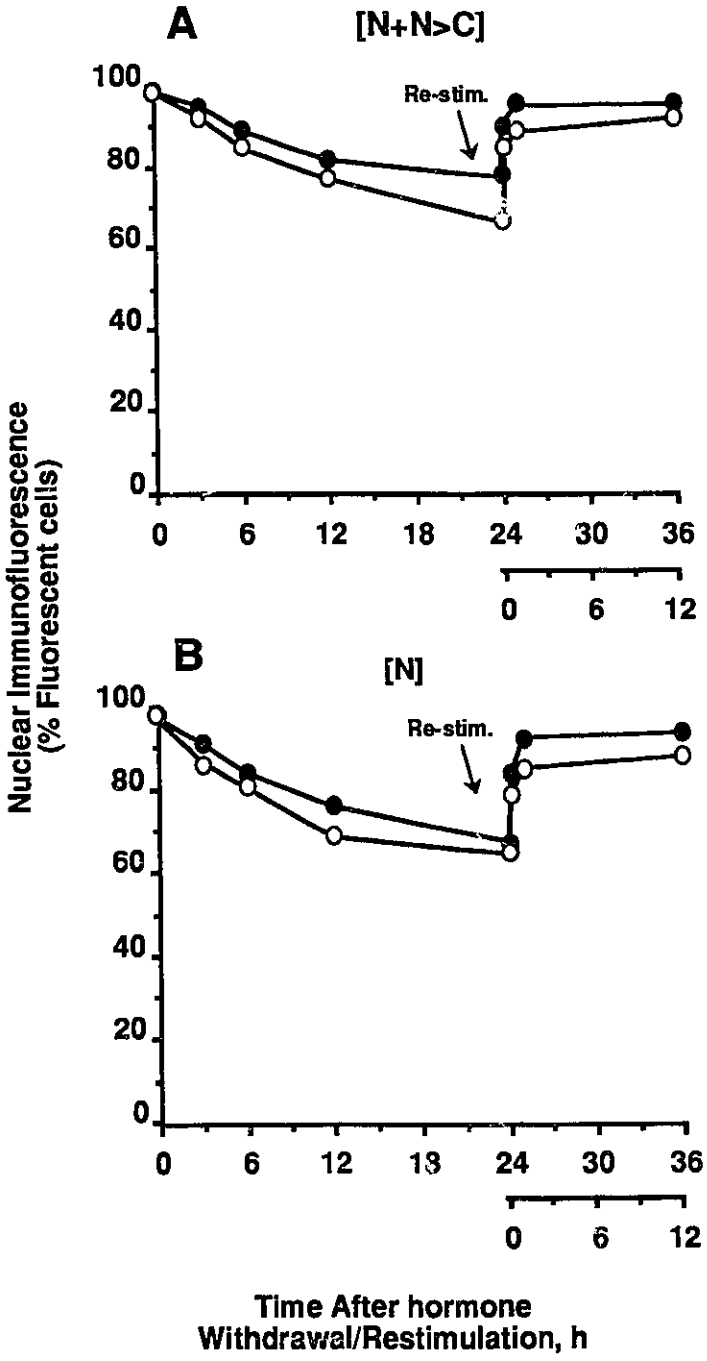

A
[N+N>C]
Re-stim.
100
80
60
40
20
0
0 6 12 18 24 30 36
0 6 12
B
[N]
Re-stim.
100
80
60
40
20
0
0 6 12 18 24 30 36
0 6 12
Nuclear Immunofluorescence (% Fluorescent cells)
Time After hormone Withdrawal/Restimulation, h

Figure 23

Figure 24. Effect of Simultaneous Stimulation by TPA and Forskolin on Nuclear Uptake of Mouse Wild Type Receptor and Arg484-His Mutant at Physiological Concentrations of Dex. COS-7 cells were transfected with WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection, WT and mutant receptors were pretreated for 4 h with a combination of final concentrations of 100 nM TPA and 25 μM forskolin, or 50 nM TPA alone or 25 μM forskolin alone, followed by stimulation with 1 nM dex. Nuclear uptake was monitored for 12 h. At the times indicated on the kinetic curve, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 10. **A** and **B**, mWT; **C** and **D**, Arg484-His. ●mWT or Arg484-His controls □100 nM TPA, ■25 μM forskolin, ○Forskolin /TPA. For both WT and Arg484-His, whilst the forskolin or TPA alone increased the rate of nuclear import the simultaneous treatment of forskolin and TPA slowed the rate of uptake to control levels. The error bars represent standard errors determined from 3-5 transfection experiments.

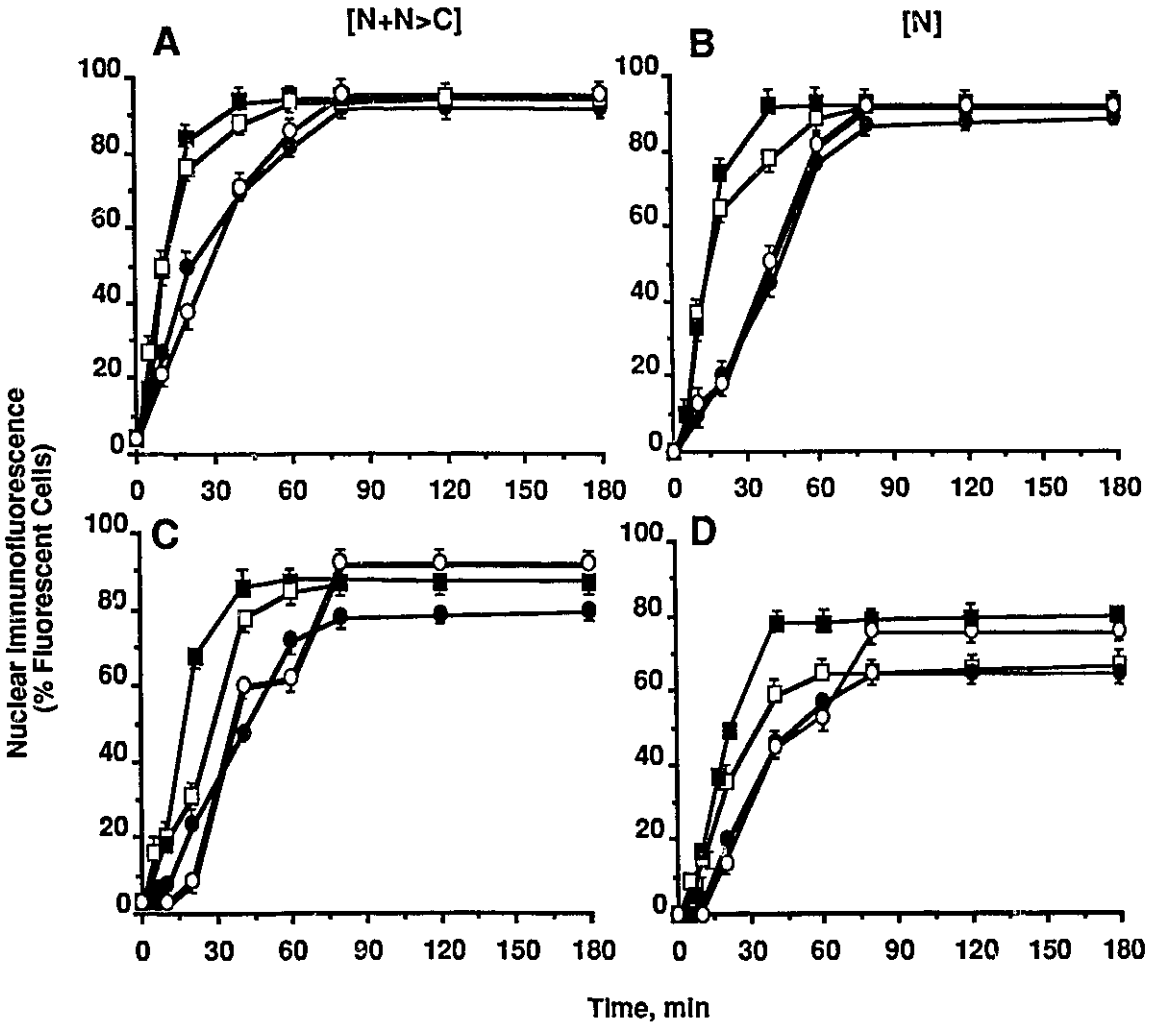

[N+N>C]
[N]
A
B
C
D
Nuclear Iminunofluorescence
(% Fluorescent Cells)
Time, min
0
20
40
60
80
100
0
30
60
90
120
150
180

Figure 24

Figure 25. Effect of Simultaneous Stimulation with TPA and Forskolin on the Kinetics of Nuclear Uptake of Mouse Wild Type and Arg484-His Mutant at Sub-physiological Concentration of Dex. COS-7 cells were transfected with mWT or Arg484-His mutant expression vectors. Forty-two hours postransfection, WT and mutant receptor were pretreated for 4 h with a combination of 100 nM TPA and 25 μM forskolin, or TPA alone or forskolin alone, followed by stimulation with 0.1 nM dex. The kinetics of nuclear uptake was monitored for 12 h. At the times indicated on the kinetic curve, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. Cell Counting and plotting of graphs were as described in legend to Figure 10. **A** and **B**, WT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls □100 nM TPA, ■25 μM forskolin, ○Forskolin /TPA and Δforskolin/4α-phorbol ester. For both WT and Arg484-His, there was no significant increase in the rate of uptake when cells were treated with either TPA or forskolin alone. However, cells treated with a combination of forskolin/TPA resulted in the WT receptor demonstrating 40-50 % reduction of total nuclear and 60 % of exclusively nuclear steady state levels by 6 h of fluorescent cells, but nuclear levels began to rise again by 6-12 h. The Arg484-His mutant receptor showed a decrease in its rate of uptake but did not demonstrate a reduction in steady state levels of receptor.

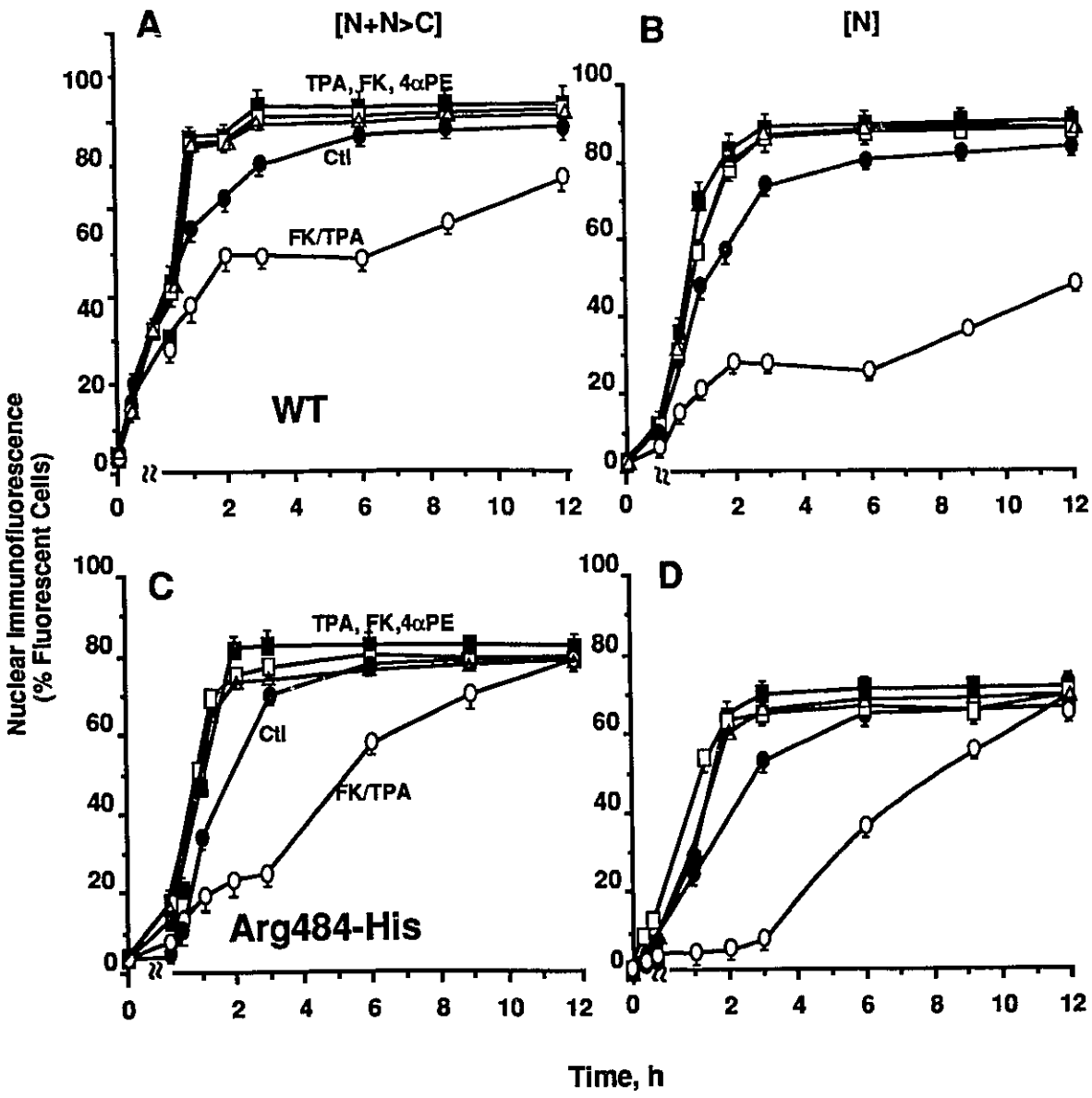

A
[N+N>C]
B
[N]
TPA, FK, 4αPE
Ctl
FK/TPA
WT
C
D
Arg484-His
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time, h

Figure 25

Figure 26. Effect of Simultaneous Treatment with TPA and Forskolin on the Kinetics of Cytoplasmic Redistribution of Mouse Wild Type and DNA Binding Mutant Nuclear GRs. COS-7 cells were transfected with mouse WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours postransfection cells containing WT or mutant receptors were stimulated with 1 μM cortisol for 1 h. After removal of hormone, cells were incubated with withdrawal medium supplemented with a combination of a final concentrations of 50 nM TPA/25 μM forskolin or TPA or forskolin alone. The kinetics of loss of nuclear receptor to the cytoplasm was monitored for 24 h. At the times indicated, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. Cell counting and plotting of graphs were as described in legend to Figure 12. **A** and **B**, WT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls □50 nM TPA ○25 μM forskolin and ■Forskolin/TPA. The combined treatment of TPA/ forskolin produced two distinct effects on WT receptor and Arg484-His mutant. WT receptor demonstrated an additive effect of forskolin and TPA, whilst Arg484-His showed an effect identical to TPA alone treatment. The error bars represent standard errors determined from 3-5 transfection experiments.

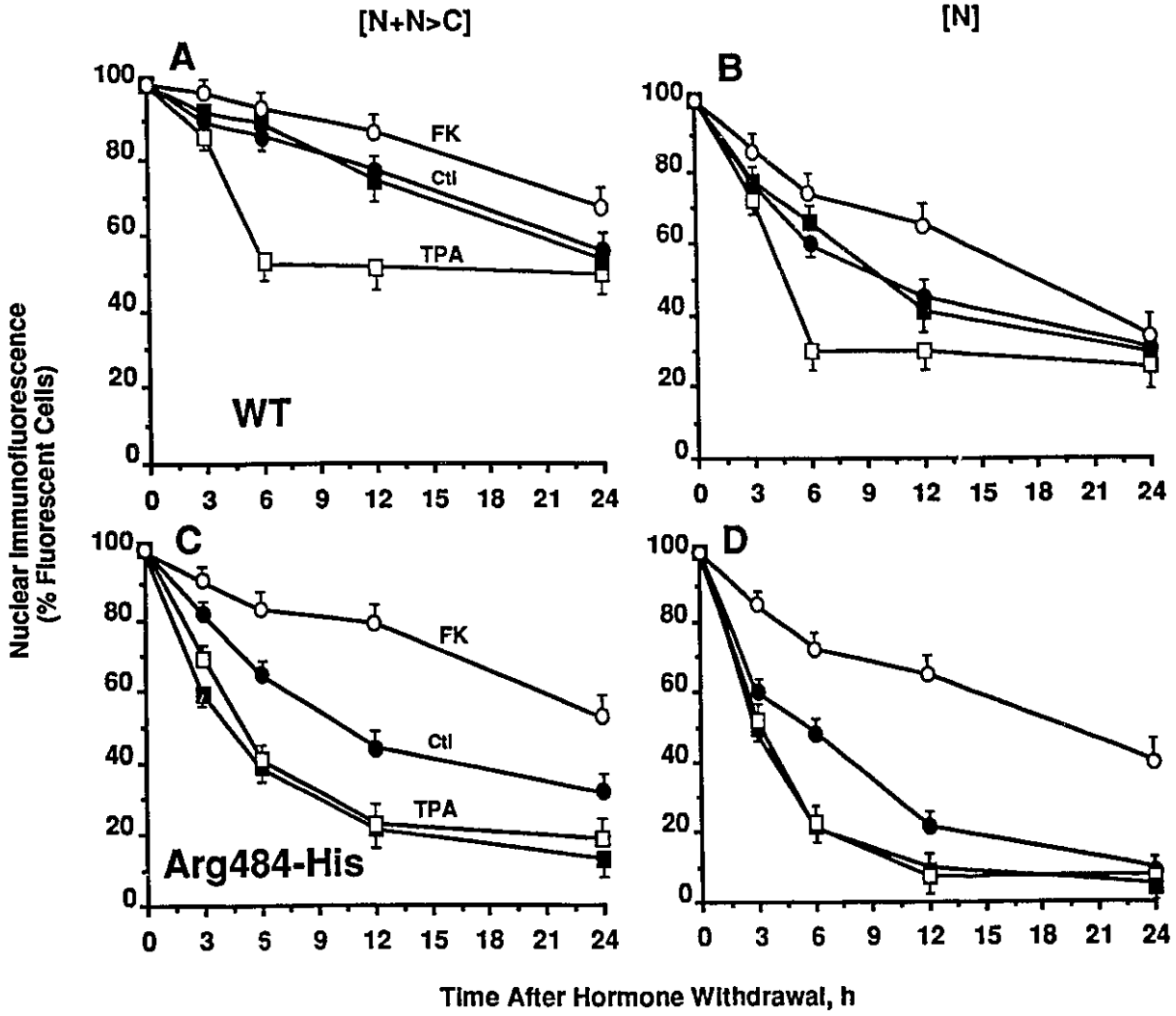

[N+N>C]
[N]
A
B
C
D
FK
Ctl
TPA
WT
Arg484-His
Nuclear Immunofluorescence (% Fluorescent Cells)
Time After Hormone Withdrawal, h

Figure 26

Figure 27. Effect of Simultaneous Inhibition of Protein Phosphatase 1/2A Activities and Stimulation of cAMP Signaling Pathway on the Kinetics of Nuclear Uptake of Mouse Wild Type and DNA Binding Mutant. COS-7 cells were transfected with mouse WT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours postransfection cells containing WT or mutant receptors were pretreated for 4 h with a combination of 25 μM forskolin and 100 nM OA, or forskolin or OA alone, followed by stimulation with1 nM dex. Nuclear uptake was monitored for 3 h. At the times indicated, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 10. **A** and **B**, WT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls ■25 μM forskolin, □100 nM OA OForskolin/OA. Whilst the combined effect of forskolin/OA on the WT partially cancelled each other, the effect on Arg484-His resulted in reduced rate of import. The error bars represent standard errors determined from 3-5 transfection experiments.

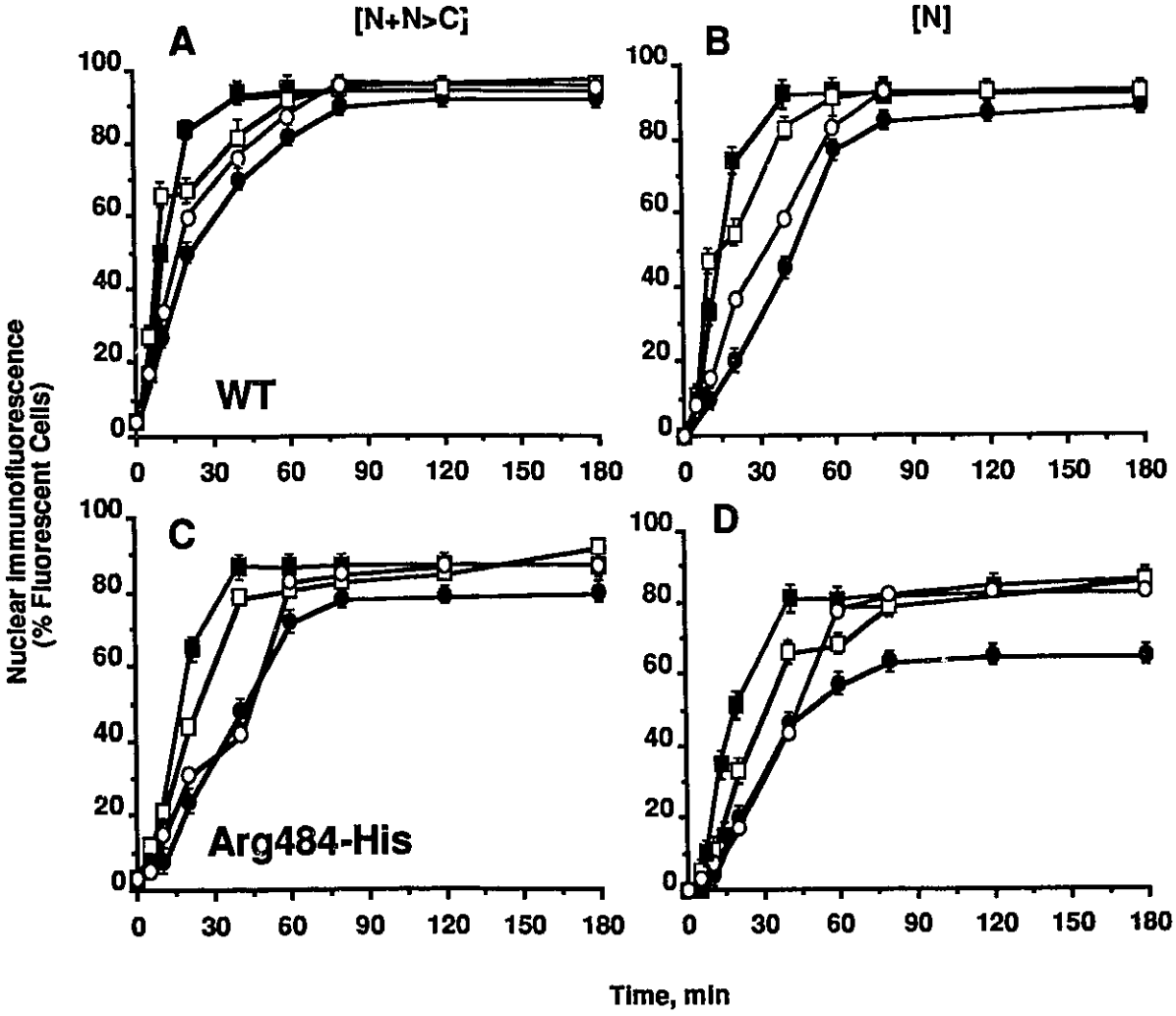

[N+N>C]
[N]
A
B
C
D
WT
Arg484-His
Nuclear Immunofluorescence (% Fluorescent Cells)
Time, min
0
20
40
60
80
100
0
30
60
90
120
150
180

Figure 27

Figure 28. Effect of Simultaneous Treatment with Forskolin and OA on the Kinetics of Cytoplasmic Redistribution of Mouse Wild Type and DNA Binding Mutant Nuclear GRs. COS-7 cells were transfected with mWT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours postransfection cells containing WT or mutant receptors were stimulated with 1 μM cortisol for 1 h. After removal of hormone, cells were incubated with withdrawal medium supplemented with a combination of 25 μM forskolin/100 nM OA or forskolin or OA alone. The loss of nuclear receptors to the cytoplasm was monitored for 24 h. At the times indicated on the kinetic curve, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence, as described in Materials and Methods. The counting of cells and plotting of graphs were as described in legend to Figure 12. **A** and **B**, mWT; **C** and **D**, Arg484-His. ●WT or Arg484-His controls ○25 μM forskolin, ■100 nM OA and □Forskolin/OA. The combined treatment of forskolin/OA produced two distinct effect on WT and Arg484-His mutant. WT receptor demonstrated an additive effect, whilst Arg484-His displayed kinetic curve identical to forskolin alone treatment. The error bars represent standard errors determined from 3-5 transfection experiments.

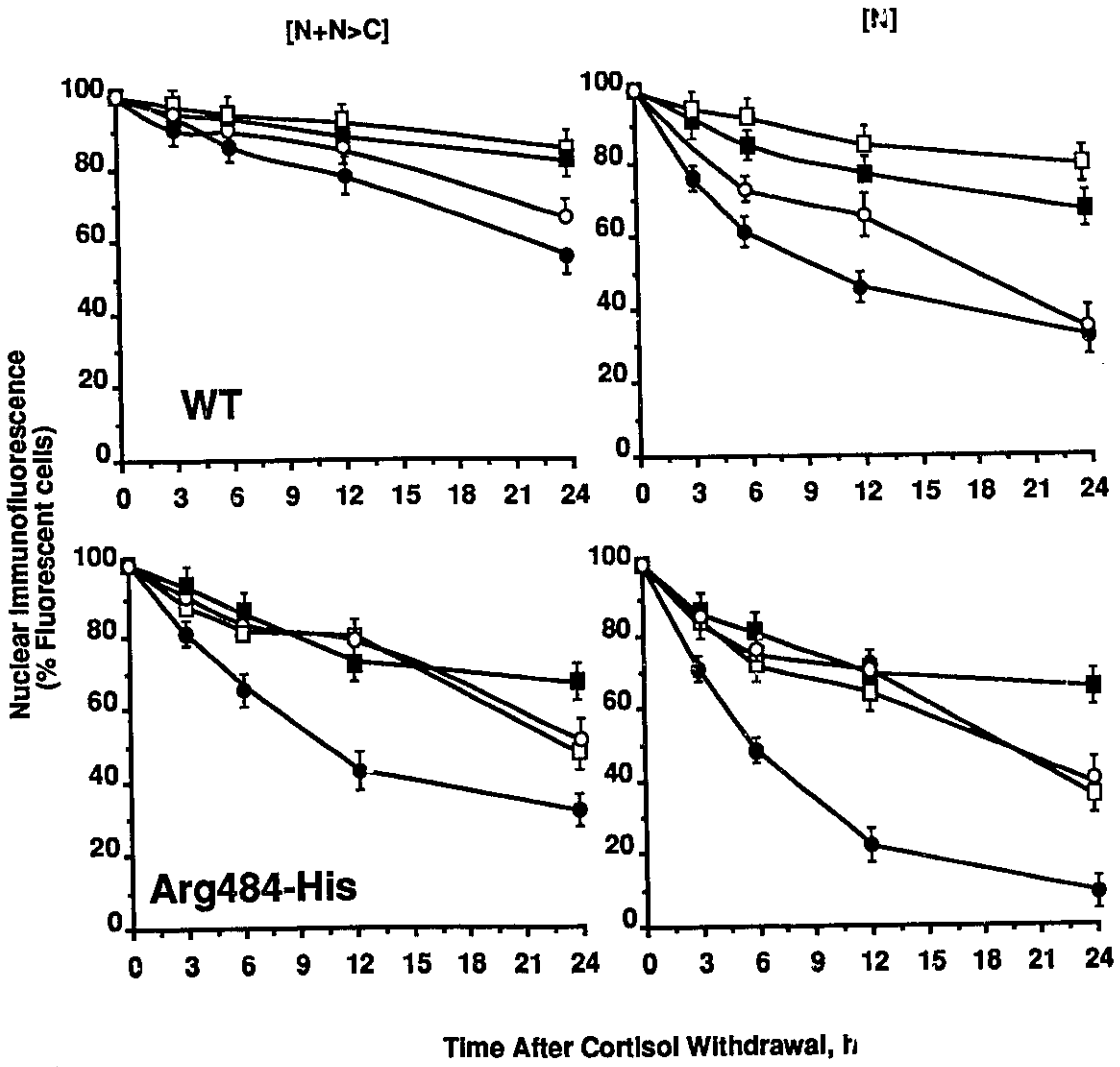

[N+N>C]
[N]
WT
Arg484-His
Nuclear Immunofluorescence (% Fluorescent cells)
Time After Cortisol Withdrawal, h

Figure 28

Figure 29. Effect of Energy Depletion on Efflux of WT and DNA Binding Mutant GRs From the Nucleus After Hormone Withdrawal. COS-7 cells were transfected with mWT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection, cells containing WT and mutant receptors were stimulated with 1 μM cortisol or RU486 for 1 h. After removal of hormone, cells were incubated with withdrawal medium supplemented with a final concentration of 6 mM 2-deoxyglucose and 50 μM oligomycin (energy depletion medium). Alternatively, 100 μM H-7 was added to the energy depletion medium, after cortisol withdrawal. The loss of nuclear receptor to the cytoplasm was monitored for 6 h. At the times indicated on the curve, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 13. **A** and **B**, mWT; **C** and **D**, Arg484-His. ●Cortisol ■Ru486 and Ocortisol/H-7. The rate of efflux of receptors from the nucleus was approximately the same for both WT and Arg484-His, under all three experimental conditions. Receptor loss from the nucleus is faster ($t_{1/2}$=2-3 h) as compared to controls (receptors from cells untreated with energy inhibitor). The error bars represent standard errors determined from 3-5 transfection experiments.

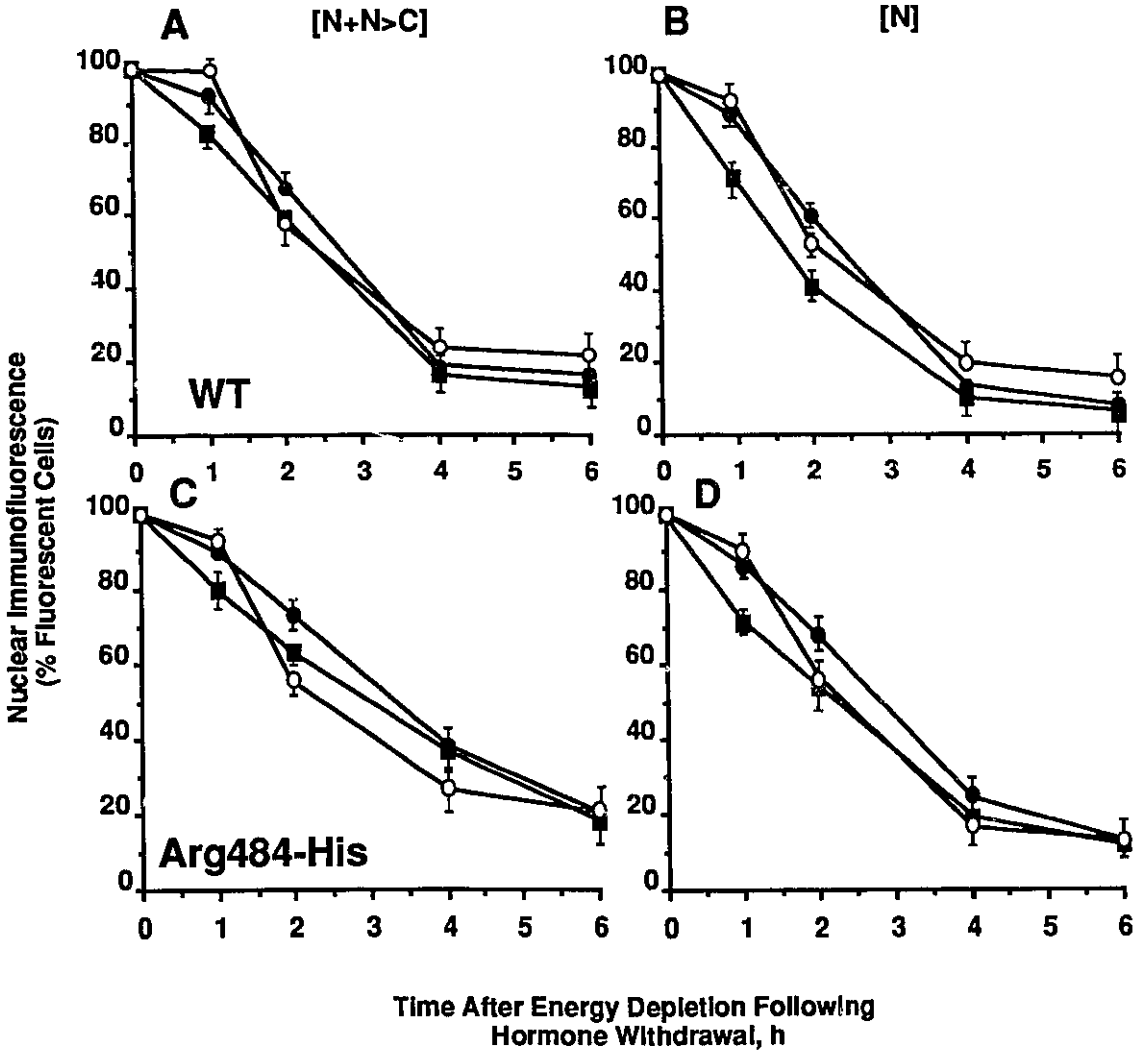

A
[N+N>C]
B
[N]
C
D
WT
Arg484-His
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time After Energy Depletion Following
Hormone Withdrawal, h

Figure 29

Figure 30. Effect of Energy Depletion on Efflux of WT Receptor and Arg484-His Mutant From the Nucleus in the Presence of Hormone. COS-7 cells were transfected with mWT or Arg484-His mutant expression vectors, as described in Materials and Methods. Forty-two hours post-transfection, cells containing WT and mutant receptors were stimulated with 1 μM cortisol or RU486. The energy depletion medium contained 6 mM 2-deoxyglucose and 100 μM oligomycin and 10-6 M cortisol, final concentration. In another set-up, 100 μM H-7 was added to the energy depletion medium. The loss of nuclear receptors to the cytoplasm was monitored for 6 h. At the times indicated, cells were fixed with 3 % paraformaldehyde and processed for indirect immunofluorescence. The counting of cells and plotting of graphs were as described in legend to Figure 13. **A** and **B**, WT; **C** and **D**, Arg484-His. ●Cortisol ■RU486 and ○cortisol/H-7. The rate of efflux of receptors from the nucleus is approximately the same for both WT and Arg484-His under all three experimental conditions. Receptor release was faster (t1/2=2-3 h), as compared to controls (receptors from cells untreated with energy inhibitor). The error bars represent standard errors determined from 3-5 transfection experiments.

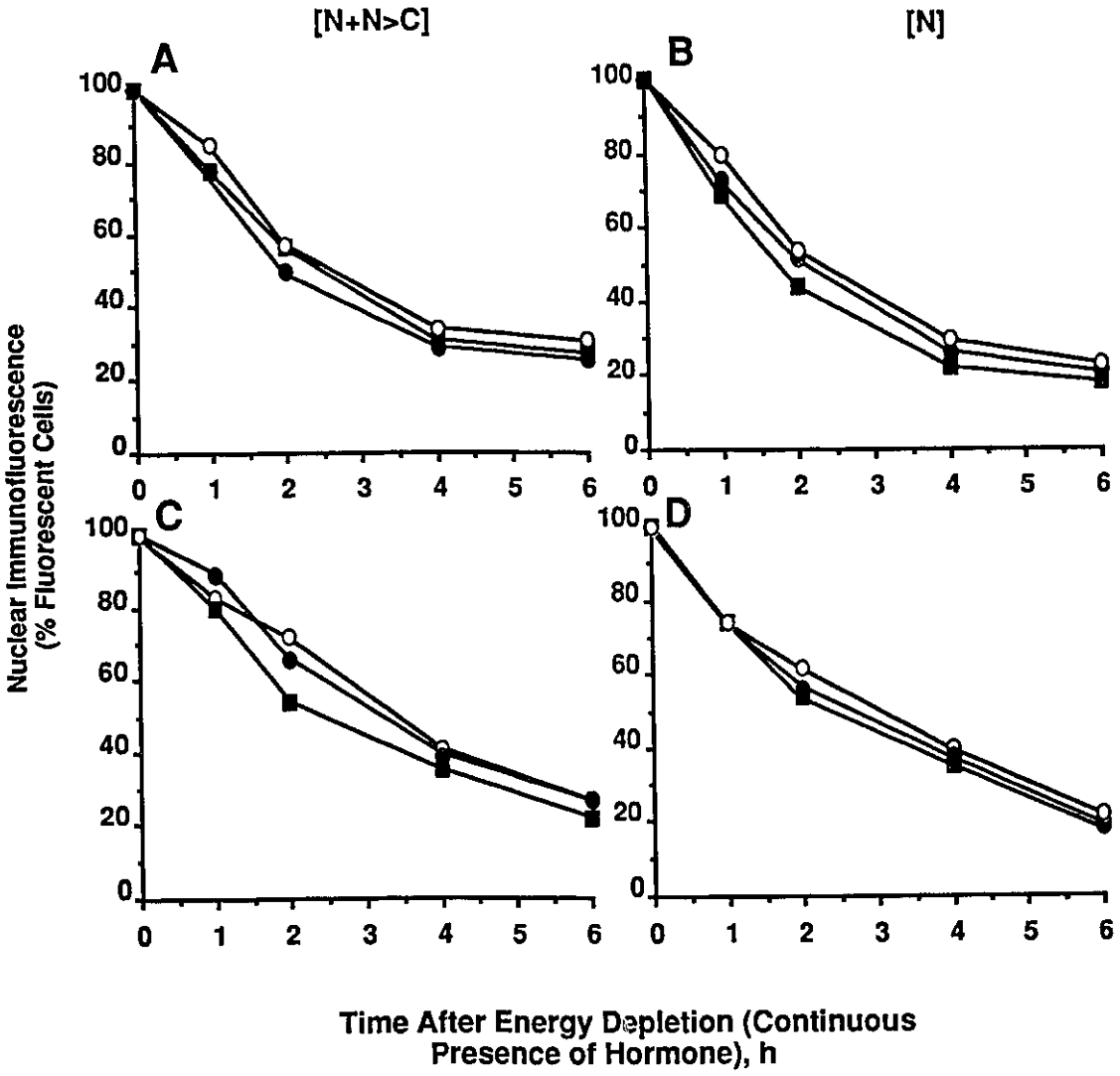

[N+N>C]
[N]
A
B
C
D
Nuclear Immunofluorescence (% Fluorescent Cells)
Time After Energy Depletion (Continuous Presence of Hormone), h

Figure 30

Figure 31. Comparison of the Rate of Cytoplasmic Redistribution of RU486 Bound and H-7 Treated Wild Type Receptors and Arg484-His Mutant After Treatment With or Without an Energy Inhibitor. The loss of WT nuclear receptor and the Arg484-His mutant to the cytoplasm after treatment of cells with RU486 and subsequently not adding (controls) or adding energy inhibitor to the cells at the time of hormone withdrawal. It is a composite graph from Figures 17 and 29 . OWT untreated control; ●WT, energy depletion; □Arg484-His, untreated control; and ■Arg484-His, energy depletion. Treatment with an energy synthesis inhibitor removes the RU486 or H-7 blockage of cytoplasmic accumulation of nuclear receptors and causes an increase in the rate of redistribution of shuttling receptors to the cytoplasm by blocking nuclear import. Composite graphs from Figures 19 and 29 which shows withdrawal from cortisol bound nuclear receptors treated with H-7 in the presence or absence of energy inhibitors are essentially the same for both WT receptors and Arg484-His mutant (not shown).

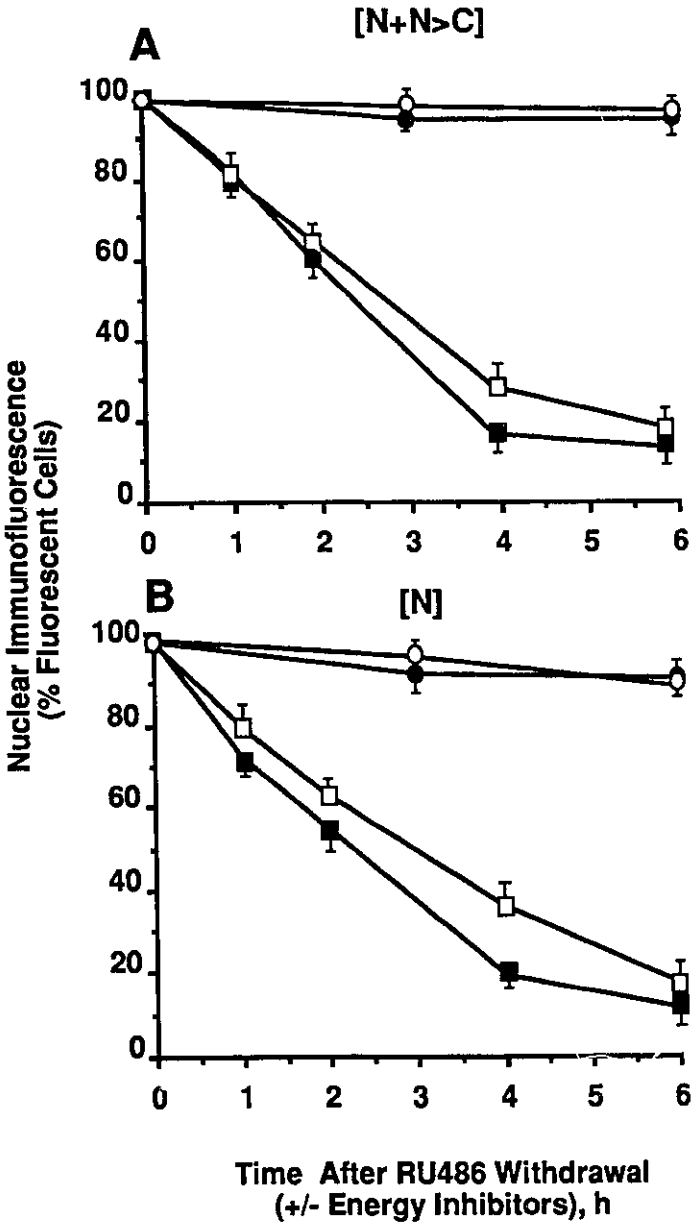

[N+N>C]
A
100
80
60
40
20
0
0
1
2
3
4
5
6
B
[N]
100
80
60
40
20
0
0
1
2
3
4
5
6
Nuclear Immunofluorescence
(% Fluorescent Cells)
Time After RU486 Withdrawal
(+/- Energy Inhibitors), h

Figure 31

Figure 32. Functional Domains of the Human Glucocorticoid Receptor Showing the Positions of the Two Nuclear Localization Signals. The Nuclear localization signals, NL-1 and NL-2 were originally mapped by Picard and Yamamoto in the rat GR[17]. The entire NL-1 sequence is conserved in the human and the mouse GRs. The core sequence of NL-1 shares 70 % homology with that of the SV40 large T-antigen[117] as indicated by italics. The N-terminal flanking region sequence which appears to be very important in determining the rate of nuclear accumulation of the receptor is indicated in the Figure.

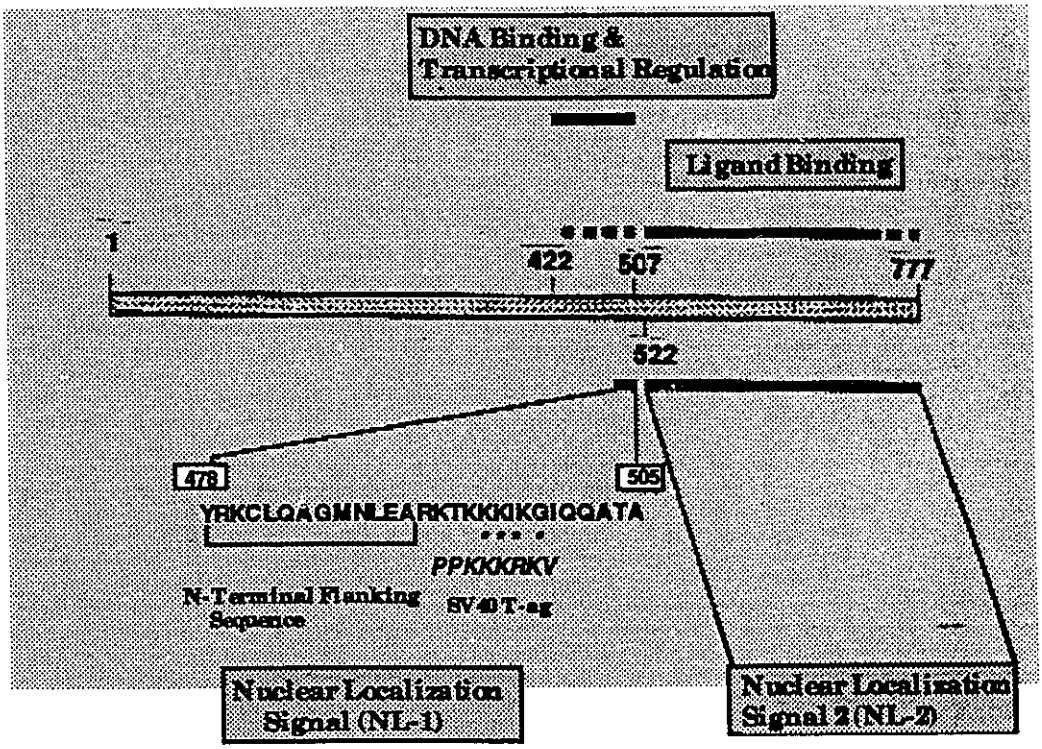
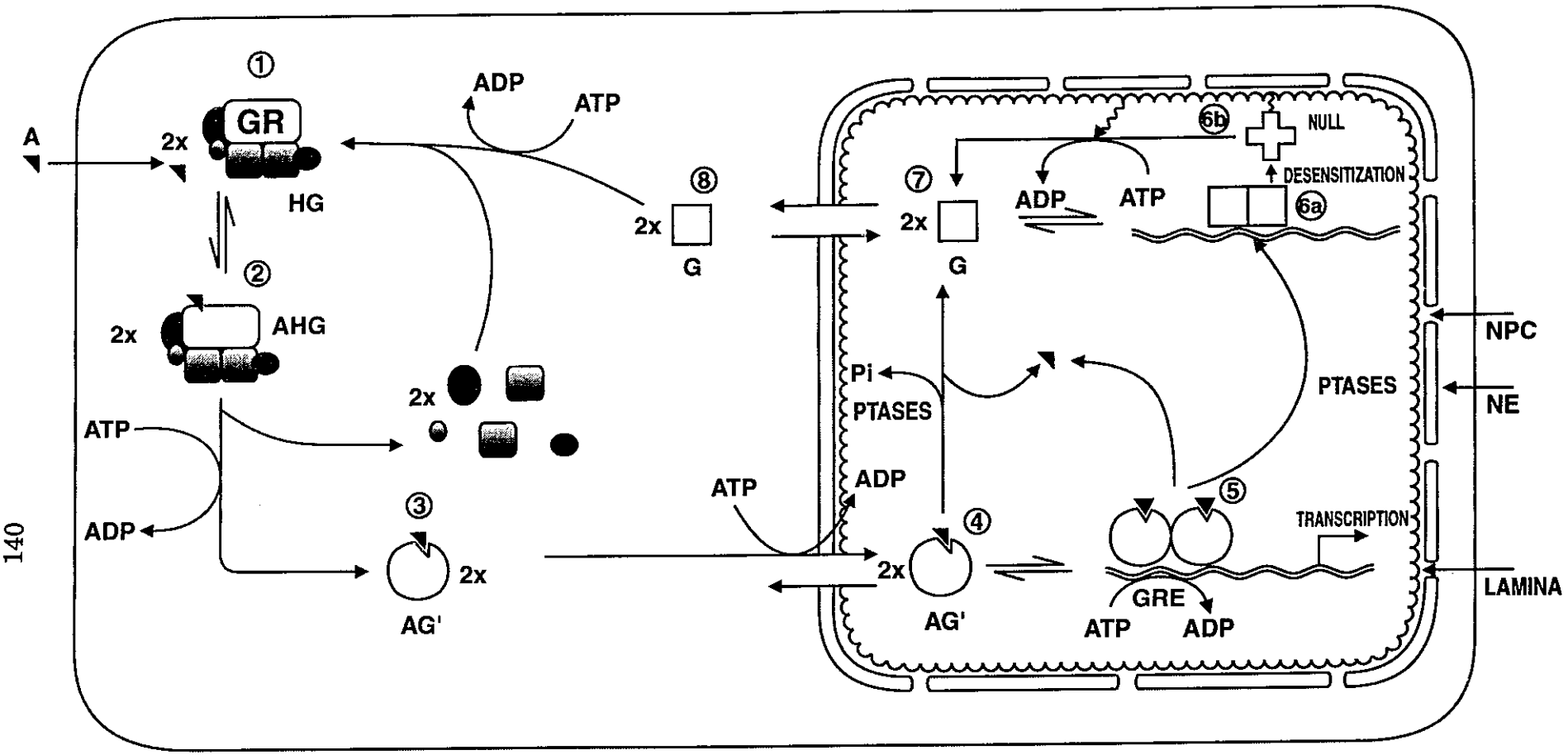

DNA Binding &
Transcriptional Regulation
Ligand Binding
1
422
507
777
522
478
505
YRKCLQAGMNLEARKTKKKIKGIQQATA
PPKKKRKV
N-Terminal Flanking
Sequence
SV40 T-ag
Nuclear Localization
Signal (NL-1)
Nuclear Localization
Signal 2 (NL-2)

Figure 32

Figure 33A. A Model of Nucleocytoplasmic Trafficking Events of the Glucocorticoid Receptor Upon Agonist Binding. The cytoplasmic GR in an inactive state is associated with hsps and other factors in an 8-9S multiprotein complex (HG). Following agonist binding, a liganded inactive complex is produced (AHG). Subsequently the hsps are dissociated followed by activation of the receptor (AG; steps 1 and 2) with rapid entry into the nucleus by an active transport process that is dependent on ATP and is inhibited by energy inhibitors. In the nucleus AG interacts with many components, including site specific high affinity DNA binding sites, the glucocorticoid response elements (GRE) of target genes that sequester receptor onto DNA (step 3). However, DNA binding mutants are unable to associate with DNA and thus increase the nuclear pool of receptor. AG' liganded receptor molecules are trapped in the nucleus upon receptor activation and either bind to DNA in the form of homodimers or are dephosphorylated by phosphatases to generate free unliganded receptor. Following loss of ligand, the free unliganded receptor (G) shuttles between the nucleus and the cytoplasm. Receptor mutants unable to bind DNA are more readily available for shuttling which shifts the steady-state distribution of the receptor towards the cytoplasm. Free G is also accessible to the re-cycling machinery which eventually results in the association into the 8-9S heterocomplex (HG; step 8 to 1).

The null receptor is generated through desensitization mechanisms involving loss of ligand from the receptor and dephosphorylation (steps 5 to 6B). It is underphosphorylated as compared to the 4S unliganded receptor and remains tightly bound to the nuclear matrix and may require rephosphorylation to generate the 4S Free G receptor (steps 6b to 7).



A Model of Nucleocytoplasmic Shuttling and Cycling of GR.

Based on Orti et al [218, 246,247]

F.N.A. Sackey (1994)©

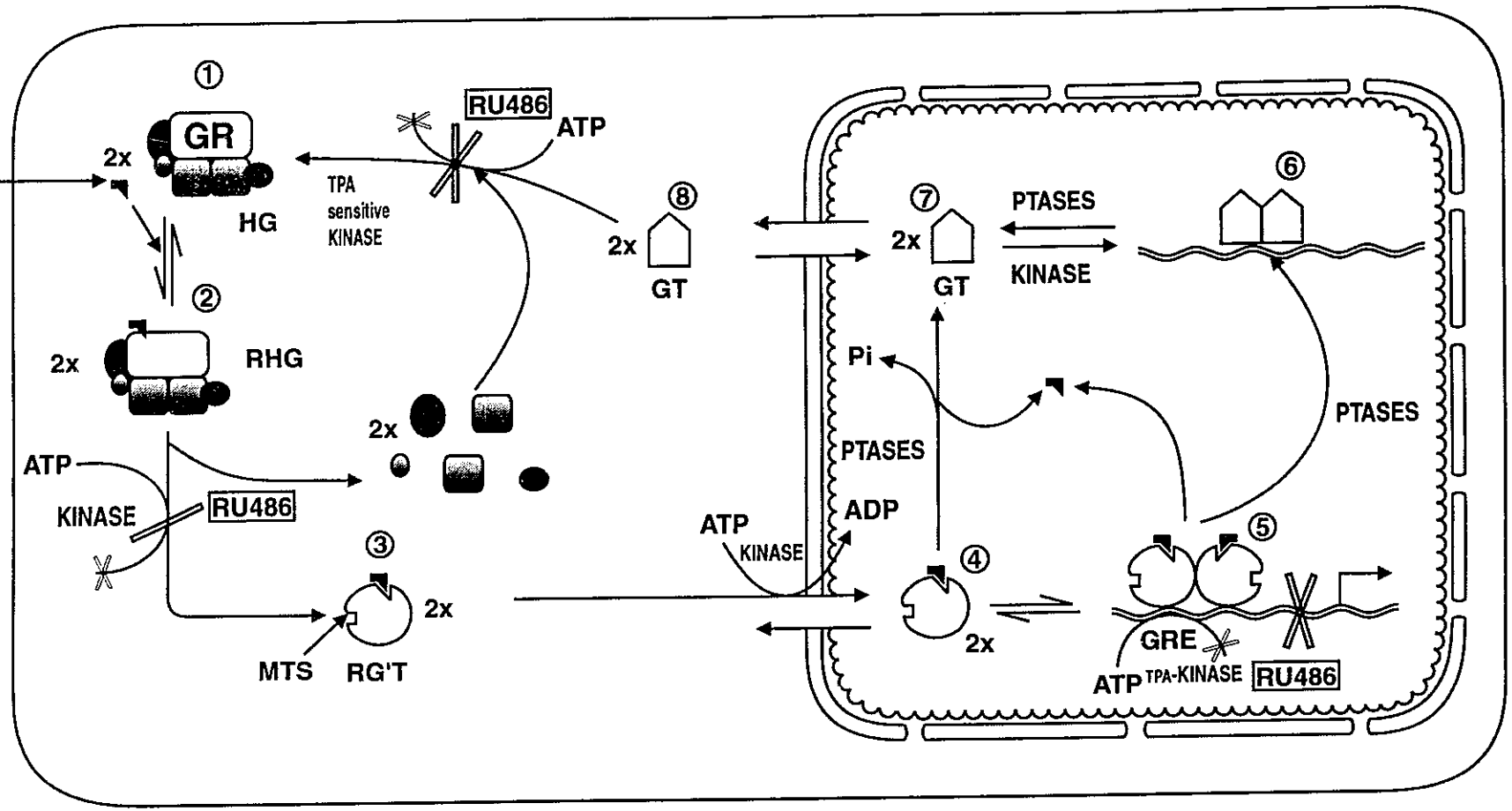
GR = Glucocorticoid Receptor
HG = GR-Heat Shock Proteins-Complex,
A = Agoinst
AHG = Agonist Bound Inactivated Receptor,

AG' = Activated Receptor
G = Free GR
GRE = Glucocor ticoid Response Element

Figure 33A

Figure 33B. A Simplified Schematic Presentation of a Model of Nucleocytoplasmic Trafficking Events of the Glucocorticoid Receptor Upon Binding to the Antagonist RU486. The cytoplasmic GR in an inactive state is associated with hsps and other factors in an 8-9S multiprotein complex (HG), as described in Figure 33A. Following RU486 binding, an RU486 inactive complex is produced (RHG). Subsequently the hsps are dissociated at a much slower (50 % reduction) rate than from the hormone inactive complex, because of an induction of C-terminal altered conformation of the activated receptor (RGT; steps 1 and 2). The entry of RG' into the nucleus is by the same active transport process, as described for AG. In the nucleus RG' can also interact with many components, but more importantly, site specific, high affinity DNA binding sites (GRE), as does the agonist bound receptor. However, it does not alter the transcription of target genes (step 3), possibly due to the altered conformation which prevents it from interacting with other transcription factors required for transcriptional activity.

Following loss of ligand, the Free unliganded altered receptor (GT) shuttles between nucleus and cytoplasm. Although, GT is also accessible to the re-cycling machinery it does not form 8-9S heterocomplex (RHG) , because it does not serve as a substrate for the TPA-sensitive kinase. Thus RU486 affects 3-steps in the cycling process: a) it inhibits dissociation of receptor from hsps; b) it blocks transcription because of an altered conformation of a phosphorylation site; c) the altered 4S unliganded receptor, GT, is not a substrate for reassembly of 8-9S heterocomplex because of the masked phosphorylation site.



Proposed Model Showing the Effects of RU486 on GR Cycling.

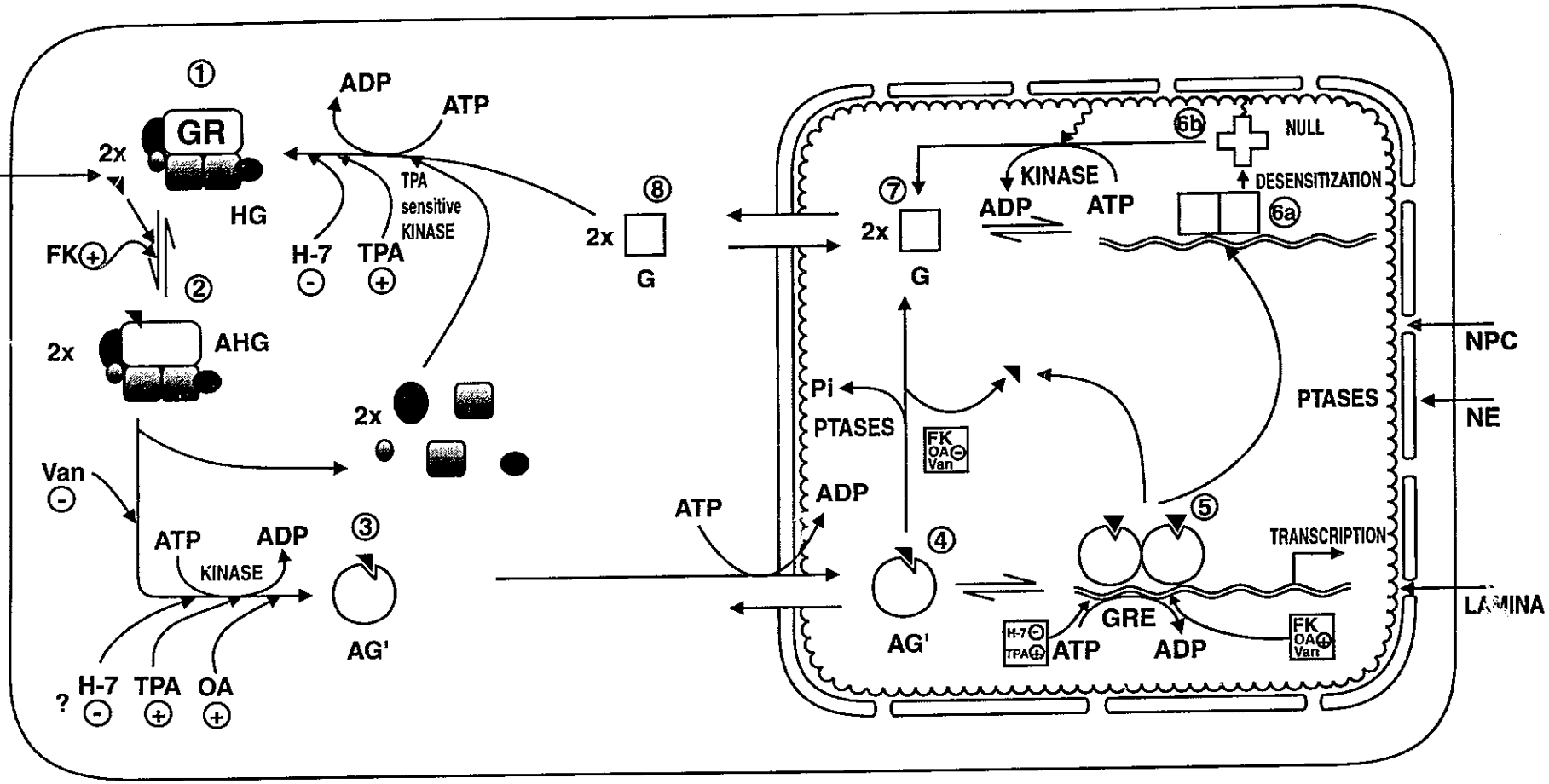
GR = Glucocorticoid Receptor (GR)
HG = GR-Heat Shock Proteins Heterocomplex
RG'T = Activated RU486 Bound GR with Altered Conformation
GRE = Glucocorticoid Response Element
GT = Altered 4S Receptor
R = RU486

MTS = Masked TPA-Sensitive Kinase Site
PTASES = Phosphatase(s)
/ = Inhibited
X = Blocked

Figure 33B

Figure 33C. A Model of Nucleocytoplasmic Trafficking Events of the Glucocorticoid Receptor Upon Agonist Binding, Highlighting the Effects of H-7, TPA and other Modulators of Protein Phosphorylation. The cytoplasmic GR in an inactive state is associated with hsps and other factors in an 8-9S multiprotein complex (HG), and after ligand binding it undergoes the same cycling pathway as described in Figure 33A (steps 1-8). H-7 decreases the rate of nuclear import by inhibiting receptor dissociation from the 8-9S heterocomplex and/or inhibit the ATP-dependent translocation step at the nuclear pore. H-7 also inhibits transactivation and blocks receptor re-cycling by blocking the action of a TPA sensitive kinase whose activity necessary for re-cycling. TPA stimulates the action of this enzyme thereby increasing the rate of re-cycling into the 8-9S heterocomplex.

Modulators such as forskolin and OA effect a moderate increase in the rate of nuclear import, whilst vanadate reduces the import rate by inhibiting receptor dissociation from the 8-9S heterocomplex. All three agents increase the apparent nuclear retention of the receptor. Dissociation of ligand from the receptor may be accompanied by dephosphorylation of the receptor (steps 4 and 7). Thus one possibility by which these agents may increase nuclear retention is to enhance site-specific phosphorylation of the receptor and to suppress the dephosphorylation mechanism carried out by phosphatases, (step 4 and step 7), in which case the receptor will be maintained in the ligand bound form thereby shifting the equilibrium of occupied receptor towards DNA binding (step 4 to step 5) and subsequently to enhance transcription.



Proposed Model Showing the Effects of Modulators of Phosphorylation on GR Cycling.

GR = Glucocorticoid Receptor (GR)
HG = GR-Heat Shock Proteins Heterocomplex
AHG = Agonist Bound Inactivated Receptor
AG' = Activated Receptor
G = Free GR
GRE = Glucocorticoid Response Element
FK = Forskolin

Van = Vanadate
OA = Okadaic Acid
⊕ = Effect is Stimulatory
⊖ = Effect is Inhibitory

Figure 33C

IV. DISCUSSION

The goals of this research were to further characterize the nuclear transfer defect of a mutant GR isolated from the mouse S49 lymphoma, by determining the single amino acid substitution mutation(s) that is/are responsible for the nuclear transfer phenotype and to investigate the effect of phosphorylation on nucleocytoplasmic transport of the GR. I determined: a) the kinetics of nuclear uptake of expressed cytoplasmic receptors after exposure to hormone; b) the kinetics of return of GR to the cytoplasm after hormone withdrawal; c) the effects of modulators of protein phosphorylation on (a) and (b). My results show that the Arg484-His substituted mutation results in reduced equilibrium levels of nuclear receptor. I confirmed that the observed lowered nuclear equilibrium levels were due to lack of DNA binding by obtaining similar results with another DNA binding mutant. The nuclear equilibrium levels of GR mutants that are defective in DNA binding was reduced by up to ~ 40 %, which suggested that DNA binding is important in nuclear retention of the receptors. The Tyr770-Asn mutant however, displayed exactly the same rate of nuclear uptake and similar steady state distribution as the WT GR.

Hormonal restimulation of nuclear receptors returned to the cytoplasm after hormone withdrawal resulted in rapid re-entry of the receptor into the nucleus, suggesting that after hormone withdrawal, GRs accumulate in the cytoplasm as inactive 8-9S hsps-heterocomplexes rather than as 4S unliganded receptors.

The kinetic studies with the modulators of protein phosphorylation and the GR antagonist, RU486, showed that whilst the initial rate of loss of nuclear receptors to the cytoplasm was markedly enhanced by TPA,

return to the cytoplasm was completely blocked by nuclear receptors that had been bound to RU486 or that had been treated with H-7. This suggested that RU486 and H-7 treatment might have produced a form of receptor that could not accumulate in the cytoplasm and that an active TPA-sensitive kinase activity was important for the cytoplasmic return of nuclear receptors. Further, by monitoring the kinetics of loss of nuclear RU486 and H-7 treated receptors to the cytoplasm in the presence of an energy synthesis inhibitor, I distinguished between the effects of these agents on exit of the receptor from the nucleus and receptor re-cycling, that is, re-incorporation of the receptor into an inactive hsps heterocomplex competent to re-bind ligand. My results showed that when cells are deprived of energy the blockage by RU486 and H-7 treated receptors in redistribution to the cytoplasm is overcome. Since energy is required for import and not for export of steroid receptors and certain proteins my results suggest that RU486 or H-7 treated nuclear receptors are able to exit from the nucleus and hence, retain their ability to shuttle between the nucleus and cytoplasm. Thus it is the re-cycling process that appears to have been blocked by treatment with these two agents and not shuttling. My results from the TPA and H-7 experiments demonstrated that re-cycling requires the action of kinase(s) that is/are inhibited by H-7 and stimulated by TPA.

Kinetic measurements of cytoplasmic return of nuclear receptors after hormone withdrawal, using modulators in combination such as forskolin and TPA or forskolin and OA demonstrated a cancellation effect on apparent nuclear retention of WT receptor. However, the Arg484-His GR mutant in both cases only demonstrated the effects of either the TPA alone or the forskolin alone respectively. This provided preliminary

evidence that phosphorylation events may play a very important role in GR DNA binding and the subsequent transcriptional activity.

A. Establishment of Conditions for Quantitative Indirect Immunofluorescence Assay

The ability of lipofectin to facilitate DNA uptake into cells was compared with another commonly used method of transfection, electroporation. When considering the best conditions for transfection, I assessed expressed β-gal activity (transfection signal) and cell death. I determined that the lipofectin to DNA ratio and not the amount of DNA is the major determinant of the efficiency of transfection. Consistent with the observation made by Felgner et al. who showed by lipofection that transfection efficiency is relative to a broad range of DNA concentration[276]. Further, the concentration of lipofectin also affected the transfection efficiency; I was able to obtain satisfactory transfection with lipofectin levels between 5 to 10 μg. At 10-15 μg the transfection signal was reduced but the cells remained viable. However, at 20 μg of lipofectin about 50 % of cell death occurred, suggesting that while at this amount of lipofectin there may be an increase in the specific activity of β-gal in the cell extract, which is an index of the transfection efficiency, significant toxicity also occurs. This observation is also consistent with a previous report which showed that high levels of lipofectin were toxic to the cells[277]. However, toxicity was obtained at a level much higher (100 μg) than what I obtained. The toxicity varied with density of the cell culture: dense cultures of approximately 70-80 % confluency were more resistant to the toxicity effect than less dense cultures.

The transfection of DNA by electroporation was also studied under different conditions. Previous reports have shown that transfection efficiency increased with increased amount of plasmid DNA up to 80 μg, and that denatured salmon sperm DNA could be used to make up the DNA requirement for a high transfection efficiency[279]. I therefore used 50 μg of denatured salmon sperm DNA as a carrier, and maintained the plasmid construct levels at 20 μg. Voltage was a critical parameter for electroporation; for a given capacitance, buffer and number of cells, there was a sharply defined voltage for optimal transfection efficiency. The transfection signal rose with increasing voltage until it was offset by a marked decrease in cell viability. Thus application of this procedure to other cell lines should include a careful measurement of transfection efficiency over a range of voltages. Electroporation efficiency was also tested at two values of capacitance, 800 μF and 1080 μF. Transfection was more efficient at the lower capacitance than at the higher capacitance if the voltage was kept constant. This may be due to greater percentage (> 90 %) of cell death at the higher capacitance. The number of cells used in the transfection experiments is also a determinant of transfection efficiency: if cell number is increased there is a higher voltage requirement for optimal signal if capacitance is kept at 800 μF. In contrast, Chu et al. demonstrated in primary human fibroblast, CV-1 and NIH 3T3 cell lines that electroporation was more efficient at the higher capacitance and that the level of expression was independent of the number of cells used for electroporation[279].

Using the optimal conditions determined for electroporation yielded a 4-fold increase in β-gal expression per μg of protein relative to the β-gal levels obtained by using optimal conditions of lipofection. However,

when the same electroporation conditions were used to transfect the GR plasmid construct, a plasmid recombinant with an SV40 replication origin, the rate of GR expression was negligible, as observed by Western immunoblotting analysis, of COS-7 cell extracts. By contrast, when the established optimal transfection conditions for lipofection of the β-gal plasmid construct were used to carry out transfections with plasmid constructs of GR in COS-7 cells, a high level of GR expression was observed. The most likely explanation is that because of the high rate of replication, the expression of the GR construct after electroporation was too high. A threshold might have been reached at which GR was inhibiting its own expression by sequestering limiting transcriptional coactivators (squelching) of its own gene. Consistent with this explanation, I also found that to obtain optimal expression by lipofection using the GR plasmid construct, I needed to use sub-optimal amounts, i.e. 10:3 (μg) lipofectin to DNA ratio instead of the 10:6 (μg) we obtained with β-gal construct. This may be because the GR expression vector, unlike the β-gal expression construct has the ability to replicate to a high copy number.

Whole cell steroid binding assays were performed to confirm that the overexpressed WT receptor was capable of binding hormone and also to estimate receptor number in the transfected COS-7 cells. The detection and assay of steroid hormone receptors were based on measurement of high affinity, low capacity (saturable), 'specific' binding sites for the hormone that are distinguishable from low affinity, high capacity (non-saturable), 'non-specific' binding to other cell constituents that are always present. As observed from the hormone binding

saturation curve, the quantity of non-specific binding was very high compared to the total binding. The high level of non-specific binding in this experiment was most probably due to one factor: because only limited amounts of expressed GR were available after transfection by lipofection, cell extracts were prepared by sonication. Sonication results in membrane disruption and the release of membrane phospholipids and proteins which are known to bind steroid[290]. Others have also shown high levels of non-specific binding in the presence of membranes[291]. Thus a rough estimation of the affinity constant, Kd, and receptor number, n, was made from this experiment. However, the estimated Kd (2.2×10^{-9} M) and receptor molecules per cell (1.2×10^{6}) were comparable with what have been quoted in the literature for overexpressed receptors (2.0×10^{-9} M and $1\text{-}3 \times 10^{6}$ respectively)[32,311].

B. Influence of Receptor Mutants Deficient in Hormone and DNA Binding on Nucleocytoplasmic Transport of GR

Nuclear import occurs after ligand binding to the cytoplasmic inactive GR-hsps-heterocomplex which results in dissociation of the hsps from the receptor-complex. A change in conformation of the receptor and unmasking of the nuclear localization signals makes it accessible for interaction with NBPs. Subsequent docking of the receptor at the nuclear pore occurs which is followed by the translocation of the ligand-receptor complex into the nucleus in an ATP-dependent manner. Our studies on kinetics of nucleocytoplasmic transport of the single amino acid substitution mutations of the nt^{-} GR showed that the Tyr770-Asn substitution did not have any influence on nucleocytoplasmic transport of the mutant receptor. Although Danielsen and co-workers previously

reported a 50 % decrease in this receptor's affinity for steroid[14], I did not observe any difference in the rate of nuclear uptake as compared with the WT receptor even at low doses of dex. In addition, the Tyr770-Asn substitution did not affect the cytoplasmic redistribution of the mutant receptor. Although since this receptor mutant has been reported to result in decreased affinity for steroid I had expected it to at least be less sensitive to residual concentrations of glucocorticoids in the cell. Consistent with our findings however, the Tyr770-Asn substitution has been shown to result in only a 15 % decrease in transcriptional activity of the glucocorticoid inducible GR-mediated MMTV-CAT reporter gene[14].

Our results on kinetics of nuclear uptake of the Arg484-His substitution indicated that this substitution does not have any appreciable effect on the initial rate of nuclear uptake following dex treatment, but rather resulted in ~ 40 % decrease in the steady state nuclear distribution of the mutant receptor. Previous X-ray crystallographic studies suggest that the Arg484-His substitution could distort a carboxy terminal α-helical structure of the second finger between the helix and the GRE base specific phosphate contact[24]. Thus this mutation, predicted to disrupt contact with the DNA phosphate backbone of the GR DNA binding site results in a receptor with defective DNA binding. We therefore reasoned that the reduced steady state levels attained by this mutant could be attributed to the mutant receptors' inability to bind DNA with subsequent increased rate of export from the nucleus. We substantiated this claim by investigating the effect of another DNA binding receptor mutant with a deletion of the first finger (Δ420-451). It too demonstrated relative to its WT counterpart, lower steady state nuclear levels.

A previous report by Picard and Yamamoto[17] suggested that NL-2 could compensate for NL-1 in directing nuclear entry of the GR, whilst Cadepond et al. have reported that NL-2 may not compensate for NL-1[312]. To clarify this controversy, and to further demonstrate that mutations that disrupt DNA binding do not affect the rate of nuclear entry but only result in reduced equilibrium levels of nuclear receptor, I investigated the import kinetics of two other human GR mutants; a second zinc finger mutant which also had a deletion of the N-terminal region of NL-1 and two zinc finger mutants with N-terminal region deletions of NL-1. In contrast to the first zinc finger mutant tested previously which did not show an altered rate but rather showed reduced equilibrium levels of nuclear receptor, these two mutants displayed both marked impairment of the rate of import into the nucleus as well as reduction in equilibrium levels of nuclear receptor. Normal equilibrium levels were not attained even after 24 h period. Previous subcellular localization studies with a human GR construct lacking the NL-1 core sequence also demonstrated a marked reduction in nuclear immunostaining[312]. As mentioned above, the Arg484-His mutant which also displayed only reduced equilibrium levels of nuclear receptor is a second zinc finger mutant. Since all of the human receptor mutants have an intact NL-2, the results of these kinetic studies suggest that for GR both the N-terminal flanking region sequence and the core sequence of NL-1, shown in Figure 32, are important for efficient nuclear entry of the receptor. Further, both zinc fingers of GR are probably required to provide normal equilibrium levels, i.e., to maintain nuclear retention of the receptor. Whether or not part of the second zinc finger structure also contributes in determining the rate of nuclear uptake is difficult to

assess in the absence of import kinetic studies using stepwise second zinc finger, and NL-1, deleted mutants of the receptor. Thus future studies would address this question by preparation of stepwise GR second zinc finger and NL-1 deletion mutant constructs for use in the studies of import kinetics. An alternate approach would be to generate substituted point mutants of the second zinc finger and to use them in nuclear uptake studies.

All previous investigations by cytoimmunofluorescence on the effect of DNA binding on nuclear localization of steroid receptors have led to a conclusion that the DNA binding ability of the steroid receptor does not grossly affect nuclear localization [128,129,209,312]. My data is the first to show that the nuclear occupancy of the receptor is reduced by mutations that impair DNA binding *in vitro* . The differences in nuclear occupancy between WT GR and DNA binding mutants would not have been detected by previous protocols used because quantitation of nuclear localization was not carried out. However, my data are consistent with previous results obtained by subcellular fractionation and dex binding studies which showed that the single amino acid change (Arg484-His) at the carboxy terminus of the second zinc finger reduces nuclear association of the receptor by close to 40 %[14]. Subsequently this substitution mutation was shown to completely abolish the hormone-inducible GR-mediated transcriptional response. My present data also show an approximately 40 % reduction in equilibrium nuclear distribution of the receptor. However, my kinetic studies have distinguished between a defect in nuclear entry and a defect due to impaired association with a nuclear structure; nuclear association studies on subcellular fractions do not distinguish between these two possibilities.

I then showed that the lowered steady state distribution of the Arg484-His substitution of the nt⁻ GR was due to the fact that the mutant receptor is defective in DNA binding and thus was made more accessible for export. After hormone withdrawal this receptor mutant and other DNA binding mutants were returned to the cytoplasmic compartment ~ 2 to 3-fold faster, relative to their WT counterparts. Similar observations on the effect of nuclear retention in decreasing export rate have been reported by Schmidt-Zachmann et al[187]. They demonstrated that an artificial nuclear reporter protein derived from cytoplasmic pyruvate kinase (PK) fused to the NLS, RNA binding motifs (RNP) and Gly-Arg rich (GA) domains of nucleolin (NLS-PK-RNP/GA) exhibited < 10 % of the export rate from the nucleus in 8 h whereas NLS-PK which lacked the RNP and GA domains displayed a much faster cytoplasmic distribution (45 %) within the same time. Subsequently, biochemical data illustrating the influence of the nucleolin GA domain on extractability of transfected nucleolin also demonstrated that upon gentle fractionation of transfected HeLa cells approximately 90 % of a ΔGA mutant was released into the supernatant while only 20 % release of the WT nucleolin was observed. This strongly supported the increased export property of the NLS-PK being largely due to the absence of the GA domain.

With this present data it is difficult to explain why the export rate of hWT was faster than the mWT. The major difference between these two proteins is a stretch of twelve polyglutamine residues in the amino terminal domain of the mouse GR[14] which is absent in the human GR[13]. Perhaps these amino acid residues with basic side chains

interact with a component(s) of the nucleus and thereby cause greater retention of the mWT receptor.

Thus my present data and other reports lead to the conclusion that, although nuclear import is determined by the availability of a functional NLS, attainment of normal equilibrium levels may be determined by specific binding to intranuclear structures once a protein has been transported into the nucleus. This careful quantitative kinetic analysis of nuclear occupancy of DNA binding mutants of GR, contrary to the claims others have made in more gross analysis, demonstrated a role of DNA binding in nuclear retention and draws an attention to export being the aberrant process in nuclear transfer-deficient DNA-binding mutants of GR.

The role of tight binding of proteins to intranuclear structures in nuclear retention and in determining at least in part the export rate and shuttling ability of PR has also been confirmed in the recent report by Guiochon-Mantel et al.[189]. Their report suggested a second mechanism that controls the export of shuttling proteins. Their studies showed that a large protein devoid of an NLS cannot cross the nuclear membrane in either direction. However, an NLS-β-galactosidase fusion protein not only accumulates in the nucleus but also continuously shuttles between the nucleus and the cytoplasm, suggesting that all proteins that carry karyophilic signals should shuttle between the nucleus and cytoplasm. Such a conclusion is however, contradictory to observations made by Schmidt-Zachmann et al.[187,313], which demonstrated that the tagging of an SV40 large T-antigen NLS to another cytoplasmic protein, pyruvate kinase, rather withheld the protein in the nucleus as compared to its untagged counterpart. The most likely explanation for the discrepancy in

the findings of the two groups was suggested to be due to the fact that they had used different proteins in their study. The export rate at least in part depends on the ability of a protein to bind tightly to intranuclear structures and the fraction of unbound protein that remains able to shuttle between the nucleus and the cytoplasm. In heterokaryons between COS-7 cells containing SV40 large T-antigen and mouse L- cells devoid of this protein, the transfer of the large T-antigen was not detectable at 6 h but was readily observed at 24 h. On the other hand the transfer of NLS-β-galactosidase fusion protein was already maximal by 6 h. Thus the karyophilic signal of the large T-antigen does impart the ability to shuttle to heterologous proteins. However, in the context of the SV40 large T-antigen itself, which is a DNA-binding protein, this phenomenon was slow and thus difficult to observe.

Conclusions of our findings on the effects of DNA binding on nuclear import and export are: a) GR mutants that are impaired in site specific DNA binding are not altered in their rates of uptake but rather are more poorly maintained in the nucleus; b) DNA binding slows nuclear export by increasing nuclear retention of the receptor; and c) nuclear retention is determined at least in part by the strength of interaction of GR with DNA.

C. The Nuclear GRs that are Redistributed to the Cytoplasm after Hormone Withdrawal are Competent for Ligand Binding

Our data showed that the $t_{1/2}$ for nuclear import of WT receptor was 4-5 min. Previous studies have also shown that the $t_{1/2}$ for loss of ligand from the receptor is < 10 min[211]. However, our measurements of export kinetics demonstrated long $t_{1/2}$ (>12-18 h) for redistribution of GR

to the cytoplasm. We wondered whether the long $t_{1/2}$ for this process was due to the necessity for reformation of the inactive GR-hsps-complex before GR could be detected in the cytoplasm.

As mentioned in the Introduction, the cytoplasmic GR before exposure to hormone is in the form of 8-9S GR-hsps-heterocomplex. The assembly of this heterocomplex has been extensively studied in a cell-free system, the rabbit reticulocyte lysate, by Pratt and co-workers[326-340]. In this system hsp90 associates with the GR via the HBD, at the termination of receptor translation[326] to form a complex with hsp90. Hsp90 association represses the DNA-binding activity of the receptor[328,329]. In addition, this untransformed state is required for the high affinity steroid binding conformation of the GR. This scheme has been called the 'docking model' of steroid hormone action because GR is 'docked' to the hsps90 in the cytoplasm.

Three lines of evidence support the notion that hsp90 plays a biologically important role in the steroid receptor activity. First, in the case of the GR[327,328] PR[331] and ER[332], mutant receptors that are constitutively active are not bound to hsp90 whereas receptors that are steroid-inducible are bound to hsp90. Second, the glucocorticoid response is defective (requiring very high steroid concentrations) in yeast cells that produce reduced levels (50 %) of hsp90 in the signal transduction pathway[333]. The third line of evidence is indirect and evolves from studies of fusion proteins where the HBD of steroid receptors was shown to confer hormonal control onto other proteins[333].

Recently, it has been shown by studies involving selective depletion of the reticulocyte lysate system of hsp70 by affinity ATP-agarose chromatography, and readdition of purified hsp70 to the system that

hsp70 is also required for heterocomplex assembly of GR[334,335]. A model was proposed in which hsp70 provides a protein unfolding activity required for the opening up of hsp90 binding region to the HBD of the receptor. In addition, it has been reported that hsp56 which has been shown to be an immunophilin of the FK506 binding class, forms a component of the heterocomplex in an hsp70-dependent manner[337-339,346]. The immunophilins are proteins that bind immunosuppressive agents like cyclosporin A, FK506 and rapamycin[340]. The existence of three hsps in a complex implies that they may act in a coordinated manner, and it has been proposed that the hsps heterocomplex may function as a protein folding/trafficking structure[348]. Upon ligand binding or heat treatment, hsps dissociate from the 8-9S heterocomplex, thereby transforming the receptor into hsps-free 4S receptor, competent for DNA binding. It is also clear that the re-assembly process requires ATP/Mg^{2+} and other unidentified factors[336,349], (see Section D3 below).

The nuclear re-uptake experiments performed by re-stimulation of cytoplasmic redistributed nuclear receptors indicated that receptor molecules returned to the cytoplasm are competent for re-entry into the nucleus. This result could only be obtained if the redistribution of immunofluorescence reflected the cytoplasmic accumulation of GR in a state competent for ligand binding, i.e., in the hsps-bound complex form. This experiment may suggest that GRs accumulate in the cytoplasm after hormone withdrawal as inactive 8-9S hsp90 heterocomplexes, rather than the 4S unliganded receptor. Formation of the 8-9S hsps-complex may thus be rate-limiting for redistribution of GR to the cytoplasm. Further, since cytoplasmic redistribution of the WT GR was slower than for the DNA binding mutants, one may conclude that DNA binding by GR

in the nucleus plays a significant role in promoting nuclear occupancy of GR and inhibits reformation of the inactive GR-hsps complex following loss of hormone.

D. Influence of Glucocorticoid Receptor Antagonist and the Roles of Protein Phosphorylation Signaling Pathways in the Regulation of Nucleocytoplasmic Transport of the Receptor

Phosphorylation is rapidly emerging as an important mechanism in regulating the activities of many transcription factors[228-244,315-317]. Several studies involving cooperative interaction of different kinases to multiply phosphorylate transcription factors to induce synergistic enhancement of their transcription activity have been well documented[315-317]. For example, the cAMP response element binding protein and activating transcription factor 1 (CREB/ATF) family of proteins are phosphorylated at specific sites in the protein by different kinases to increase their DNA binding and/or their transcriptional activities[315,316]. Several kinase phosphorylation sites have been mapped in GR including a CK-II site in the N-terminal domain, which upon phosphorylation may stabilise the active conformation of GR[318], and a $p34^{cdc-2}$ kinase site in the N-terminal domain, which upon phosphorylation may control the function of GR in a cell cycle dependent manner[247,319]. As indicated earlier (Section I. C. 2), there are putative PKC and protein tyrosine kinase (PTK) sites present in the receptor. I identified both PKC and PTK sites in the hormone binding domain through a search for kinase phosphorylation site sequence and consensus motifs. It has also been demonstrated previously that GR may serve as a substrate for both kinases[226,320].

Similar multiple phosphorylation in the human progesterone and vitamin D receptors (hPR and hVDR) have been observed. hPR is phosphorylated at multiple serine residues, at a basal level in the unliganded receptor and then hyperphosphorylated in a hormone induced step with a subsequent enhancement of both DNA-binding and transcriptional activities[321]. Likewise, several protein kinase phosphorylation sites have been mapped in the human vitamin D receptor (hVDR)[322].

In addition, recent studies have shown that the glucocorticoid regulated GR-mediated transcription can be modulated by the activation or expression of certain oncogenes, including *mos*, *fos*, *jun* and *ras*[30-32,323,324]. Since these glucocorticoid response-modulating oncoproteins include a cytoplasmic protein kinase (*mos*)[323,324], transcription factors (*jun and fos*),[30-32] and GTP-binding protein (*ras*)[323,324], together with the fact that sequential protein phosphorylation is observed with SHRs, it seems likely that cooperativity of various signal transduction pathways some of which could lead to multiple site phosphorylation of the receptor in a progressive cascade, could impact upon different functions of SHRs. This present study on the kinetics of nucleocytoplasmic transport with RU486, TPA, H-7 and other modulators of protein phosphorylation provides evidence that these compounds regulate nucleocytoplasmic distribution of the GR which subsequently may modulate the GR mediated inducible effects on target gene transcription.

1. *Influence of RU486 on the Kinetics of Nucleocytoplasmic Transport and Transcriptional Activity of GR*

This section discusses my data on the effects of RU486 on nucleocytoplasmic transport together with other previous reports on the mechanism of action of RU486 which allowed me to propose a model for future studies.

My studies on the kinetics of nuclear uptake of GR using dex and RU486 demonstrated that dex was more efficient in promoting cytoplasmic depletion and nuclear accumulation of GR than RU486, with the $t_{1/2}$ for nuclear uptake of RU486 bound receptor being increased by two-fold.

As indicated earlier in the Introduction, it has been previously demonstrated from metabolic labeling studies with $^{32}P_i$ that whilst the phosphorylation state of RU486-treated PR or GR remains unaltered, agonist-treated receptors become hyperphosphorylated. This finding has led to a suggestion that agonist/antagonist treatment of the receptors could result in marked differences in their phosphorylation states and hence could be used as models to study the effects of phosphorylation on receptor action[325].

In vitro and *in vivo*, RU486 bound to GR exerts some degree of impairment of receptor transformation, stabilizing GR in an inactive 8-9S hsps heterocomplex form and thus subsequently, inhibiting GR-DNA binding[325,350]. Sucrose gradient sedimentation analysis, after treatment of GR-containing cells with agonist showed very little or no 8-9S GR hsps complexes detected in the cytosol, i.e., they were all transformed to the 4S liganded DNA binding state. However, it has been reported that in GR-containing cells incubated with RU486, more GR-RU486 complexes

remained in the cytosol and sedimented as 8-9S and less were transformed to the salt extractable nuclear fraction, where they also sedimented as 4S, compared to agonist treated cells[250,339]. The reported extent of inhibition has varied considerably[325,351,353], and in two instances it has been reported not to inhibit transformation of GR at all[352,353]. Investigators who reported partial transformation of RU486 bound receptor also reported that the portion of the receptors that were transformed were capable of binding DNA. Reduced affinity of GR-RU486 complexes for specific MMTV DNA and non-specific DNA compared to GR agonist complexes has also been reported[351]. However, other reports suggest that transformed GR-RU486 complexes bind specific and non-specific DNA with affinity equal to that of agonist complexes[350,352,353].

Similar differential sedimentation properties and transcriptional effects of RU486 have been reported for PR[354-361]. PR-RU486 bound complexes sediment more as 6S and fewer as 4S[354-357]. However, other reports have indicated that RU486 generally promotes dissociation of the PR from hsps, receptor dimerization and receptor DNA binding as effectively as the progestin agonist, R5020, and have proposed that possibly it is the protein-protein interaction of the receptor with other transcription factors that might be affected, leading to the blockage of transcription activation[354,355]. In support of this finding Guiochon-Mantel et al. have reported that an abortive complex formation of PR-RU486 complexes occurs at the composite PRE so that this complex no longer induce the transcription of progesterone target genes[357].

In summary, these studies led to the proposal of two general mechanisms for the action of RU486[262-265]. One hypothesis is that

RU486 binding to either GR or PR traps the receptor in the non transformed state, possibly through stabilization of the receptor-hsp90 interaction and thus inhibits receptor transformation. A second hypothesis is that RU486 promotes the steps of hsp90 dissociation and DNA-binding, but fails to stimulate downstream events involving interaction of the DNA bound receptor with the transcription complex.

Recent studies by DeFranco and co-workers demonstrated yet another step in GR or PR signal transduction pathway that was influenced by RU486 treatment. This report demonstrated that RU486 treatment of cells generates receptors that were unable to re-accumulate in the cytoplasm upon antagonist withdrawal[248].

I investigated the kinetics of exit of RU486-treated WT receptor and the Arg484-His mutant from the nucleus, after withdrawal of the antagonist. This kinetic data is consistent with the previous observation by DeFranco et al. which demonstrated that indeed the RU486 treated receptors are unable to accumulate in the cytoplasm upon antagonist withdrawal. However, I also demonstrated, using the Arg484-His receptor mutant that the RU486 effect on promoting apparent nuclear retention is independent of any DNA dependent receptor transformation.

I showed in export kinetic studies that TPA could accelerate the return of nuclear bound agonist receptors to the cytoplasm (see next section) but TPA could not reverse the blockage of cytoplasmic redistribution of RU486 nuclear-bound receptors. Interestingly, previous studies on transcription involving simultaneous treatment of cells with RU486 and activators of cAMP-dependent protein kinase or activators of PKC-dependent kinases to determine antagonist mediated transcriptional inducibility on hormone responsive reporter genes demonstrated that in

the presence of a stimulator of cAMP-dependent protein kinase, the RU486 effect on abrogation of transcriptional activity is partially overcome and 50 % of agonist activity is induced[300]. Likewise, the ability of RU486 to block the action of the glucocorticoid agonist, dex, is compromised by concomitant treatment with cAMP activator[300]. In support of this finding, the RU486 treated PR has also been shown to gain partial agonist transcription activity upon activation of cAMP stimulated pathways[239]. In contrast, activators of IP3/DAG signaling pathway failed to elicit induction of partial agonist activity in both GR and cPR[300]. This later finding is consistent with our present observation made on the kinetics of cytoplasmic return of RU486 treated nuclear receptors.

Studies by O'Malley and co-workers based on proteolytic analysis of *in vitro* translated PR suggest that the agonist treatment of GR induces a dramatic conformational change of the receptor[366]. They also showed that RU486 treatment induces an equally dramatic (as observed with agonist treated receptor), but distinct, structural alteration at the carboxy terminus of the ligand binding domain of the PR which centers upon the last 30-40 amino acids[366]. It was suggested that the transcriptional inactivation of GR and PR by RU486 could involve an induction of an inappropriate structural conformation at the extreme carboxy terminus of the ligand binding domain which is unaffected by the presence or removal of hsps[366].

Although the mechanism underlying the antagonistic effect of RU486 remains controversial, my present report on the effect of RU486 treated receptor on nuclear import supports some of the observations made previously on partial stabilization of the heterocomplex of RU486

bound receptor and its effect in promoting nuclear retention. The summary of all the observable effects of RU486, shown in Figure 33B, leads to a suggestion that RU486 exerts its antagonistic effects by acting at various levels of GR action: a) prevention of complete GR transformation; b) alteration of a step subsequent to GR DNA binding; and c) blockage of cytoplasmic return of nuclear receptors. It is therefore plausible to speculate that upon RU486 interaction with receptor, some kind of altered structural conformation at the carboxy terminus of the receptor is induced which may block a PKC site. Indeed I had previously identified a PKC site between amino acid residues 769-771 of the mouse GR, which lies in the region of the altered conformation.

The observations that RU486 treatment blocks receptor phosphorylation, that PKA stimulators but not stimulators of PKC induce partial transcriptional activity in RU486 treated cells and that RU486 treatment results in a blockage of the TPA stimulatory effect on redistribution of nuclear receptors to the cytoplasm, lead me to propose that the altered structural conformation assumed by RU486 receptor complexes still exposes cAMP-dependent kinase phosphorylation site(s) but masks a TPA sensitive kinase site present at the carboxy terminal domain of the receptor thereby rendering this site unavailable for phosphorylation by the kinase. Therefore the RU486 bound receptor with altered conformation is no longer a substrate for the TPA-sensitive kinase, whose action may play a distinctive role in receptor re-cycling. At the level of transcription, blockage of this same site may prevent protein-protein interaction with other transcription factors, a step which would have been otherwise required for the transcription activity of GR and PR.

2. *Effects of TPA and H-7 on Nucleocytoplasmic Transport.*

As indicated earlier, recent studies have examined the effect of phosphorylation on steroid receptor action on transcription mainly by the use of pharmacological agents which modulate kinase/phosphatase pathways[228,243]. Kido et al. earlier demonstrated that TPA enhanced the induction of tyrosine amino transferase gene expression[367]. In *in vitro* binding studies they demonstrated that H-7 treatment inhibited nuclear accumulation of [^{3}H]-dex-GR complexes in rat hepatocytes[368]. To date very little is known about the effect of these modulators of phosphorylation on other parameters of receptor function. In order to examine quantitatively how phosphorylation cascades could influence nuclear uptake and receptor cycling, I monitored the rate of nuclear import after pretreatment of cells with these modulators, and the rate of loss of nuclear receptors to the cytoplasm over 24 h, after the addition of the modulators at the time of hormone withdrawal. I found that protein phosphorylation mechanism(s) play a significant role in regulating nucleocytoplasmic transport.

TPA treatment increased the rate of nuclear uptake for both WT GR and the DNA binding mutant by ~ 2-fold. In contrast, H-7 slowed the import rate by ~ 2.5-fold. Further, for both WT and Arg484-His receptors, treatment with TPA markedly increased the initial rate of loss of nuclear GR to the cytoplasm after hormone withdrawal. By contrast, treatment with the mixed protein kinase inhibitor, H-7, completely blocked cytoplasmic redistribution of nuclear receptors. These experiments suggested that a TPA sensitive kinase activity is required in the process of return of nuclear receptors to the cytoplasm and that the activity of this enzyme may be blocked by H-7.

3. *After Hormone Withdrawal Unliganded Receptors Shuttle Between the Nucleus and Cytoplasm*

We reasoned that if free unliganded GR is predominantly nuclear, it may retain the ability to shuttle between nucleus and cytoplasm as has been reported to occur for its liganded counterpart[185]. Thus the exit of nuclear free 4S receptor to the cytoplasm would be observed if nuclear import were blocked. My studies with energy synthesis inhibitors which blocked nuclear import but allowed rapid efflux of free unliganded receptor molecules to the cytoplasm enabled us to distinguish between receptor re-cycling, a process that includes re-incorporation of receptor into an inactive hsps-containing complex, and shuttling, a process of rapid circulation of receptors between nucleus and cytoplasm, in which the receptor only appears transiently in the cytoplasm and thus is not detected by my present assay procedure. Upon energy depletion, the efflux of nuclear RU486 or H-7 treated receptors was dramatically accelerated to a rate equal to controls (i.e., untreated with RU486 or H-7). Thus the blockage of cytoplasmic accumulation of nuclear receptors by RU486 and H-7 may not be due to a blockage of nuclear export and indicated that the exit of receptor molecules from the nucleus to the cytoplasm may not be ATP dependent.

We conclude that in the absence of an energy inhibitor, when cytoplasmic accumulation of nuclear receptors is blocked by RU486 or H-7, these receptors remain as unliganded 4S molecules and continuously shuttle between the nucleus and cytoplasm, possibly because in the cytoplasm there is no retention site for the 4S free receptor until it forms the 8-9S hsps heterocomplex, (i.e., there may be no cytoplasmic anchorage mechanism achieved through hsps association

with the receptor and the subsequent interaction with the cytoskeleton unless the receptor is incorporated into the hsps heterocomplex) . Thus it is the re-cycling of the receptors that is blocked by the effects of H-7 and RU486. TPA may increase re-cycling of the receptor by increasing the activity of the kinase, whilst as mentioned earlier, RU486 may produce a form of receptor with an altered carboxy terminal conformation which may block a TPA sensitive phosphorylation site. In H-7 treated cells a normal unoccupied 4S receptor required for re-cycling is produced but H-7 inactivates the enzyme required for this process. H-7 has been shown to abrogate hormone-induced GR and PR mediated transcription [235,238]. Thus it appears that all the three distinct stages of GR function, i.e., dissociation of hsps from the receptor with its subsequent transformation, transcriptional activity and re-cycling, that are affected by RU486 are also inhibited in the same manner by H-7 and stimulated by TPA.

In support of the view that a TPA-sensitive kinase activity is required for re-cycling, Pratt and his colleagues have demonstrated, in addition to three hsps, the requirement of certain factors in heterocomplex assembly[336,349]. It is clear that the assembly process is ATP/Mg^{2+}-dependent and has a strict requirement for the presence of a monovalent cation, such as K^+, NH_4^+ or Rb^+, with Na^+ and Li^+ being inactive. It has been postulated that the monovalent cation may be required for the protein unfoldase activity of the hsp70. In addition to the hsps and the above mentioned factors, other as yet unidentified factors in reticulocyte lysate are required for a stable receptor heterocomplex assembly[345]. What has not yet been investigated is the possibility of the role of protein phosphorylation in heterocomplex

assembly. Kinases involved in protein phosphorylation reactions utilize ATP as a co-substrate and the kinase reaction is dependent on Mg^{2+}. In addition, one or more of the unidentified factors in reticulocyte lysate required in the assembly process may well be (a) protein kinase(s). An ATP/Mg^{2+}/Kinase dependent association of the unoccupied receptor with hsps in heterocomplex assembly may render the untransformed GR-hsps-complex receptor competent to bind hormone. As Munck and co-workers demonstrated earlier, there is no change in the phosphate content of hsps when they associate with GR[127]. They have also suggested that the unoccupied receptor must be phosphorylated to maintain its hormone binding capacity (HBC)[213,220]. Thus in the process of GR-hsps assembly the receptor must be the target for direct phosphorylation.

An important question is whether heterocomplex assembly is a nuclear or a cytoplasmic event. In fact whether any hsp90 is found in the nucleus is controversial[342,343]. Munck's kinetic model has suggested cytoplasmic reincorporation of the 4S unliganded receptor into the heterocomplex[211,218,244]. My experiments did not provide any evidence to suggest where heterocomplex assembly occurs. If the 8-9S heterocomplex is formed soon after receptor is released from DNA in the nucleus, any factor that would block assembly would result in an increase in the level of GR molecules remaining as shuttling 4S molecules. In the presence of energy synthesis inhibitors these 4S receptors would all accumulate in the cytoplasm because the second step of nuclear import which is energy dependent would be blocked, so there would be no nuclear re-uptake. If heterocomplex assembly is cytoplasmic similar observations would be made. In the absence of

energy inhibitors a portion of the 4S receptors that are released to the cytoplasm would rapidly associate with hsps to form the 8-9S heterocomplex and a portion would also be available for shuttling. In the presence of energy inhibitors the 4S receptors returned to the cytoplasm would not re-cycle nor shuttle (because of the requirement of ATP in heterocomplex assembly and blockage of nuclear import by energy inhibitors respectively) and hence would be retained in the cytoplasm.

Figure 33 is a simplified schematic presentation of a model of nucleocytoplasmic trafficking of the GR. B) Highlights the effects of the Glucocorticoid antagonist, RU486 on nucleocytoplasmic transport of GR. C) Highlights the effects of TPA, H-7 and other modulators of phosphorylation on nucleocytoplasmic transport of GR.

4. *Effects of Forskolin and OA on Nucleocytoplasmic Transport*

Increasing the activity of cAMP dependent protein kinase with forskolin, or the inhibition of PP-1/2A with OA had only a minor effect on the nuclear import of GR. However, both agents promoted nuclear retention of the receptor. These agents have also been shown to synergise with agonist to enhance transactivation of both GR and PR[228-236]. In T47D cells, 8-bromo-cAMP (another activator of c-AMP-dependent protein kinase) or OA in the absence of progestin augments cPR target gene transcription by 3-4-fold. Both agents also synergise with the hormone to further enhance transcription[269].

DeFranco and co-workers have previously shown that nucleocytoplasmic trafficking of GR is inhibited in *v-mos* transformed and in non transformed 6m2 cells treated with OA under normal cell proliferating conditions[236-237]. They found the defect to be due to inability of the

cytoplasmic hormone withdrawn GR to re-enter the nucleus upon readdition of hormone. It was suggested that the cytoplasmic redistributed receptor is desensitized due to alteration in its phosphorylation status[297]. Interestingly, by contrast to what was observed with OA in 6m2 non transformed cells, my data on cytoplasmic return of nuclear receptors in OA treated cells showed that the nuclear GR is sufficiently retained in COS-7 cells. I also showed in nuclear re-uptake experiments that the nuclear receptors returned to the cytoplasm in OA withdrawn COS-7 cells are competent for re-entry into the nucleus after a secondary hormonal response. These experiments and those of DeFranco's were done under different conditions: a) different cell lines were used in the two different experiments and b) in our experiments, cells were maintained at G_0 by serum deprivation, whilst in the previous report the experiment had been done in the presence of serum. The differences in results could reflect differences in cell type specific kinases/phosphatases. An alternate explanation is that under conditions in which there was serum in the cultured medium, serum factors could stimulate the induction of certain kinases whose action could be further stabilized by inhibiting the activity of PP1/PP-2A with OA[371]. Another possibility is that in COS-7 cells a higher concentration of OA may be required to induce the desensitization effect leading to the cytoplasmic retention of the receptors. These possibilities could be distinguished in the future by repeating these experiments in COS-7 cells with added serum in the withdrawal medium and at higher concentrations of OA.

5. *Effect of Vanadate on Nucleocytoplasmic Transport*

Vanadate, a phosphotyrosine phosphatase inhibitor, has been very useful in examining the role of tyrosine phosphorylation /dephosphorylation in biological functions. It has been reported that treatment of cells with vanadate enhanced phosphotyrosine kinase (PTK) activity of some membrane bound receptors that require tyrosine phosphorylation for activation up to about 40-fold, as well as reducing phosphotyrosine phosphatase activity[372-379]. This suggests that vanadate could both mimic and potentiate the effects of growth factors such as EGF. It has previously been demonstrated in ER- and PR-mediated transcription assays that vanadate or EGF induced receptor-mediated transcription, even in the absence of ligand[241,243]. In order to test whether signals originating in pathways requiring tyrosine phosphorylation influence GR nucleocytoplasmic transport, the effect of vanadate on nuclear import and receptor re-cycling were examined. My data show that vanadate slows the rate of nuclear uptake of both WT GR and the DNA-binding mutant by ~ 2-fold. Further, vanadate increases nuclear retention of both WT GR and the DNA-binding mutant following hormone withdrawal, suggesting the role of PTK in cytoplasmic accumulation of nuclear GRs. I interpret my data on the vanadate effect on nuclear import being a purely transition metal oxyanion effect, for reasons which I will discuss below. However, vanadate effect on nuclear retention may be linked with signaling pathways involving tyrosine phosphorylation.

Vanadate, like molybdate or tungstate, has also been reported previously to inhibit receptor transformation to the DNA binding form, thereby stabilizing the complex between the steroid receptor and

hsp90[380-382]. Similarly observations have been made from sedimentation analysis of molybdate treated GH1 cells containing PR and other SHRs[383-386] which showed that molybdate stabilizes the hsps associated complex. These observations are consistent with the interpretation that molybdate affects the rapidly exchanging subunit equilibrium between 8-9S and 4S cytosolic form by slowing the rate of 8-9S receptor dissociation. In contrast Munck and co-workers have proposed a cyclic model for the GR in which activation is an irreversible step[211,219]. The effect of vanadate on receptor dissociation from the 8-9S heat-shock protein complex could explain why our data showed that treatment of cells with vanadate inhibited the rate of nuclear accumulation of dex occupied GR.

It is not known how the transition oxyanion property of molybdate or vanadate results in an increase in the stability of hsps association with the receptor nor the mechanism of interaction of these metal ions with component(s) of the receptor-hsps heterocomplex. Three mechanisms were originally proposed by which transition metal oxyanions could inhibit GR transformation: a) interaction of the oxyanion exclusively with the steroid binding protein to increase its affinity for other components of the 8-9S complex[387]; b) formation of a bridge structure between the steroid binding protein and hsp90[388,389]; c) mimicking the action of an endogenous inhibitor of transformation with low molecular weight which upon removal transformation rapidly occurs[390-406].

Pratt and co-workers have identified the functional groups that may be required for the interaction of the transition anions with the receptor[390-392]. Their studies with sulphydryl modifying agents such as

methyl methane sulphonate or N-ethylmaleimide prior to the addition of molybdate-peroxide resulted in prevention of covalent modification of the receptor, suggesting that cysteine moieties are most likely the site of attack. These residues were mapped to Cys628, Cys649, Cys671 and Cys742[389,391,392]. This region of the receptor is known to contain the sites of interaction for both hsp90 and molybdate or vanadate, with the latter having a well established avidity for sulfur. Studies by Hollenberg and co-workers, using mutant with deletions in this region have shown that the metal anion stabilization site is situated outside the hormone binding region and suggested that it is most probably towards the carboxy terminal of the HBD[393,394].

Vanadate and molybdate have been shown to also induce a conformational change in purified hsp90, apparently through binding to an ATP site[249,404]. This raises a possibility that these metal ions could also interact directly with hsp90. If there is a metal oxyanion binding site for both GR and hsp90, then a more likely explanation for the metal oxyanions effect is that possibly they would interact with both the receptor and hsp90. Thus the proposal that a bridge structure is formed between the receptor and hsp90 via sulfhydryl moieties by these metal ions is a more likely mechanism by which transition metal oxyanions may stabilize the GR heterocomplex.

On the other hand, it has been proposed that vanadate or molybdate acts similarly to a small heat stable, metal chelating ubiquitous factor present in cytosol preparations which stabilizes the GR in its untransformed state in association with hsp90, thereby inhibiting receptor transformation to the DNA binding form[395-397]. This

endogenous factor also stabilizes cytosolic ER, PR and AR[398,399], suggesting that it may perform a general role in regulating steroid receptor function *in vivo*. The factor has been purified extensively from boiled rat liver cytosol in a 4-step procedure terminating with high performance liquid chromatography (HPLC) on an Ion-110 anion exchange column, which is particularly useful for separating metal anions[400,401]. Subsequent characterization of this ubiquitous factor showed that it has physical properties, i.e., high thermal stability, charge and chelation properties[386,396,397] of a metal oxyanion. It is clear that this endogenous factor normally stabilizes the GR-hsp90 complex in cytosol because removal of the factor by passage of cytosol through a metal chelating matrix (chelex-100) makes the GR unstable, thus markedly facilitating its dissociation from hsp90 and its subsequent transformation to the DNA binding state[401]. It was suggested that vanadate or molybdate may affect steroid receptor complexes by interacting with a metal anion binding site that is normally occupied by this endogenous receptor stabilizing factor[401].

Thus the vanadate effect on the rate of nuclear import may be purely a transition metal oxyanion effect, rather than its common effect on stimulating signal transduction pathways involving tyrosine phosphorylation, and I could not use the vanadate effect to substantiate a link between tyrosine phosphorylation and GR nuclear import.

Treatment of cells with vanadate after hormone withdrawal resulted in an increase of nuclear retention of both WT receptor and the Arg484-His mutant. Vanadate has also been shown to synergise with the hormone inducible SHRs mediated target gene transcription. Vanadate is a phosphotyrosine phosphatase inhibitor and its effect results in an

increase in the phosphorylation state of tyrosine residues of cellular proteins. Thus the vanadate effect on nuclear retention may serve to promote the hormone inducible transcription response of target genes primarily resulting from signals initiated by protein tyrosine kinases (PTK)[407-409] which subsequently promotes transactivation of the receptor. There are numerous kinase cascades that are activated by binding of numerous ligands to membrane receptors which are associated with a tyrosine kinase activity (e.g. EGFR; insulin-like growth factor receptor, IGF-1R; or platelet derived growth factor receptor, PDGFR)[407,408]. One of these ligands, e.g., the EGF previously shown to synergise with steroids in certain steroid receptor mediated transactivation may do so by increasing the nuclear retention property of these SHRs.

How PTKs induce their physiological responses have been intensely investigated. It is clear that autophosphorylation of the receptors is important for the signal transducing capabilities of PTKs[415,416]. Autophosphorylation on tyrosine residues in PTKs results in these receptors being able to recruit and interact with other proteins containing domains designated SH2 (for *src* homology region 2), a conserved protein module of approximately 100 amino acids, which is found in a markably diverse group of cytoplasmic signaling proteins and plays a pivotal role in their interactions with receptor tyrosine kinases[417,423]. Several SH2-containing proteins are known to be involved in the transduction of intracellular signals[417-428]. Examples are the RAS GTPase activating protein (GAP)[420], phosphatidyl inositol-3-kinase (PI3K)[421,427-430], adaptor protein p^{85}, phospholipase c-γ (PLC-γ)[425] and most non-receptor class PTKs, including *src*. Proteins with

SH2 domains frequently possess a distinct sequence of about 50 amino acid residues, the SH3 domain, which is also implicated in the regulation of protein-protein interactions during signal transduction. Thus SH2 and SH3 domains may serve as adaptors to link PTK to specific target proteins. The phosphorylation of these non catalytic domains subsequently modulates intracellular responses to growth factor stimulation. Two of the SH2 containing proteins shown to interact with PTKs are phospholipid metabolizing enzymes (PI3K and PLC-γ). Thus association of PTKs with proteins that metabolize phospholipids suggests that the activities of many tyrosine kinases ultimately could lead to the activation of Ser/Thr kinases and subsequently phosphorylation of certain proteins on serines and/or threonines. Hence the downstream consequences of the action of PTKs may result in signaling events similar to those produced by activators of Ser/Thr kinases[436,437]. Thus vanadate effect may not necessarily lead to tyrosine phosphorylated residues in GR but rather, may ultimately lead to phosphorylation of serine or threonine residues which would subsequently enhance nuclear retention of the receptor.

Vanadate, forskolin and OA may increase nuclear retention by increasing the phosphorylation status of GR through suppression of the activity of a phosphatase(s) either directly (OA and vanadate) or indirectly (forskolin and vanadate) through reversible phosphorylation mechanisms. The loss of ligand from the receptor may be accompanied by a dephosphorylation step. Thus the action of a phosphatase(s) may be important in the formation of the unliganded 4S receptor from the liganded 4S (Steps 4 and 7 of Figure 33C).

6. *Mechanisms by Which Dual Phosphorylation Signaling Pathways May Regulate Nucleocytoplasmic Transport of GR*

Given the complexity of the regulation of cellular processes by the second messengers cAMP, Ca^{2+}, and diacylglycerides and the remarkable diversity of agents that influence cellular function and metabolism through these pathways, it is not surprising that cross-talk between these signaling systems should occur so as to bring about diversity and the generation of multi-signaling cascade systems with common control points[303-308,440-443]. This will in turn bring about high sensitivity and 'fine tuning' of the various cellular processes which they regulate.

Several recent reports suggest that phorbol esters such as TPA or PMA modulate the cAMP response[302-308,457,458]. Depending on the cell type investigated and on the specific nature of the cAMP-elevating stimulus, PKC has been shown to either augment or inhibit cAMP levels by stimulating or diminishing forskolin stimulated adenylate cyclase activity respectively[457,458]. In one report, this inhibitory PKC effect was shown to be reversed by H-7[303]. Since both forskolin alone [228,231] and TPA alone [224,235] act synergistically to enhance the hormone inducible GR-mediated transcription, I tested the effects of simultaneous addition of forskolin and TPA on nucleocytoplasmic transport of the GR.

a) Effects on nuclear import

Unlike forskolin or TPA alone which each increased the rate of nuclear import by about two-fold, forskolin and TPA in combination at physiological concentrations of dex reduced the rate of nuclear uptake for both WT receptor and Arg484-His mutant to control, untreated levels. The effect of combined forskolin and TPA treatment on nuclear import of

the receptors is difficult to explain. However, similar observations have been made in the β-adrenergic receptor systems. In these systems it has been shown that the stimulatory effect of agonist binding to the β-adrenergic receptor could be interfered with the IP3/DAG stimulatory pathway(s) to result in transmodulation of agonist-receptor induced adenylate cyclase activity. Similar observation has been made with cells treated with combined forskolin and OA. As it is to be expected, forskolin treatment alone increased the cAMP levels with a resultant increase in agonist (epinephrine) binding. However, OA treatment could reduce the forskolin induced agonist activation.

Receptor mediated activation of both adenylate cyclase and phosphatidyl inositol 4,5-bisphosphate hydrolysis occurs through guanine nucleotide regulatory proteins (GTP-binding proteins or G-proteins)[440-442]. These are a family of heterotrimeric GTP-binding, membrane bound proteins (essential components of the transduction mechanisms underlying cellular signaling), through which signals from the surface of the plasma membrane are transduced leading ultimately to specific activation of either Ca^{2+}/ phospholipid dependent PKC or cAMP-dependent PKA.

Stimulation of the cAMP-dependent protein kinase pathway occurs when the interaction of an agonist with its receptor at the cellular membrane ultimately leads to the stimulation of adenylate cyclase and subsequently an increase in intracellular cAMP levels. It is well established that adenylate cyclase is controlled by the stimulatory G-protein, G_S, and inhibitory G-protein, G_i[443,444]. G_S, like all G-proteins, is composed of three peptide chains subunits: α, β and γ. The α-subunit binds GTP and GDP. $G\alpha$ is found in a complex with $G\beta$ and $G\gamma$, and in

this condition adenylate cyclase is not activated. However, binding of an agonist to its receptor leads to a replacement of GDP by GTP with a resultant dissociation of $G\alpha$ from $G\beta G_\gamma$ subunits. GTP-bound G_α binds to adenylate cyclase which in turn catalyzes the synthesis of cAMP, a second messenger that activates a number of cAMP-dependent kinase activities. Thus $G\alpha$ functions to couple membrane receptors and adenylate cyclase. G_i possesses the same β and γ subunits as G_S but has a different G_α. In contrast to G_S, G_i causes inhibition of adenylate cyclase activity. Forskolin and other PKA stimulating agents have also been shown to directly stimulate adenylate cyclase activity thereby increasing intracellular levels of cAMP.

Likewise PKC activation is achieved through membrane phosphatidyl inositol-4,5-bisphosphate (PIP2) hydrolysis. A principal component of the phosphoinositol system leading to activation of PKC is phosphoinositidase C, (also known as phospholipase C; PLC) a receptor-activated enzyme, with the activation occurring via a PLC specific G-protein (G_p). The cleavage of PIP2 by this enzyme liberates diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3)[441,442,445-450]. The DAG stimulates PKC by increasing its affinity for Ca^{2+}, whilst IP3 stimulates the release of calcium from intracellular calcium stores such as the ER and proposed calcium containing organelles in non muscle cells referred to as calciosomes[450,455] which subsequently also stimulates PKC. TPA is a potent analog of DAG and acts in a similar manner in stimulating PKC activity[440-442].

The actual mechanism by which TPA or OA cause desensitization in the presence of forskolin is not well defined. However, it has been shown that the stimulatory effect of TPA on certain kinases could bring

about phosphorylation of the receptor or Gs, thereby uncoupling receptor interaction(s) with Gs. The consequence is to diminish adenylate cyclase activity with a subsequent decrease in intracellular cAMP levels. Thus the outcome of combined forskolin and TPA effect on nuclear uptake in COS-7 cells is similar to the β-adrenergic system described above. Further, in the SHR systems forskolin has been shown to increase dex binding, with subsequent enhancement of transcriptional activity[228,229]. However, since GR is not a plasma membrane receptor but an intracellular receptor, I am not able to suggest the molecular basis of my observation without much further investigation.

Interestingly, another mode of regulatory mechanism was observed upon simultaneous treatment of cells with forskolin and TPA at sub-physiological doses of dex. A DNA-dependent down regulation of GR levels was observed. At hormone concentrations of 10^{-6} to 10^{-9} there was no observable down regulation in my experiments. However, down regulation of GR at very low concentrations of ligand has been reported previously[387,464,465,467]. Because its clinical significance is particularly relevant with respect to its occurrence during extended therapeutic glucocorticoid treatment with subsequent glucocorticoid resistance, it has become important to identify a defined mechanism(s) leading to down regulation.

The mechanisms underlying down regulation are not understood. The majority of evidence to date indicates that the receptor itself is an integral part of this process. As mentioned above, the process is dependent upon low hormone concentration[32]. Further, it has been shown to occur in the presence of inhibitors of protein synthesis[32].

Previous experiments based on analysis on the effects of ligand on receptor protein and mRNA levels suggested that at least two mechanisms may be responsible for down regulation: a) a reduction in the rate of mRNA synthesis[467] and/or b) a decrease in the half life of the GR protein. The effect of down regulation on receptor protein levels is more profound than on receptor mRNA.

Whilst in all of my other kinetic experiments, either in the presence or absence of cycloheximide (CHD) receptor levels were maintained throughout the 24 h time course studies, with the forskolin and TPA combination experiments, at very low levels of dex, I noticed that the amount of fluorescence of WT samples were reduced in both cytoplasmic and nuclear compartments between 2 h and 12 h, as compared to the control samples. This effect was not seen with the Arg484-His mutant, and again may be DNA- or intact DNA binding domain-dependent.

I monitored the nuclear accumulation of the receptors for 12 h at 10^{-10} M dex, after pretreatment of cells with a combined forskolin and TPA. My data demonstrated a reduction in the rate of uptake of both WT and Arg484-His. In addition, the WT receptor but not the Arg484-His mutant demonstrated a marked reduction in nuclear equilibrium levels in the presence of these two agents. Therefore, as indicated in previous reports on transcription studies involving repression of GR by AP-1[30-32], the DNA binding domain of the receptor is required for the reduced nuclear equilibrium levels. At 10^{-10} M dex, forskolin or TPA alone did not have an appreciable effect on the rate of nuclear accumulation and did not affect the level of nuclear receptors. Further, simultaneous treatment with forskolin and 4α-phorbol ester, the inactive analog of TPA, produced an effect identical to that of forskolin alone.

TPA induces both *c-Jun* and *c-fos* levels, whilst forskolin induces *c-Jun* levels[463]. *c-Jun* and *c-fos* together constitute AP-1. In all reports it has been indicated that repression of GR by AP-1 requires an intact DNA binding domain of GR which may account for the difference in nuclear accumulation of the WT and the Arg484-His mutant. Thus one could postulate that the combined TPA and forskolin treatment increases the levels of AP-1 factor which heterodimerizes effectively with the WT GR and since it has been suggested that down regulation of GR may result from its faster rate of degradation[468] one could postulate that at the protein level, heterodimerization of the Jun factor of AP-1 with GR may lead to a faster rate of degradation of the receptor. This could account for the reason why I observed reduced levels of immunofluorescent signal in WT and not Arg484-His combined forskolin and TPA treated cells. The rate of nuclear accumulation of the Arg484-His binding mutant was slowed by 3-5-fold. This may be due to the fact that the N-terminal- and the DNA binding domain contribute to trans-repression and since these domains are intact in the Arg484-His mutant, they may have weak interaction with AP-1 and this slows the rate of nuclear accumulation. Another possibility is that in the presence of both forskolin and TPA the rate of nuclear uptake of both WT and Arg484-His mutant may be slowed by a mechanism that may result in lowering affinity for dex binding, as indicated above, in addition to the repression effect suggested for the WT receptor.

b) Effects on cytoplasmic return of nuclear receptors

I also investigated the effect on the rate of loss of nuclear receptors to the cytoplasm after treatment of cells with a combination of forskolin and TPA after withdrawal from 10^{-6} M cortisol. My results showed that whilst this treatment resulted in a cancellation effect on the apparent nuclear retention of the WT receptor, the Arg484-His mutant displayed a distribution identical to that observed with TPA alone. Recent reports suggest that nuclear signaling activities initiated by treatment of cells with forskolin or TPA may effect changes in receptor function and the function of other transcription factors in the nucleus.

Several observations have provided evidence for a nuclear signaling system involving PIP2 hydrolysis. Purified nuclei, *in vitro* synthesize PIP2 and phosphatidyl inositol-4-phosphate[478]. Further, there is transient decrease in the mass of these phospholipids in the nucleus when Swiss 3T3 cells are cultured in the presence of growth factors with a concomitant increase in nuclear DAG levels[479]. These data suggest that a signal reaches the nucleus as a result of stimulation of the growth factor receptor and that the signal stimulates a nuclear phosphoinositidase C (PIC) enzyme with a subsequent hydrolysis of PIP2 to generate two products, one of which is DAG[480].

In addition, although substrates of PKC have always been considered to be plasma membrane and cytoplasmic proteins[484,485], recent reports demonstrate translocation of certain PKC isozymes to the perinuclear region and the nuclear interior in a number of cell types, after stimulation with growth factors or tumor promoters[476]. This translocation correlates with an increase in the nuclear pool of a number of nuclear targeted proteins including lamin B. For example, studies

involving indirect immunofluorescence and Western Immunoblotting of isolated nuclei, have demonstrated that phorbol ester activation of PKC-α leads to its rapid redistribution to the NE, which renders the enzyme more active. It has been suggested that the activated enzyme functions by phosphorylating regulatory NE proteins. Thus, the α-isozyme may play a role in mediating PKC-induced changes in gene expression. If an analogous mechanism exists at the nuclear membrane, then there is the possibility that it may influence gene expression at the nuclear level. Indeed, studies have shown that elevation of intracellular cAMP levels by forskolin produces also a rapid and reversible nuclear translocation of the catalytic subunit of type II cAMP-dependent protein kinase whilst the regulatory subunit remains associated with the Golgi apparatus[207]. If nuclear phosphorylation signaling systems are dependent on stimulation of phosphorylating pathways with modulators of protein phosphorylation then nuclear events such as binding of transcription factors to DNA and/or transcriptional activity should be further enhanced upon stimulation of cells with these agents.

Forskolin, in addition to increasing dex binding also stimulates PKA activity which enhances DNA binding activity of GR for its response element by approximately 20-fold, with a subsequent hormone dependent transactivation[230]. Since there is no PKA consensus site in the DNA binding domain of GR, Cavanaugh et al. proposed that phosphorylation of a low molecular weight factor in rat HTC cells that enhances the DNA binding activity of GR[487] may lead to more efficient interaction of the factor with GR and better DNA binding. However, my present data demonstrate that forskolin acts by promoting the nuclear retention of GR and is independent of DNA. A possible mechanism for

the forskolin effect on increasing nuclear retention is that it may: a) increase the phosphorylation status of the receptor and b) suppress the action of a phosphatase(s) which may play a role in loss of ligand from the receptor thereby maintaining the receptor in the nucleus (Figure 33C). TPA increases re-incorporation of the GR into the 8-9S heterocomplex and the subsequent cycling of the receptor, thus the simultaneous treatment of forskolin and TPA results in an export rate which is intermediate of the two effects. The Arg484-His mutant is impaired in DNA binding. One may suggest that the forskolin effect in lowering the rate of formation of 4S free receptor from 4S liganded receptor is overcome by TPA, once the receptor remains unbound to DNA.

Similarly, my kinetic studies revealed that simultaneous pretreatment of cells with forskolin and OA prior to hormonal stimulus reduced the rate of the hormone induced GR mediated nuclear import on both WT and Arg484-His receptor. Although the effect was marginal, again statistical analysis of this data also indicated that the differences were significant. This might have also resulted from apparent decrease in the potency of the hormonal activation. Such a mechanism has also been reported in the β-adrenergic system by Clark et al.[290].

Further, like the forskolin and TPA combined treatment, simultaneous addition of forskolin and OA at the time of hormone withdrawal produced an effect similar to what I observed with forskolin and TPA combined treatment. The effect on WT receptor was additive and partially cancelled out, whilst the effect on the Arg484-His mutant was identical to that of forskolin alone.

The fact that the WT receptors demonstrated a partially cancellation effect on nuclear retention by the combined treatment of forskolin and OA provides additional evidence for phosphorylation signaling activities being involved in DNA-binding of the GR.

V. CONCLUDING REMARKS AND DIRECTIONS FOR FUTURE RESEARCH

By investigating the role of DNA binding and protein phosphorylation in nucleocytoplasmic transport of the GR my work has contributed the following new information: a) DNA binding at least partially regulates nuclear occupancy of the receptor; b) after hormone withdrawal, redistribution of the receptor to the cytoplasm may be dependent upon the formation of the 8-9S receptor heterocomplex; c) after hormone withdrawal and before the formation of the GR-8-9S hsps-heterocomplex, 4S unliganded receptor which is detected in the nucleus may be in fact constantly shuttling between the nucleus and the cytoplasm and thus remains able to re-enter the nucleus; d) there are important factors other than those previously elucidated, involving protein phosphorylation mechanisms that influence the reformation of the 8-9S heterocomplex; e) phosphorylation mechanisms could also enhance a tighter binding of the receptor onto DNA which may subsequently further influence the re-cycling or the shuttling process.

1. *nt⁻ Mutant GRs Defective at a Step Distal to Nuclear Import*

Our interest in the substitution mutations that affect DNA and hormone binding of the mutant GR isolated from the S49 lymphoma nuclear transfer deficient (nt^-) cell line arose because of the correlation between hormone unresponsiveness and lack of nuclear occupancy. A defect in nuclear occupancy could be the result of defective: a) hormone binding; b) NLS; c) nuclear import; d) nuclear retention; or e) export from the nucleus. It would perhaps be most obvious that the defect would be in

nuclear import. However, I found that it was not. Rather, the defect is at a step distal to nuclear import. Studies involving measurements of cytoplasmic return of nuclear receptors either after hormone withdrawal or in the continuous presence of hormone have provided three other models of defective nuclear occupancy; okadaic acid treated fibroblasts, which demonstrated that OA treatment at a dosage that inhibits PP1/PP2A leads to inefficient nuclear retention of agonist bound GRs[236]. This was suggested to be due to changes in the phosphorylation status of the receptor; *v-mos* transformed cells[237], which demonstrated that hormone insensitivity in these cells is associated with inefficient nuclear retention of GR because of the generation of a novel desensitized receptor which is trapped in the cytoplasm unable to recycle into the nucleus; and cells in G2[297], which demonstrated that upon hormone stimulus, GRs that translocate to the nucleus are not sufficiently retained in G2 phase of the cell cycle and redistribute to the cytoplasmic compartment. In all three cases a defect in the import process was not detected, rather the defect seems to be distal to the initial entry of GR into the nucleus, consistent with our results. With the emerging knowledge of the process of nuclear import, it seems that although import is an active process it uses general import proteins[142,144,156]. Thus it is perhaps not surprising for control to be exerted at a level other than the initial nuclear import, i.e., on retention and re-entry.

2. *Mechanisms of Export of Proteins from the Nucleus*

The mechanism of export of proteins from the nucleus is to a large extent unknown but the most fundamental question to be answered is whether export is a regulated or a passive process. An active process

would require the existence of export signals and cellular components to respond to those signals and possibly the availability of energy, whereas in a passive process all proteins may shuttle between the nucleus and the cytoplasm with the only determining factor being the affinity of the protein for distinct nuclear components. Thus nuclear occupancy as a passive process would be governed solely by binding of the protein to intranuclear components. The recent report by Schmidt-Zachmann et al. reached the conclusion that there are probably no specific export signals and that in the absence of binding to nuclear components all proteins able to get into the nucleus by virtue of their native or tagged NLS would shuttle between the nucleus and the cytoplasmic compartments[187].

I capitalized on the opportunity afforded by steroid receptors to measure export initiated by the withdrawal of hormone from cells in which WT, Arg484-His and another DNA binding mutant of GR had been shifted into the nucleus upon hormonal stimulus. I showed that the association of steroid receptor with DNA is a determinant of the rate of export from the nucleus of the receptor. Thus binding to DNA of GR influences the nuclear occupancy of the receptor. My results are consistent with the conclusions reached by Nigg and co-workers[187], as return of the GR to the cytoplasm was negatively correlated with the ability of the receptor to bind a nuclear component, DNA. The DNA binding domain can in fact be regarded as a nuclear retention signal.

Apart from binding to DNA, several other nuclear binding sites have been identified for the steroid receptor, including non histone proteins such as transcription factors[498] and RNA[499]. Munck and co-workers have identified two populations of nuclear receptors, which differ in their extractability from the nucleus, presumably because they bind to different

nuclear sites[464]. My present report provides evidence that the presence of the polyglutamyl stretch in the mouse GR may play a role in further increasing nuclear retention of the mouse receptor over the human WT which does not contain this stretch. My studies while confirming the role of DNA binding in the export process, do not exclude an export signal mediated mechanism nor an active export mechanism regulated by other signaling pathways, (see Section V. 6. below).

3. *Redistribution of Cytoplasmic GR may be Due to Formation of 8-9S Heterocomplex*

In the kinetic analysis of loss of nuclear receptors to the cytoplasm after hormone withdrawal, we ruled out the possibility of hormone dissociation from the receptor being an important determinant of the rate of export. We reached this conclusion based on $t_{1/2}$ measurements of loss of dex binding from GR[211], which were much shorter (< 10 min) than the long $t_{1/2}$ of loss of nuclear receptors to the cytoplasm (> 12 h). We hypothesized that the difference in the rates of dissociation of dex and return of receptors in the cytoplasm may lead to a consideration that the time required for nuclear GR to appear in the cytoplasm after hormone withdrawal may represent the rate of reformation of the inactive GR-hsps-complex. We called the process of reformation into the 8-9S complex re-cycling.

Munck and Holbrook's kinetic experiments proved that before receptor can bind hormone it must reform the 8-9S complex in the cytoplasm[211]. I showed this in nuclear re-uptake experiments in which I restimulated with agonist, nuclear receptors that had been returned to the cytoplasm at different time points along the withdrawal curve (for both WT

receptor and the DNA binding mutant) demonstrated a rapid re-uptake of receptor molecules into the nucleus at a rate which was not different from the primary hormonal stimulus. This strongly suggests that all of the accumulated cytoplasmic GRs were in the 8-9S heterocomplex. We also speculated that the formation of the heterocomplex may be a rate-limiting step in steroid hormone action. As Munk and co-workers earlier suggested, the formation of the inactive GR-hsps-complex is critical for the subsequent events that proceed during GR signal transduction[127], thus such an important process may well represent a rate-limiting step in the overall signal transduction pathway of GR.

4. *The 4S Unliganded GR Shuttles Even if Re-cycling is Blocked*

Previous investigators have reported nucleocytoplasmic shuttling of agonist bound PR[186] and GR[185] in heterokaryon assays. My studies on cytoplasmic redistribution of nuclear receptors after hormone withdrawal using RU486 or H-7 caused a complete blockage of cytoplasmic redistribution of the receptors after hormone withdrawal. I showed, using the Arg484-His mutant that these effects were independent of DNA binding. We were able to distinguish between the effects of H-7 and RU486 on shuttling or re-cycling of the receptor in energy depletion experiments. I demonstrated that when H-7 or RU486 treated cells were depleted of energy, there was a rapid efflux of nuclear receptors to the cytoplasm. Thus it is the re-cycling process which is blocked by H-7 and RU486 treatment. The 4S unliganded receptor still remains able to shuttle between the nucleus and cytoplasm.

5. *Possible Roles of Shuttling of Proteins*

Since 1989 when transient heterokaryon technology was first developed[183], it has become clear that many proteins shuttle between the nucleus and the cytoplasm. These shuttling proteins include the steroid receptors. It is reasonable to assume an important role of this shuttling process in steroid hormone action. Although in previous reports it was suggested that this process most likely coordinates nuclear and cytoplasmic events[183,187], there is no evidence as yet for the function of shuttling. Shuttling proteins may allow for the nuclear transport of other proteins which do not themselves carry an NLS in their primary sequence. For example, the adenovirus DNA polymerase shuttles between the nucleus and cytoplasm and a piggyback mechanism for its nuclear transport has been observed[492,493]. Another possibility is that during steroid receptor mediated inducible transcriptional activity, the correct signals needed for the modulation of the transcriptional activity of the target gene are required to be released in discrete quantities (i.e., in the form of quanta), as is observed with Ca^{2+} oscillation signaling[495]. Thus the transactivation machinery has to be desensitized after the first round of transcription and subsequently the receptor be shuttled or re-cycled for the subsequent round of transcription induction. If such a mechanism operates, it might allow a 'finer control' of the transcriptional activity.

6. *Effect of Modulators of Phosphorylation on Nucleocytoplasmic Transport of GR*

A large body of evidence indicates that the GR, as well as other members of steroid receptor superfamily, are phosphoproteins *in vivo*[211-218]. *In vitro* they have been shown to be substrates for protein kinases[226-320]. Further, stimulation of phosphorylation pathways has an often strikingly positive influence on the steroid hormone response[232]. However, compelling evidence that directly links steroid receptor phosphorylation and/or dephosphorylation to specific functions is lacking. Studies that have sought to identify receptor phosphorylation sites whose modification correlated with increased transcriptional activity have produced mixed results, as often no direct link could be observed[500]. Thus some groups have indicated that GR itself may not be the target for phosphorylation but rather factors other than the receptor itself involved with the signal transduction pathway may be phosphorylated to alter receptor function[231,232].

My studies on the influence of modulators of protein phosphorylation on nucleocytoplasmic trafficking suggest that for nuclear import it appears the effects of these modulators may either augment or inhibit the rate of uptake but none of them plays an obligatory role in controlling uptake. Thus while they produce minor changes in the rate of nuclear uptake, other factors such as NLSs and their binding proteins may play by far a more essential role. On the other hand kinetic studies on the rate of loss of nuclear receptors to the cytoplasm upon hormone withdrawal in the presence of these modulators showed that some produced complete blockage in cytoplasmic accumulation of GR. We identified this block as a blockage in the formation of the 8-9S GR-hsps-

heterocomplex and called this process re-cycling. Re-cycling of SHRs, which could be the rate-limiting step in steroid hormone action may serve as a key controlling point for certain phosphorylation cascades in the regulation of nucleocytoplasmic trafficking of the GR.

Studies by Munck and co-workers have also provided evidence that the receptors continuously traverse an ATP-dependent cycle between soluble and nuclear bound forms whether or not hormone is present[211,244,246,247], suggesting that the phosphorylation status of the receptor is subject to changes during its signal transduction events. Although our studies and others[186,189] suggest that nuclear export may not be energy dependent, Munck and co-workers have also postulated, based on experimental evidence, the existence of a null receptor tightly bound to a nuclear component, with only 2 out of 3 of the phosphate content, as compared to the cytoplasmic intermediate that associate with hsps[219,245,247]. The null receptor was suggested to result from desensitization of transcriptional event. If this were true then it would require phosphorylation to render it competent for re-cycling. Thus an involvement of a nuclear kinase may be important in regulating cytoplasmic accumulation of the null receptor. Modulators such as phorbol esters activate certain isoforms of PKC, aid PKC translocation to the nucleus and render it NE bound. Similarly, forskolin activates cytoplasmic anchored PKA by effecting dissociation of its catalytic subunit from the regulatory subunit and allows its translocation into the nucleus. It is therefore possible that an active reversible kinase activity may exist either at the nuclear envelope or in the nucleus to catalyze phosphorylation reactions, one of which may phosphorylate the null receptor.

Our data from nuclear re-uptake experiments with the WT and the DNA binding mutants suggest that binding to DNA per se may not be a necessary prerequisite to other receptor modification events involved in regulating export of the receptor from the nucleus. However, the results obtained from treatment of cells with combination of forskolin and TPA or forskolin and OA suggested that certain phosphorylation signaling pathways when simultaneously 'turned on' may lead to multiple phosphorylation events which are important for DNA binding and the subsequent transcriptional event.

To date it is not clear whether the GR itself is the relevant substrate for PKA or PKC mediated phosphorylation. In fact, OA-induced increases in GR-mediated gene expression are not accompanied by discernible changes in receptor phosphorylation[231]. However, it has been reported that there is a DNA binding dependent phosphorylation of PR[497], which suggests that there may be direct phosphorylation of steroid receptors at the level of transcription. That GR itself is phosphorylated is supported by the fact that Munck and co-workers through metabolic labeling of cells with ^{32}Pi, cell fractionation and subsequent phosphate content measurements, have isolated different phosphorylated forms of the receptor. It seems likely that the receptors' association with heat shock proteins is one process in which phosphorylation could play an important role.

The criteria for establishing the mechanism of regulation of cellular processes by a protein kinase have been expressed clearly by Krebs and Beavo[501]. One important criterion is that the site(s) of phosphorylation of the protein in question be demonstrated in the intact cell. Previous reports by Bodwell et al. have utilized the techniques of phosphopeptide mapping

and phosphoamino acid analysis to identify seven phosphorylation sites in the amino terminal domain of the mouse GR[222]. Subsequently, studies in which site directed mutagenesis had been carried out, substituting the serine phosphorylation residues with alanine to determine the role of these sites in transcriptional activity reached a surprising conclusion that these sites are not an important determinant of transactivation at the MMTV-LTR promoter[500]. Even receptors containing mutations at all seven phosphorylation sites exhibited only a modest decrease (30 %) in transcriptional activity. However, it must be noted that these phosphorylated serines and threonine sites were not hormone inducible phosphorylation sites.

I have identified other putative protein kinase phosphorylation sites in the GR by comparing sequences in the receptor with protein kinase consensus motifs. Ser771 represents a potential PKC phosphorylated residue and Tyr770 may represent a tyrosine kinase phosphorylated residue provided Ser771 is initially phosphorylated to mimic the structure of aspartate. I originally postulated that a mechanism may operate to sequentially phosphorylate these two sites in the mouse GR. Although, my present data show that the Tyr770-Asn mutation does not affect nucleocytoplasmic transport of the GR, the effect of the Ser771 on nucleocytoplasmic transport was not investigated. I initially mapped these sites in the receptor because of our interest in the Tyr770-Asn substitution in the nt$^-$ S49 lymphoma and found it to be conserved among the different GR species. However, the consensus motif for PKC (**K/RX$_{770}$S*/T***) suggests that although the Tyr770-Asn substitution is not conservative, the substitution of tyrosine residue to asparagine should not affect phosphorylation of this site by PKC, i.e., the '**X**' in the consensus motif

could be any amino acid. Thus although my data indicated that the Tyr770 may not be directly phosphorylated to alter receptor function, phosphorylation of the Ser771 residue may still be important for the function of the receptor. Since the Tyr770-Asn substitution has been shown to slightly impair the transcription activity of the GR, it is possible that the mutation may have an effect on phosphorylation of the Ser771; a serine residue which upon phosphorylation may play a critical role on the transcriptional activity. Interestingly this PKC site is within the carboxy terminal 30-40 amino acid residues that is altered in RU486 treated PR, which would be analogous to the region of GR that would be altered upon RU486 treatment. Thus the phosphorylation of the Ser771 and its effect on transcription is worth investigating.

Other hormone inducible protein phosphorylation sites exist in the receptor that have not yet been identified by the current mapping and phosphoamino acid analysis procedures. Perhaps phosphorylation of these sites may occur very transiently and may be sub-stoichiometric, such that they are difficult to measure or identify. Future work lies in carrying out site directed mutagenesis of the hormone inducible phosphorylation sites in the receptor, including the Ser771, changing the serine residues to alanine and aspartate to identify which of the protein kinase site(s) is/are required for dissociation of receptor from the 8-9S heterocomplex, DNA binding and re-cycling of the receptor, using my present assay. Some of these phosphorylation sites may not be a direct PKC or PKA site but because of the network between these phosphorylation pathways it is possible that the activation of PKC by TPA could bring about a cascade of events leading to the activation of other

kinases and subsequently phosphorylation of sites other than PKC or PKA site.

It would also be interesting to further examine the direct role of modulators of kinases in re-formation of the 8-9S heterocomplex, using the cell-free system developed by Pratt and co-workers. The development of appropriate techniques and experimental procedures for molecular and biochemical characterization of these phosphorylation sites and also the identification and characterization of the kinases involved which have not yet been mapped are also to be considered. Further, the contributions that cellular phosphatases must make in the reversible modification of steroid receptors by phosphorylation also need to be recognized by accelerating efforts in their identification and characterization.

When considering these issues I could not rule out the possibility that the influence of these agents on nucleocytoplasmic trafficking of the GR and also in the hormonal inducibility of target gene transcription may not in all cases be directly linked to covalent modification on the receptor. The overall signal transduction of the GR may at least in part be markedly enhanced or slowed as a result of synergistic interactions between the GR and other factors that could be modified by protein kinases whose activities are stimulated by these agents. Previous reports have demonstrated that TPA, forskolin, OA and vanadate enhance hormone dependent transcriptional activity of the GR. These findings are consistent with our present data which demonstrate the role of these agents in augmenting the rate of nuclear import and in promoting re-cycling (TPA) and nuclear retention (forskolin, OA and vanadate), the consequence of which may result in an increase in transcriptional activity.

My present data on the effect of modulators of protein phosphorylation on nucleocytoplasmic trafficking gives more insight into the role of these protein phosphorylation agents in the regulation of GR function and demonstrate that they may generate a cascade of signal transduction network that underpins the hormonal inducible response of the GR and other steroid hormone receptors. Thus, one may conclude that these agents affect different aspects of receptor function to result in a concerted mechanism for the production of the hormonal responsiveness.

Finally, by coupling two phosphorylation signaling pathways with the signal transduction of GR to study nucleocytoplasmic trafficking of the receptor I demonstrated that receptor function may be regulated through additive effects of two modulators that stimulate the pathways independently, and via desensitization mechanisms (down-regulation and trans-modulation). Our data on the rate of cytoplasmic return of nuclear receptors in the presence of the combined agents after hormone withdrawal provided evidence for the role of phosphorylation signaling activities on DNA binding and in the regulation of nucleocytoplasmic transport of the GR.

In conclusion, my present report compared the kinetics of nucleocytoplasmic trafficking of WT, nt^- and the two substitution mutations in the nt^- GR. Further, I have studied the influence of modulation of protein phosphorylation in this process. My results show that the DNA binding mutants bearing the Arg484-His mutation demonstrated reduced equilibrium levels of nuclear receptor following glucocorticoid treatment. My data highlights the importance of quantitation of immunocytochemical procedure to distinguish between receptor defects in resistant lymphoid (and leukemic) and sensitive cells.

Perhaps the ability of GRs in human lymphomas and leukemias to traffic between the nucleus and the cytoplasm should be assessed to help select those which will be responsive to glucocorticoid therapy, see below. Further, we have used the same quantitative immunocytochemical techniques to investigate the role of modulators of protein phosphorylation in the nucleocytoplasmic trafficking of the GR and I have proposed that receptor site specific phosphorylation involving a TPA sensitive kinase controls receptor re-cycling. This observation provides evidence that an active enzymatically mediated protein phosphorylation mechanism(s) is/are involved in the receptor association with hsps. Another important finding was the observation made with the energy depletion experiments with cells in which cytoplasmic return of nuclear receptors had been blocked by H-7 and RU486 treatment. This showed that the blockage I was observing might be only apparent because the receptors could still shuttle between the nucleus and cytoplasm. My studies provide new insights on how steroid hormone action may be controlled through regulation of a process in which unoccupied receptors are re-cycled to the cytoplasm in the ligand binding form. The mutagenesis studies and the studies on effects of protein kinase modulators that need to be done, in addition to our present finding would provide an important extension to the understanding of the mechanism of heterocomplex assembly and subsequently how the regulation of this process affects GR action.

7. *The Biological Significance of some of my Findings*

I indicated in the introductory part of this Thesis the use of glucocorticoids in many chemotherapeutic regimens because of their antiproliferative and cytolytic effects on some population of lymphocytes. Studies have shown that some lymphocyte cell lines are also killed by elevated levels of cAMP in a manner which is morphologically similar to that observed in glucocorticoid mediated death[502-504]. Indeed a group of investigators have demonstrated that in some 'deathless' lymphoma cell lines the isolated GRs became significantly resistant to glucocorticoids through the loss of cAMP-dependent protein kinase function. This led to the suggestion that cAMP-dependent protein kinase activity has the potential to modulate steroid sensitivity in lymphoma cells, by affecting the levels of GR expression, as well as the receptors' efficiency in producing a cytolytic response in murine T-lymphoma cells. However, in some steroid resistant S49 and WEHI 7.2 'deathless' cell lines the receptor variants that were isolated although demonstrated identical steroid binding and nuclear translocation properties as their WT counterparts they were shown to be resistant to cAMP treatment and yet the cAMP-dependent kinase activities determined were normal[505-506]. Since my present kinetic studies with modulators of protein phosphorylation demonstrated the involvement of different phosphorylation signaling pathways in the nucleocytoplasmic trafficking of GR, it would be interesting to examine in these mutant cell lines the possibility of a defect distal to the cAMP-dependent protein kinase. The determination of responsiveness of these receptor variants to the TPA-dependent hormone sensitive kinase activity would provide an information on re-cycling ability of the receptors in these resistant cell

lines. This would then stimulate investigators to persue future studies in the isolation, molecular cloning and direct characterization of the kinase.

REFERENCES

1. Ezdinli, E.Z. et al. (1969) Corticosteroid Therapy for Lymphomas and Chronic Lympholytic Leukemia. *Cancer* **23**, 900-907

2. DeVita, V.I. et al. (1981) The Chemotherapy of Lymphomas: Looking Back Moving Forward. *Cancer Res* **47**, 5810-5824

3. Goldin, A et al. (1971) The Chemotherapy of Human and Animal Acute Leukemia. *Cancer chemotherapy Rep* **55**, 309-507

4. Bourgeois, S. et al. (1978) Glucocorticoid Resistance in Murine Lymphoma and Thymoma Lines. *Cancer Res.* **38**, 4279-4284

5. Thompson, E.B. et al. (1988) In Lipman, M.E. (ed) *Growth and Regulation of Cancer* **74,** New York 221

6. Powers, J.H. et al. (1993) Cloning and Expression of mutant Glucocorticoid Receptors from Glucocorticoid-Sensitive and -Resistant Human Leukemia Cells. *Cancer Res.* **53,** 4059-4065

7. Sibley, H.C. and Tomkins, G.M. (1974) Mechanisms of Steroid Resistance. *Cell* **2,** 221-227

8. Sibley, H.C. and Tomkins, G.M. (1974) Isolation of Lymphoma Cell Variants Resistant to Killing by Glucocorticoids. *Cell* **2,** 213-220

9. Zawydiwski, R. (1983) Glucocorticoid Resistant in Human Acute Lymphoblastic Leukemia Cell Line With Functional Receptor. *Cancer Res.* **43**, 3865-3873

10. Segnitz, B. and Gehring, U. (1990) Mechanisms of Action of a Steroidal Antiglucocorticoids in Lymphoid Cells. *J. of Biol. Chem.* **265**, 2789-2796

11. Maoli, P.A. et al. (1992) A Mechanism of Resistance to Glucocorticoids in Multiple myeloma. *Blood* **79**, 213-222

12. Evans, R.M. (1988) The Steroid and Thyroid Hormone Receptor Superfamily. *Science* **240,** 889-895

13. Hollenberg, S.M. et al. (1985) Primary Structure and Expression of a Functional Glucocorticoid Receptor cDNA. *Nature* **318**, 635-641

14. Danielsen, M. et al. (1986) The Mouse Glucocorticoid Receptor: Mapping of Functional Domains by Cloning, Sequencing, and Expression of the Wild Type and Mutant Receptor Proteins. *EMBO J.* **5**, 2513-2522

15. Gigure, V. et al. (1986) Functional Domains of the Human Glucocorticoid Receptor. *Cell* **46**, 645-652

16. Encio, I.J. (1991) The Genomic Structure of the Human Glucocorticoid Receptor. *J. of Biol. Chem.* **266**, 7182-7188

17. Picard, D. and Yamamoto, K.R. (1987) Two Signals Mediate Hormone Dependent Nuclear Localization of the Glucocorticoid Receptor. *EMBO J.* **6**, 3333-3340

18. Hollenberg, S.M. et al. (1987) Colocalization of the DNA Binding and Transcriptional Activation Functions in the Human Glucocorticoid Receptor. *Cell* **49**, 39-46

19. Dalman, F.C. et al. (1991) Localization of the 90 kDa Heat Shock Protein-Binding Site Within the Hormone Binding Domain of the Glucocorticoid Receptor by Peptide Competition. *J. of Biol Chem* **266**, 3482-3490

20. Sanchez, E.R. (1990) The 56, 59 kDa Protein Identified in Untransformed Steroid Receptor Complexes is a Unique Protein that Exists in Cytosol in a Complex With Both 70 and 90 kDa Heat Shock Proteins. *Biochemistry* **29**, 5145-5152

21. Pratt, W.B. (1990) Glucocorticoid Receptor Structure and Initial Events in Signal Transduction. *Progr. Clin. Biol. Res.* **322**, 119-132

22. Wikstrom, A.N. et al. (1987) Intracellular Localization of the Glucocorticoid Receptor: Evidence for Cytoplasmic and Nuclear Localization. *Endocrinology* **120**, 1232-1242

23. Schimdt, T.J. and Litwark, G. (1982) Activation of the Glucocorticoid Receptor Complex. *Physiol. Rev.* **62**, 1131-1192

24. Luisi, B.F. et al. (1991) Crystallographic Analysis of the Interaction of the Glucocorticoid receptor With DNA. *Nature* **352**, 497-505

25. Schule, R. (1986) Cooperativity of the Glucocorticoid Receptor and the CACCC-Box Binding Factor. *Nature* **332**, 87-90

26. Simmons, S.S. (1992) Higher Levels of Control: Modulation of Steroid Regulated Gene Transcription *Mol. Endocrinology* **6**, 995-1002

27. Diamond, N.I. et al. (1990) Transcription Factor Interactions:

Selectors of Positive or Negative Regulation from a Single DNA Element. *Science* **249,** 1266-1272

28. Sakai, D.D. et al.(1988) Hormone-Mediated Repression of Transcription: A Negative Glucocorticoid Response Element From the Bovine Prolactin Gene. *Genes and Development* **2,** 1144-1154

29. Akerblom, I.W. (1988) Negative Regulation of Glucocorticoid Through Interference with a cAMP Response Enhancer. *Science* **241,** 3503

30. Yang-Yen, H.F. et al. (1990) Transcriptional Interference Between c-*Jun* and the Glucocorticoid Receptor: Mutual Inhibition of DNA Binding Due to Direct Protein-Protein Interaction. *Cell* **62,** 1205-1215

31. Schule, R. et al. (1990) Functional Antagonism Between Oncoprotein c-*Jun* and Glucocorticoid Receptor. *Cell* **62,** 1217-1226

32. Jonat, C. et al. (1990) Antitumor Promotion and Anti-Inflammation: Down Modulation of AP-1 (*Fos/Jun*) Activity by Glucocorticoid Hormone. *Cell* **62,** 1189-1204

33. Compton, M.M. (1987) Identification of a Glucocorticoid Induced Nuclease in Thymocytes: A Potential Lysis Gene Product. *J. of Biol. Chem.* **262,** 8288-8292

34. Thompson, E.B. and Harmon, J.M. (1986) Glucocorticoid receptors and Glucocorticoid Resistance in Human Leukemia *In Vitro* and *In Vivo* . In: Chrousos, G.P. et al. (eds) Advances In Experimental Medicine in Biology. **196,** 111-127 NY: Plenum Publishing Corp.

35. Thompson, E.B. et al. (1986) Glucocorticoid Action in Human Acute Lymphoblastic and Leukemia T cell line : A Model system for Understanding Steroid Therapy. In: Lacobelli et al (eds), *Hormones and Cancer* 89-98 NY Raven Press

36. Rabindran, S.K. et al. (1987) Glucocorticoid Resistant Lymphoma Cell Variants that Contain Functional Glucocorticoid Receptors. *Mol. Cell. Biol.* **7,** 4211

37. Lijinsky, W. (1986) Mutagenesiss, Carcinogenesis and Alkylating Properties of Nitrosamines and Related Compounds. *Progress in Clinical and biological Res.* **209B,** 141-148

38. Darzynkiewicz, Z. et al (1989) Effect of Derivatives of Chrysophanol, a New Type of Potential Anitumor Agents of

Athraquinone Family, on Growth and Cell Cycle of L120 Leukemic Cells. *Cancer Letters* **46,** 181-187

39. Faqua, S.A.W. et al. (1993) Estrogen Receptor Mutations in Breast Cancer. *J. of Cellular Biochem.* **51,** 135-139

40. Newport, J.W. and Forbes, D.J. (1987) The Nucleus: Structure, Function and Dynamics. *Ann. Rev. Biochem.* **56,** 535-565

41. Franke, W.W. et al. (1981) The Nuclear Envelope and Architecture of the Nuclear periphery. *J. of Cell Biol.* **91,** 39-50

42. Dingwall, C. and Lasky, R. (1992) The Nuclear Membrane. *Science* **258,** 942-947

43. Aaronson, R.P. and Blobel, G. (1974) On the Attachment of the Nuclear Pore Complex. *J. Cell Biol.* **62,** 746-754

44. Dwyer, N. and Blobel, G. (1976) A Modified Procedure for the Isolation of a Pore Complex-Lamina Fraction From the Pore Complex Nuclei. *J. Cell. Biol.* **70,** 581-591

45. Jenkins, H. et al. (1993) Nuclei that Lack Lamina Accumulate Karyophilic Proteins and Assemble a Nuclear Matrix. *J. Cell Science* **106,** 275-285

46. Aebi, U. et al. (1986) The Nuclear Lamina is a Meshwork of Intermediate-Type Filaments. *Nature* **323,** 560-564

47. McKeon, F.D. (1986) Homologies in Both Primary and Secondary Structure Between Nuclear Envelope and Intermediate Filament Proteins. *Nature* **319,** 463-468

48. Doring, V. and Stick, R. (1990) Gene Structure of Nuclear Lamin, L_{III} of *Xenopus Laevis*: A Model for Evolution of Intermediate Filament Proteins from a Lamin-Like Ancestor. *EMBO J.* **9,** 4073-4081

49. Stewart, C. and Burke, B (1987) Teratocarcinoma Stem Cells and Early Mouse Embryos Contain Only a Single Major Lamin Poly-Peptide Resembling Lamin B. *Cell* **51,** 385-392

50. Weber, K. et al. (1988) Amino Acid Sequences and Homopolymer Forming Ability of the Intermediate Filament Proteins From an Invertibrate Epithelium. *EMBO J.* **7,** 2995-3001

51. Vorburger, K. (1989) A Second Higher Vertibrate B-type Lamin: cDNA Sequence Determination and *in vitro* Processing of Chicken Lamin B2. *J. Mol. Biol.* **208,** 405-415

52. Lehner, C.F. et al. (1986) The Nuclear Lamin Protein Family in Higher Vertibrates: Identification of Quantitatively Minor Lamin Proteins by Monoclonal Antibodies. *J. of Biol Chem.* **261,** 13293-13301

53. Rober, R.A. et al. (1989) Differential Timing of Nuclear Lamin A/C Expression in the Various Organs of the Mouse Embryo and the Young Animal: a Developmental Study. *Development* **105,** 365-378

54. McKeon, F.D. et al. (1991) Nuclear Lamin Proteins: Domains Required for Nuclear Targetting Assembly. *Curr. Opin. Cell Biol.* **3,** 82-86

55. Peter, M. et al. (1990) *In Vitro* Disassembly of the Nuclear Lamina and M-Phase Specific Phosphorylation of Lamins by CDC-2 Kinase. *Cell* **61,** 591-602

56. Luscher, B. et al. (1991) A Role for $p34^{cdc-2}$ Kinase and Phosphatases in the Regulation of Phosphorylation and Disassembly of Lamin B_2 During the Cell Cycle. *EMBO J.* **10,** 865-875

57. Dvecha, H. et al. (1991) The Polyphosphoinositide Cycle Exists in the Nuclei of Swiss 3T3 Cells Under the Control of a Receptor (for IGF-1) in the Plasma Membrane, and Stimulation of the Cycle Increases Nuclear Diacyglycerol and Apparently Induces Translocation of PKC to the Nucleus. *EMBO J.* **10,** 3207

58. Panté, N. and Aebi, U (1993) The Nuclear Pore Complex. *Journal Biol Chem.* **122,** 977-984

59. Feldherr, C.M. (1984) Movement of Karyophilic Proteins through The Nuclear Pores of Oocytes. *J. Cell. Biol.* **99,** 2216-2222

60. Gerrace, L. (1992) Molecular Trafficking Across The Nuclear Pore Complex. *Curr. Opin. in Cell Biol.* **4,** 637-645

61. Akey, C.W. (1992) The Nuclear Pore Complex. *Curr. Opin. Struct. Biol.* **2,** 258-263

62. Forbes, D.J. (1992) Structure and Function of the Nuclear Pore Complex. *Ann. Rev. of Cell Biol.* **8,** 495-527

63. Stewart, M. (1992) Nuclear Pore Structure and Function. *Seminar in Cell Biol.* **3,** 267-277

64. Hinshaw, J.E. et al. (1992) Architecture and Design of the Nuclear Pore Complex. *Cell* **69,** 1133-1141

65. Akey, C.W. and Radermacher, M. (1993) Architecture of the *Xenopus* Nuclear Pore Complex Revealed by a 3-Dimensional Cryo-Electron Microscopy. *J. Cell. Biol.* **121,** 1-19

66. Newmeyer, D.D. (1993) The Nuclear Pore Complex and Nucleocytoplasmic Transport. *Curr. Opin. in Cell Biol.* **5,** 395-407

67. Reichelt, et al. (1990) Correlation Between Structure and Mass Distribution of the Nuclear pore Complex and the Distinct Pore Complex Components. *J. Cell Biol.* **110,** 883-894

68. Akey, C.W. (1989) Interactions and Structure of the Nuclear Pore Complex Revealed by Cryo-Electron Microscopy. *J. Cell. Biol.* **109,** 955-970

69. Rihs, H. (1989) Three Dimensional Imaging of Cell Ultrastructure with High Resolution Low Voltage Scanning Electron Microscopy. *Inst. Phys. Conf. Sem.* **98,** 651-662

70. Rihs, H. (1991) Three Dimensional Structure of the Nuclear Pore Complex as Seen by High Voltage Electron Microscopy and High Resolution Low Voltage Scanning Electron Microscopy *EMSA Bull.* **21,** 54-56

71. Goldberg, M.W. and Allen, T.D. (1992) High Resolution Scanning Electron Microscopy of the Nuclear Envelope: Demonstration of a New, Regular, Fibrous Lattice Attached to the Baskets of the Nucleoplasmic Face of the Nuclear Pore. *J. Cell Biol.* **119,** 1429-1440

72. Golberg, M.W. et al. (1992) The Use of Field Emmision In Lens-Scanning Electron Microscopy to Study the Steps of Assembly of the Nuclear Envelope *In Vitro*. *J. of Struct. Biol.* **108,** 257-268

73. Jarnik, M. and Aebi, U. (1991) Toward a More Complete 3-D Structure of the Nuclear Pore Complex. *J. of Struct. Biol.* **107,** 291-308

74. Unwin, P.N.T. and Milligan, M. (1982) A Large Particle Associated With the Perimeter of the Nuclear pore Complex. *J. Cell. Biol.* **93,** 63-75

75. Akey, C.W. (1990) Visualization of transporter-Related . Configuration of the Nuclear Pore Transporter. *Biophys. J.* **58,** 341-355

76. Gerrace, L. et al. (1982) Identification of a Major Polypeptide of the Nuclear pore Complex. *J. Cell Biol.* **95,** 826-837

77. Berrois, M. et al. (1983) A 174 kDa ATPases /dATPase Polypeptide and Glycoprotein of Apparently Identical Molecular Weight are Common but Distinct Components of Higher Eukaryotic Nuclear Structural Protein Subfractions. *J. of Biol. Chem.* **258,** 13384-13390

78. Wozniac, R.W. et al. (1989) Primary Structure Analysis of an Integral Membrane Protein of the Nuclear Pore. *J. Cell Biol.* **108,** 2083-2092

79. Grebber, U.F. and Gerrace, L (1992) Nuclear Protein Import is Inhibited by an Antibody to a Lumenal Epitope of a Nuclear Pore Complex Glycoprotein. *J. Cell Biol.* **116,** 15-30

80. Grebber, U.F. (1990) A Major Glycoprotein of the Nuclear Pore Complex is a Membrane Spanning Polypeptide With a Large Lumenal Domain and a Small Cytoplasmic Tail. *EMBO J.* **19,** 1495-1502

81. Snow, C.M. et al. (1987) Monoclonal Antibodies Identify a Group of Nuclear Pore Complex Glycoproteins. *J. Cell. Biol.* **104,** 1147-1156

82. Davis, L.I. and Blobel, G. (1987) The Nuclear Pore Complex Contains a Family of Glycoproteins That Includes p^{62} : Glycosylation Through a Previously Unidentified Cellular Pathway. *Proc. of Natl. Acad. of Sc.* **84,** 7552-7556

83. Davis, L.I. and Fink, G.R. (1990) The NUP1 Gene Encodes an Essential Component of the Yeast Nuclear Pore Complex. *Cell* **61,** 965-978

84. Nehrbass; U. et al. (1990) NSP: A Yeast Nuclear Envelope Protein Localized at the Nuclear Pores Exerts its Essential Function by the Carboxy Terminal Domain. *Cell* **61,** 979-989

85. Wente, S.R. et al. (1992) A New Family of Yeast Nuclear Pore Complex Proteins. *J. Cell Biol.* **119,** 705-723

86. Pandey, S. (1994) Inhibition of Nuclear Protein Import by a Monoclonal Anibody Against a Novel Class of Nuclear Pore Proteins. *Exptal. Cell Res.* **212,** 243-254

87. Wente, S.R. and Blobel. G. (1993) A Temperature Sensitive NUP116 Null Mutant Forms a Nuclear Envelope Seal Over the

Yeast Nuclear Pore Complex Thereby Blocking Nucleocytoplasmic Transport. *J. Cell Biol.* **123**, 275-284

88. Sukegawa, J. and Blobel, G. (1993) A Nuclear Pore Complex Protein that Contain Zinc Finger Motifs, Binds DNA and Faces the Nucleoplasm. *Cell* **72**, 29-38

89. Richardson, W.D. et al. (1988) Nuclear Protein Migration Involves Two Steps: Rapid Binding to the Nuclear Envelope Followed by Slower Translocation Through the Nuclear Pores. *Cell* **52**, 655-664

90. Paine, P.L. et al. (1975) Nucleocytoplasmic Movement of Fluorescent Traces Microinjected into Living Salivary Gland Cells. *Nature* **254**, 109-114

91. Dingwall, C et al. (1982) A Polypeptide Domain that Specifies Migration of Nucleoplasmin into the Nucleus. *Cell* **30**, 449-458

92. Garcia-Bustos, J.F. et al. (1991) Nuclear Protein Localization. *Biochimica et Biophysica Acta* **1071**, 83-101

93. Finlay, et al. (1987) Inhibition of *In Vitro* Nuclear Import by a Lectin that Binds to Nuclear Pores. *J. Cell Biol.* **104**, 189-200

94. Dabauvalle, M.C. et al. (1988) Inhibition of Nuclear Accumulation of Karyophilic Proteins in Living Cells by Microinjection of the Lectin Wheat Germ Agglutinin. *Exptal. Cell Res.* **174**, 291-296

95. Yoneda, Y. et al. (1987) Reversible Inhibition of Protein Import into the Nucleus by Wheat Germ Agglutinin in Cultured Cells. *Exptal. Cell Res.* **173**, 586-595

96. Featherstone, C.M. et al. (1988) A Monoclonal Antibody Against the Nuclear Pore Complex Inhibits Nucleocytoplasmic Transport of Protein and RNA *In Vivo*. *J. Cell. Biol.* **107**, 1289-1297

97. Gerrace, L. and Burke, B. (1988) Functional Organisation of the Nuclear Envelope. *Ann. Rev. of Cell Biol.* **4**,335-374

98. Goldberg, M.W. et al. (1992) High Resolution Scannining Electron Microscopy of the Nuclear Envelope: Demonstration of a New, Regular, Fibrous Lattice Attached to the Baskets of the Nucleoplasmic Face of the Nuclear Pores. *J. of Sruct. Biol.* **108**, 257-268

99. Burke, B. and Gerrace, L (1986) A cell Free system to Study Reassembly of the Nuclear Envelope at the End of Mitosis. *Cell* **44,** 639-652

100. Miake-Lye, R. and Kirschner, M.W. (1985) Induction of early Mitotic Event in Cell-Free Systems. *Cell* **41,** 165-175

101. Lohka , M.J. and Matsui, Y. (1983) Formation *In Vitro* of Sperm Pronuclei and Chromosomes by Amphibian Ooplasmic Components. *Science* **220,** 719-721

102. Lohka, J.M. and Matsui, M. (1984) Roles of Cytosol and Cytoplasmic Particles in Nuclear Envelope Assembly and Sperm Pronuclei Formation in Cell-Free Preparations from Amphibian Eggs. *J. of Cell: Biol.* **61,** 591-602

103. Dabauvalle, M.C. and Scheer, U. (1991) Assembly of Nuclear Pore Complexes in *Xenopus* Egg Extracts. *Cell* **72,** 25-29

104. Sheenan, M.A.et al. (1988) Steps in the Assembly of Replication Competent Nuclei in Cell free Systems from *Xenopus* Eggs. *J. of Cell Biol.* **106,** 1-12

105. Newport, J. and Dumpy, W. (1992) Characterization of Membrane Binding and Fusion Events During Nuclear Envelope Assembly using Purified Components. *J. of Cell Biol.* **116,** 295

106. Boman, A.L. (1992) GTP Hydrolysis is Required for Vesicle Fusion during Nuclear Envelope Assembly *In Vitro*. *J. of Cell Biol.* **116,** 281

107. Wilson, K. and Newport, J. (1988) A Trypsin Sensitive Receptor on Membrane. *J of Cell. Biol.* **107,** 57

108. Vigers, G.P.A. and Lohke, M.J. (1991) A Distinct Vesicle Population Targets Membrane and Pore Complexes. *J of Cell Biol.* **112,** 545

109. Gerrace, L. (1986) Nuclear Lamina and Organisation of Nuclear Architecture. *Trends Biochem. Sc.* **11,** 443-446

110. Glass, J.K. and Gerrace, L. (1990) Lamin A and C Bind and Assemble at the Surface of Mitotic Chromosomes. *J. cell Biol.* **111,** 1047-1057

111. Burke, B. (1990) On the Cell Free Association of Lamin A and C with Metaphase Chromosomes. *Exptal. Cell Res.* **186,** 169-176

112. Cox, L.S. (1992) DNA Replication in Cell Free Extracts from *Xenopus* Eggs is Prevented by Disrupting Nuclear Envelope Function. *J. Cell Sc.* **101,** 43-53

113. Meier, J. et al. (1991) The Role of Lamina L Substructure in Nuclear Assembly and DNA replication, in Cell Free Extracts of *Xenopus* Eggs. *J. of Cell Sc.* **98,** 271-278

114. King, W.J. and Greene, G.L (1984) Monoclonal Antibodies Localise Estrogen Receptor in the Nuclei of Target Cells. *Nature* **307,** 745 747

115. Perrot-Aplanat. M. et al. (1985) Immunocytochemical Study of Mammalian Progesterone Receptor Using Monoclonal Antibodies. Endocrinology **116,** 1473-1484

116. Garcia-Bustos, J.F. et al. (1991) Nuclear Import Substrates Compete for a Limited number of Binding Sites. *JBC* **266,** 22303-22306

117. Kalderon, D. et al. (1984) Sequence Requirement for Nuclear Localization of Simian Virus 40 Large T-Antigen. *Nature* **311,** 33-38

118. Lanford, R.E. and Butel, J.S. (1984) Construction and Characterization of an SV40 Mutant Defective in Nuclear Transport of T-Antigen. *Cell* **37,** 801-813

119. Yoneda, Y.T. et al. (1987) Reversible Inhibition of Protein Import into the Nucleus by Wheat Germ Agglutinin Injected into Cultured Cells. Exp. *Cell Res.* **170,** 439-452

120. Colledge, W.H. et al. (1986) Extensive Mutagenesis of the Nuclear localization Signal of Simian Virus 40 large T-Antigen. *Mol. Cell Biol.* **6,** 4136-4139

121. Kalderon, D. et al. (1984) A Short Amino Acid Sequence able to Specify Nuclear Location. *Cell* **39,** 499-509

122. Hall, M.N. et al. (1984) Targetting of Ecoli β-galactosidase to the Nucleus in Yeast. *Cell* **36,** 1057-1065

123. Tratner, I. and Verma, I.M. (1991) Identification of a Nuclear Targetting Sequence in Yeast. *Oncogene* **6,** 2049-2053

124. Burghin, T.R and De Roberts, E.M. (1987) Nuclear Migration Signal of *Xenopus Laevis* Nucleoplasmin. *EMBO J.* **6,** 2617-2625

125. Dingwall, C. et al. (1988) The Nucleoplasmin Nuclear Localization Sequence is Larger and More Complex than that of SV40 Large T Antigen. *J. Cell. Biol.* **107,** 841-849

126. Roberts, B. (1989) Nuclear Location Signal-Mediated protein Transport. *Biochemica et Biophysica Acta* **1008,** 263-280

127. Orti, .E. et al. (1989) Phosphorylation of Glucocorticoid Receptors-Associated and Free Forms of the Approximately 90 kDa Heat Shock Proteins Before and After Activation. *J. Biol. Chem.* **264,** 231-237

128. Picard, D.and Yamamoto K.R. (1987) Two Signals Mediate Hormone Dependent Localization of the Glucocorticoid Receptor: . *EMBO J.* **6,** 3333-3340

129. Guiochon-Mantel, A. et al. (1989) Mechanisms of Nuclear Localization of the Progesterone Receptor: Evidence for Interaction Between Monomers*Cell* **57,** 1147-1154

130. Ylikomi, T. et al. (1992) Cooperation of Proto-Signals for Nuclear Accumulation of estrogen and Progesterone Receptors. *EMBO J.* **11,** 3681-3694

131. Simental, J.A. et al. (1991) Transactivation and Nuclear Targetting Signals of Human Androgen Receptor. *J of Biol Chem.*. 510-518

132. Lubhan, D.B. et al. (1988) The Human Androgen Receptor: cDNA Cloning, Sequence Analysis and Gene Expression in Prostate. *Mol. Endocrin.* **2,** 1265-1275

133. Zhou, Z. et al (1994) A Ligand-Dependent Bipartite Nuclear Targetting Signal in the Human Androgen Receptor. *J of Biol Chem.*. **269,** 13115-13123

134. Arriza, J.L. et al. (1987) Cloning of Human Mineralocorticoid Receptor Complementary DNA: Structural and Functional Kinship within the Glucocorticoid Receptor. *Science* **237,** 268-275

135. Benbrook, , D. and Pfahl, M. (1987) A Novel Thyroid Hormone Receptor Encoded by a cDNA Clone from a Human Testis Library. *Science*: **238,** 788-791

136. Nakai, A. et al. (1988) Characterization of a Thyroid Hormone Receptor Expressed in Human Kidney and Other Tissues. *Proc. of Natl. Acad. of Sc.* **85,** 2781-2785

137. Weinberger, C. et al. (1986) The c-*Erb*-A Gene Encodes a Thyroid Hormone Receptor. *Nature* **324,** 641-646

138. Giguere, V. et al. (1987) Identification of a Receptor for the Morphogen Retinoic Acid. *Nature* **330,** 624-629

139. Baker, A.R. et al. (1988) Cloning and Expression of Full Length cDNA Encoding Human Vitamin D Receptor. *Proc. of Natl. Acad. of Sc.* **85,** 3294-3298

140. Wang, L.H. et al. (1990) COUP Transcription Factor is a Member of Steroid Receptor Superfamily. *Nature* **340,** 163-166

141. Yoneda, Y. et al. (1988) Antibodies to Asp-Asp-Glu-Asp can Inhibit Transport of Proteins into the Nucleus. *Science* **242,** 22303-22306

142. Garcia-Bustos, J.F. et al (1991) Nuclear Import Substrates Compete for a Limited number of Binding Sites: Evidence for Different Classes of Yeast Nuclear Import Receprors. *J. of Biol. Chem.*. **266,** 22303-22306

143. Adam, S.A. et al. (1989) Identification of Specific Binding Protein for a Nuclear Location Sequence. *Nature* **323,** 560-564

144. Dworetzky, S.I. et al (1988) Translocation of RNA Coated Gold Particles Through the Nuclear Pores of Oocytes. *J. Cell Biol.* **106,** 575-584

145. LaCasse E.C. and Lefebvre. Y.A. (1991) Nuclear and Nuclear Envelope Binding Proteins of the Glucocorticoid Receptor Nuclear Localization Peptide Identified by Cross-Linking. *J. of Steroid Biochem. Mol. Biol.* **40,** 279-285

146. Yamasaki, L. (1989) Identification of Four Nuclear Transport Signal-Binding Proteins for a Nuclear Localization Sequence. *Mol. and Cell. Biol.* **9,** 3028-3036

147. Gurdon, J.B. (1970) Nuclear Transplantation and the Control of Gene Activity in Animal Development. *Proc. Royal Soc. Biochem.* **176,** 303-314

148. Goldfarb, D.S. et al. (1986) Synthetic Peptides as Nuclear Localization Sequences. *Nature* **322,** 641-654

149. Shneider, J. et al. (1988) A Mutant SV40 Large T Antigen Interferes with Nuclear Localization of Heterologous protein. *Cell* **54,** 117-125

150. Felderr, C.M.et al. (1983) Movement of Karyophilic Proteins Through the Nuclear Pores of Oocytes. *J.Cell.Biol.* **99,** 2216-2222

151. Pandey, S. and Parnaik, V.K. (1991) Identification and Characterization of Nuclear Location Signal Binding Proteins in Nuclear Envelopes. *Biochimica et Biophysica Acta* **1063,** 81-89

152. Adam, S.A. and Gerrace, L. (1991) Cytosolic Proteins that Specifically Bind Nuclear Location Signals are Receptors for Nuclear Import. *Cell* **66,** 837-847

153. Newmeyer, D.D. et al (1986) Assembly *In Vitro* of Nuclei Active in Nuclear Protein Transport: ATP is Required for Nucleoplasmin Protein Accumulation. *EMBO J.* **5,** 501-51

154. Newmeyer, D.D. and Forbes, D.J. (1990) N-Ethylmaleimide -Sensitive Cytosolic Factor Necessary for Nuclear Protein Import: Requirement in Signal Mediated Binding to the Nuclear Pore. *Cell* **52,** 641-653

155. Adam, S.A. et al (1990) Nuclear Protein Import in Permeabilised Mammalian Cells Require Soluble Cytoplasmic Factors. *J. Cell Biol.* **111,** 807-816

156. Shi, Y and Thomas, J.O. (1992) The Transport of Protein into the Nucleus Requires Heat Shock Protein 70 or its Cytosolic Cognate. *Mol. Cell. Biol.* **12,** 2186-2192

157. Moore, M.S. and Blobel, G (1992) Two Steps of Nuclear Import, Targetting to the Nuclear Envelope and Transport Through the Nuclear Pore Require Cytosolic Factors. *Cell* **69,** 939-950

158. Finlay, D. et al. (1991) A Complex of Nuclear Pore Complex Proteins Required for Nuclear Function. *J. Cell Biol.* **114,** 169

159. Imamoto, N et al. (1992) Antibodies Against 70 kDa Heat Shock Cognate Protein Inhibit Mediated Nuclear Import of Karyophilic Proteins. *J. Cell Biol.* **119,** 1047-1061

160. Pratt, W.D. (1993) Control of Steroid Receptor Function and Cytoplasmic Nuclear Transport by Karyophilic Proteins. *Bioessays* **14,** 841-848

161. Smith, D.F. (1993) Steroid Receptors and their Associated Proteins. *Mol. Endocrin.* **7,** 4-11

162. Mandell, R.B. and Feldherr, C.M. (1990) Identification of Two Heat Shock Proteins Related *Xenopus* Oocyte Proteins that are Capable of Recycling Across the Nuclear Envelope. *J. Cell Biol.* **111,** 1775-1783

163. Melchior, F. et al (1993) Inhibition of Nuclear protein Import by Non-hydroxylable Analogues of GTP and Identification of the Small GTPase, Ran/TC4 as an Essential Transport Factor. *J. Cell Biol.* **123,** 1649-1659

164. Bowman, A.L. et al. (1992) A Role for ADP-Ribosylation Factor in Nuclear Vesicle Dynamics. *Nature* **358,** 512

165. Stochaj, U. and Silver, P.A. (1992) Conserved Phosphoprotein that Specifically Binds Nuclear Localization Sequences is Involved in Nuclear Import. *J. Cell Biol.* **117,** 473-482

166. Goldfarb, D. and Michaud, (1991) Pathways for the Nuclear Transport of Proteins and RNA. *Trends in Cell Biol.* **1,** 20-24

167. Nigg, E.A. et al. (1991) Nuclear Import-Export: In Search of signals and Mechanisms. *Cell* **66,** 15-22

168. Stewart, M. (1992) Nuclear Pore Structure and Function. *Seminar in Cell Biol..* **3,** 267-277

169. Li, R. and Thomas. O. (1989) Identification of a Human Protein that Interacts with Nuclear Localization Signal. *J. of Cell Biol.* **66,** 837-841

170. Newmeyer, D.D. and Forbes, D.J. (1988) Nuclear Import can be Separated into Two Distinct Steps *In Vitro*: Nuclear Pore Binding and Translocation. *Cell:* **52,** 641-653

171. Sterne-Marr, R.T. et al. (1992) O-Linked Glycoproteins of the Nuclear Pore Complex Interacts with a Cytosolic Factor Required for Nuclear Protein Import. *J. Cell. Biol.* **116,** 271-280

172. Akey, C.W. and Goldfarb, D.S. (1989) Protein Import Through the Nuclear Pore Complex. *J. Cell. Biol.* **109,** 971-982

173. Skoglund, U. (1983) Visualisation of the Formation and Transport of a Specific hnRNP. *Cell* **34,** 847-855

174. Michaud, N. and Goldfarb, D. (1992) Microinjected U snRNPs are Imported to Oocyte Nuclei Via a Nuclear Pore Complex by a 3 Distinguishable Pathways. *J. Cell .Biol.* **116,** 851-861

175. Zasloff, M. (1983) tRNA Transport from the Nucleus in a Eukaryotic Cell: Carrier Mediated Translocation Process. *Proc. of Natl. Acad. of Sc.* **80,** 6436-6440

176. Hamm, J. and Mattaj, I.W. (1990) Monomethylated Cap Structure Facilitates RNA Export from the Nucleus. *Cell* **63,** 109-118

177. Melhin, H.B. et al (1992) Translocation of a Specific Pre-mRNA Ribonucleoprotein Particles Through the Nuclear Pore Studied with Electron Microscope Tomography. *Cell* **69,** 605-613

178. Hamm, J. et al. (1990) The Trimethyl Guanosine Cap Structure of U1 snRNA is a Component of a Bipartite Nuclear Targetting Signal. *Cell* **62,** 569-577

179. Shatkin, A.J. (1985) mRNA Cap Binding Proteins: Essential Factors for Initiating Translation. *Cell* **40,** 223-224

180. Patzelt, E. et al. (1983) Cap Binding Proteins Associate with the Nucleus. *Nucleic Acid Res.* **11,** 5821-5835

181. Goldstein, L. (1958) Localization of Nucleus Specifc Proteins as Shown by Transplantation Experiments in *Amoeba Proteus.* *Exptal. Cell.Res.* **15,** 635-637

182. Goldstein, L and Ko, C. (1981) Distribution of Proteins Between Nucleus and Cytoplasm of Amoeba Proteus. *J. cell Biol.* **88,** 516-585

183. Borer, R.A. et al. (1989) Major Nucleolar Proteins Shuttle Between Nucleus and Cytoplasm. *Cell* **56,** 379-390

184. Pinol-Roma. S. and Dreyfuss, G. (1992) Binding Proteins Between Nucleus and Cytoplasm. *Nature* **335,** 730-732

185. Madan, A.P. and DeFranco, D.B. (1993) Bidirectional Transport of Glucocorticoid Receptors Across the Nuclear Envelope. *Proc. of Natl. Acad. of Sc.* **90,** 3588-3592

186. Guiochon-Mantel, A. et al. (1991) Nucleocytoplasmic Shuttling of the Progesterone Receptor. *EMBO J.* **10,** 3851-3859

187. Schmidt-Zachmann, M.S. et al. (1993) Nuclear Export of Proteins: The Role of Nuclear Retention. *Cell* **74,** 493-504

188. Kambach, C. and Mattaj, I.W. (1992) Intracellular Distribution of the U1A Depends on an Active transport and Nuclear binding to U1 snRNA. *J. Cell Biol.* **118,** 11-21

189. Guiochon-Mantel, A. (1994) Nuclear Localization Signals Also Mediate the Outward Movement of Proteins From the Nucleus. *Proc. of Natl. Acad. of Sc.* **91,** 7179-7183

190. Whiteside, S.T. and Goodburn, S. (1993) Signal Transduction and Nuclear Targetting: Regulation of Transcription Factor Activity by Subcellular Localization. *J.of Cell Science* **104,** 949-955

191. Rihs, H.P and Peters, R. (1991) The Rate of Nuclear Cytoplasmic Protein Transport is Determined by the Casein Kinase II Site Flanking the Nuclear Localization Sequence of the SV40 Large T Antigen. *EMBO J.* **10,** 633-639

192. McVey, D. (1989) Phosphorylation by a Large Tumor T Antigen by a CDC-2 Stimulates SV40 DNA Replication. *Nature* **341,** 503-507

193. Mosialos, G. et al (1991) A protein Kinase A Recognition Sequence is Structurally Linked to Transformation by $P59^{v\text{-}rel}$ and Cytoplasmic Retention of $p68^{c\text{-}rel}$. *Mol. Cell. Biol.* **11,** 5857-5871

194. Metz, R. and Ziff, E. (1991) cAMP Stimulates the C/EBP-Related Transcription Factor, rNFIL-6 to Translocate to the Nucleus and Induce *c-Fos* Transcription. *Genes and Dev.* **5,** 1754-1766

195. Roux, P. et al. (1990) Nuclear Localization of c-Fos, but not v-Fos Proteins, is Controlled by Extracellular Signals. *Cell* **63,** 341-351

196. Chida, K. Vogt, P.K. (1992) Nuclear Translocation of Viral *Jun* but not of Cellular *Jun* is Cell Cycle Dependent. *Proc. of Natl. Acad. of Sc.* **89,** 4290-4294

197. Nasmyth, K. et al (1990) The Identification of a Second Cell Cycle Control in the HO Promoter in Yeast: Cell Cycle regulation of SWI5 Nuclear Entry. *Cell* **62,** 631-634

198. Moll, T. et al. (1991) The Role of Phosphorylation and the CDC28 Protein Kinase in Cell Cycle Regulated Nuclear Import of the S. *Cerevisae* Transcription Factor SWI5. *Cell* **66,** 743-758

199. Lee, W.H. et al. (1987) The Retinoblastoma Susceptibility Gene Encodes a Nuclear Phosphoprotein Associated with DNA binding Activity. *Nature* **329,** 642-645

200. Mittnacht, S. and Weinberg, R.A. (1991) G_1/S Phosphorylation of the Retinoblastoma Protein is Associated with Altered Affinity for the Nuclear Compartment. *Cell* **65,** 381-393

201. De Caprio, J.A. et. al. (1988) SV40 Large T Antigen Forms a Specific Complex with the Product of the Retinoblastoma Susceptibility Gene. *Cell* **54,** 275-283

202. Buchnovich, K. et al. (1989) The Retinoblastoma Protein is Phosphorylated During Specific Phases of the Cell Cycle. *Cell* **58,** 1079-1105

203. Chen, P.L. et al. (1989) Phosphorylation of the Retinoblastoma Gene Product is Modulated During the Cell Cycle and During Cellular Differentiation. *Cell* **58,** 1193-1198

204. Gosh, S. and Baltimore, D. (1990) Activation *In Vitro* of NF-κB by Phosphorylation of its Inhibitor IκB. *Nature* **344,** 678-682

205. Blank, V. et al. (1992) NF-κB and Related Proteins: Rel/Dorsal Homologies Meet Ankyrin-Like Repeats. *Trends in Biochem. Sc.* **17,** 135-140

206. Nigg, E.A. et al. (1985) Rapid and Reversible Translocation of the Catalytic Subunit of cAMP Dependent Protein Kinase Type II From the Golgi to the Nucleus. *EMBO J.* **4,** 2801-2806

207. Vandroma, M. et al. (1992) Nuclear Import of the Myogenic Factor MyoD requires cAMP-Dependent Protein Kinase Activity but not the Direct phosphorylation of MyoD. *J. of Cell Science* **107,** 613-620

208. Catelli, M.G. et al. (1985) The Common 90 kDa Protein Component of Non Transformed '8S' Steroid Receptors is a Heat Shock Protein. *EMBO J.* **4,** 3131-3135

209. Sanchez, E.R. (1990) Hormone Free Mouse Glucocorticoid Receptors Overexpressed in Chinese Hamster Ovary Cells are Localised to the Nucleus and are Associated with the Heat Shock Proteins 70 and 90. *J. of Biol. Chem.* **265,** 20123-20130

210. Wikstrom, A.C. et al. (1987) Intracellular Localization of the Glucocorticoid Receptor: Evidence for Cytoplasmic and Nuclear Localization. *Endocrinology* **120,** 1232-1242

211. Munck, A and Holbrook, N.J. (1984) Glucocorticoid Receptor Complexes in Rat Thymus Cells: Rapid Kinetic Behaviour and Cyclic Model. *J. of Biol. Chem.* **259,** 820-831

212. Dougherty, J.J. et al. (1982) Phosphorylation *In Vitro* of Chicken Oviduct Progesterone Receptor. *J. of Biol. Chem.* **257,** 14226-14230

213. Munck, A. et al. (1972) Glucocorticoid Receptor Complexes and the Earliest Step in the Action of Glucocorticoids on Thymus. *J. of Steroid Biochem.* **3,** 567-578

214. Housley, P.R. et al. (1982) Inactivation of Glucocorticoid binding Capacity by Protein Phosphorylation in the Presence of Molybdate and Complete Reactivation by Dithiothreitol. *J. of Biol. Chem.* **257,** 8615-8616

215. Chauchereau, A. et al. (1991) Phosphorylation of Transformed Wild Type and Mutated Progesterone Receptors *J. of Biol. Chem.* **266,** 18280-18286

216. Mendel, D.B. et al. (1986) Molybdate Stabilised Non-Activated Glucocorticoid Receptor Complexes Contain a 90kDa Non-Steroid Binding Phosphoprotein that is Lost on Activation. *J. of Biol. Chem.* **261,** 3758-3763

217. Hoeck, C.K. et al. (1989) Down Regulation and Phosphorylation of Glucocorticoid Receptors in Cultured Cells. *J of Biol Chem.* **264,** 14398-14402

218. Orti, E et al. (1989) Agonist-Dependent Phosphorylation and Nuclear Dephosphorylation of Glucocorticoid Receptors in Intact Cells. *J. of Biol. Chem.* **264,** 11884-11890

219. Orti, E. et al. (1989) A Dynamic Model of the Glucocorticoid Receptor: Phosphorylation and Cycling in Intact cells. *J. of Steroid Biochemistry* **34,** 85-96

220. Bell, P.A. and Munck, A. (1973) Steroid Binding Properties and Stabilization of Cytoplasmic Glucocorticoid Receptors From Rat Thymus Cells. *Biochemical J.* **136,** 77-109

221. Smith, I.L. et al. (1989) Phosphorylated Sites Within the Functional Domains of the 100 kDa Steroid Binding Subunit of Glucocorticoid Receptors. *Biochemistry* **28,** 4490-4497

222. Bodwell, J.E.et al (1991) Identification of Phosphorylation Sites in the Mouse Glucocorticoid Receptor. *J. of Biol. Chem.* **266,** 7549-7555

223. Housley, P.R. et al. (1985) The Molybdate Stabilised L-Cell Glucocorticoid Receptor Isolated by Affinity Chromatography or with a Monoclonal Antibody is Associated with a 90-92 kDa Non-Steroid Binding Phosphoprotein. *J. of Biol. Chem.* **260,** 13810-13817

224. Hoeck, W. and Groner, B. (1990) Hormone-Dependent Phosphorylation of the Glucocorticoid Receptor Occurs Mainly in the Amino-Terminal Transactivation Domain. *J. of Biol. Chem.* **265,** 5403-5408

225. Auricchio, F. (1989) Phosphorylation of Steroid receptors. *J Steroid Biochemistry* **32,** 613-622

226. Auricchio, F et al. (1987) Phosphorylation on Tyrosine of Estradiol-17β-Receptor in Uterus and Interaction of Estradiol 17β and Glucocorticoid Receptors with Anti-phosphotyrosine Antibodies. *J. Steroid Biochemistry* **27,** 245-253

227. Rao, V.S.K. and Fox, F. (1987) Epidermal Growth Factor Stimulates Tyrosine Phosphorylation. *Biochemical and Biophysical Res. Comm.* **144,** 512-519

228. Gruol, D.J. et al. (1986) cAMP-Dependent Protein Kinase Promotes Glucocorticoid Receptor Function. *J. of Biol. Chem.* **261,** 4909-4914

229. Dong, Y. et al. (1989) The Mechanism of cAMP-Induced Glucocorticoid Receptor Expression. *J. of Biol. Chem.* **264,** 13679-13683

230. Rangarajan, P.N. et al. (1992) Modulation of Glucocorticoid Receptor Function by PKA. *Mol. Endocrin.* **6,** 1451-1457

231. Somers, J.P. and DeFranco, D.B. (1992) Effects of Okadaic Acid, a Protein Phosphatase Inhibitor on Glucocorticoid Mediated Enhancement. *Mol. Endocrin.* **6,** 26-34

232. Moyer, M.L. et al. (1993) Modulation of Cell Signaling Pathways Can Enhance or Impair Glucocorticoid-Induced Gene Expression *J. of Biol. Chem.* **268,** 22933-22940

233. Zhang, H. et al. (1993) Protein Kinase A Activation of Glucocorticoid-Mediated Signaling in the Developing Retina. *Proc. of Natl. Acad. of Sc.* **90,** 3880-3884

234. Reik, A. et al. (1994) Cross-Talk Modulation of Signal Transduction Pathways: Two Mechanisms are Involved in the Control of Tyrosine Amino Transferase Gene Expression by Phorbol Esters. *Mol. Endocrin.* **8,** 490-497

235. Nordeen, S.K. et al. (1994) The Coupling of Multiple Signal Transduction Pathways with Steroid Response Mechanisms. *Endocrinology.* **134,** 1723-1732

236. DeFranco, D.B. et al. (1991) Protein Phosphatase Types 1/2A Regulate Nucleocytoplasmic Shuttling of the glucocorticoid Receptors. *Mol. Endocrin.* **5,** 1215-1228

237. Qi, M. et al. (1989) v-Mos Oncoproteins Affect the Nuclear Retention and Re-utilisation of Glucocorticoid Receptors. *Mol. Endocrin.* **3,** 1279-1288

238. Beck, C.A. et al. (1992) Effects of Hormone and Cellular Modulators of Protein Phosphorylation on Transcriptional Activity, DNA Binding, and Phosphorylation on Human Progesterone Receptors. *Mol. Endocrin.* **6,** 607-620

239. Beck, C.A. et. al. (1993) The Progesterone Antagonist RU486 Acquires Agonist Activity upon Stimulation of cAMP Signaling Pathways. *Proc. of Natl. Acad. of Sc.* **90,** 4441-4445

240. Cho, H. and Katzenellenbogen, B.S. (1993) Synergistic Action of Estrogen Receptor-Mediated Transcription by estradiol and Protein Kinase Mediated Transcription by estradiol and Protein Kinase Activators. *Mol. Endocrin.* **7,** 441-452

241. Aronica, S.M. and Katzenellenbogen, B.S. (1993) Stimulation of Estrogen Receptor-Mediated Transcription and Alteration in the Phosphorylation State of the Rat Uterine Estrogen Receptor by Estrogen, a Cyclic Adenosine Monophosphate , and Insulin-Like Growth Factor-1. *Mol. Endocrin.* **7,** 743-752

242. Huggenvik, J.I. et al. (1993) Modification of the Retinoic Acid Signaling Pathway by Catalytic Subunit of PKA. *Mol. Endocrin.* **7,** 543-550

243. Zhang, Y. et al. (1994) Multiple Signaling Pathways Activate the Chicken Progesterone Receptor. *Mol. Endocrin.* **8,** 577-584

244. Mendel, D.B. et al. (1990) Evidence for Glucocorticoid Receptor Cycle and Nuclear Dephosphorylation of the Steroid Binding Protein. *Progress in Clini. and Biol Res.* **322,** 97-117

245. Mendel, D.B. et al. (1986) Glucocorticoid Receptors Lacking Hormone-Binding Activity are Bound in Nuclei of ATP-Depleted Cells. *Nature* **324,** 478-480

246. Orti, E. et al. (1993) Kinetics of Glucocorticoid Receptor Phosphorylation in Intact Cells: Evidence for Hormone Induced Hyperphosphorylation After Activation and Recycling of Hyperphosphorylated Receptors. *J. of Biol. Chem.* **268,** 7779-7784

247. Bodwell, J.E. et al. (1993) Glucocorticoid Receptors: ATP Dependent Cycling and Hormone Dependent Hyperphosphorylation *J. of Steroid Biochem. and mol. Biol.* **47,** 1-6

248. Qi, M et al. (1990) Recycling and Desensitization of Glucocorticoid Receptors in v-mos Transformed Cells Depend on the Ability of Nuclear Receptors to Modulate Gene Expression. *Molecular Endocrin.* **4,** 455-464

249. Hutchison, K.A. et al. (1993) Regulation of Glucocorticoid receptor Function Through the Assembly of a Receptor Heat Shock Protein Complex. *Annals of New York Academy of Sciences* **684,** 35-48

250. Yamamoto, K.R.et al. (1974) Receptors From Glucocorticoid Sensitive Lymphoma Cells and two Classes of Insensitive Clones: Physical and DNA Binding Properties. *Proc. of Natl. Acad. of Sc.* **71,** 3901-3905

251. Yamamoto, K.R. et al. (1976) Genetic Approaches to Steroid Hormone Action. *Recent Progr. Horm. Res.* **32,** 3-32

252. Nordeen, S.K. et al. (1981) Photoaffinity Labelling of the Glucocorticoid Receptor. *J. of Biol. Chem.* **256,** 285-289

253. Dellweg, H.G. et al. (1982) Active Domains in Wild Type and Mutant Glucocorticoid Receptors. *EMBO J.* **1,** 285-289

254. Gehring, U. (1983) Glucocorticoid receptors of Wild Type and Variant Mouse Lymphoma Cells. *J. of Steroid Biochem.* **19,** 475-482

255. Gehring, U. et al. (1983) Photoaffinity Labelling and partial Proteolysis of Wild Type and Variant Glucocorticoid Receptors From Rat Hepatoma and Mouse Lymphoma Cells. *Biochemistry* **22,** 4013-4018

256. Clark, W. (1987) Characterization of Glucocorticoid Receptors in S49 Lymphoma Cells by Affinity Labelling With [^{3}H]-Dexamethasone-21-Mesylate. *J. of Steroid biochem.* **26,** 59-65

257. Westphal, H.M. et al (1984) Immunochemical Characterization of Wild Type and Variant Glucocorticoid Receptors by Monoclonal Antibodies. *EMBO J.* **3,** 1493-1498

258. Northrop, J.P. et al. (1985) Characterization of Wild Type and Mutant Glucocorticoid Receptors From Rat Hepatoma and Lymphoma Cells. *J. of Biol. Chem.* **260,** 6398-6403

259. Connors, M. and Lefebvre, Y.A. (1991) Unpublished data

260. Miesfield, R. et al. (1984) Characterization of a Steroid Hormone Receptor Gene and mRNA in Wild Type and Mutant Cells. *Nature* **312,** 779-781

261. Pearson, R.B. and Kemb, B.E. (1991) Protein Kinase Specific Site Sequences and Consensus specificity motifs: Tabulations. *Methods in Enzymology* **200,** 62-81

262. Robins, D.M et al. (1981) Transforming DNA Integrates Into the Host Chromosome. *Cell:* **23,** 29-39

263. Okayama, H. and Berg, P. (1983) A cDNA Cloning Vector That Permits Expression of cDNA Inserts in Mammalian Cells. *Mol. Cell. Biol.* **3,** 280-298

264. Northrorp, J.P. et al. (1986) Analysis of Glucocorticoid Unresponsive Cell Variants Using Mouse Glucocorticoid Receptor cDNA Clone. *J. of Biol. Chem.*

265. Young, R.A. and Davis, R.W. (1983) Efficient Isolation of Genes by Using Antibody Probes. *Proc. of Natl. Acad. of Sc.* **80,** 1194-1198

266. Subramani, S. et al. (1981) Expression of the Mouse Dihydrofolate Reductase cDNA in Simian Virus 40 Vectors. *Mol. Cell. Biol.* **1,** 854-864

267. Lee, F. et al. (1981) Glucocorticoids Regulate the Expression of Dihydrofolate Reductase cDNA in mouse Mammary Tumor Virus Dihydrofolate Reductase. *Nature* **294,** 228-232

268. Gluzman, Y. (1981) SV40 Transformed Simian Cells Support the Replication of SV40 Early Mutants. *Cell* **23,** 175-182

269. Sanger, F. et al. (1977) DNA sequencing with Chain Terminating Inhibitors. *Proc. of Natl. Acad. of Sc.* **74,** 5463-5467

270. Hollenberg, S.M. and Evans, R.M. (1985) Multiple and Cooperative Transactivation Domains of the Human Glucocorticoid Receptor. *Cell* **55,** 899-906

271. Gigue`re, V. et al. (1986) Functional Domains of Human Glucocorticoid Receptor. *Cell* **46,** 645-652

272. Maniatis, T. et al. (19) Molecular Cloning: A laboratory Manual. **1,** 1.33-1.110 2nd Ed *Coldspring Harbor Lab.* Coldspring Harbor N.Y.

273. Scoll, J.R. (1984) Regulation of Plasmid Replication. *Microbial. Rev.* **48,** 1

274. Cantor, C.R. and Schimmel, P.R. (1980) Biophys. Chem. Part III: The Behaviour of Biological Macromolecules. p 1251 Freeman Publications SanFrancisco.

275. Reese, J.C. and Katzenellenbogen, B.S. (1991) Differential DNA-Binding Abitities of Estrogen Receptor Occupied with 2 Classes of Antiestrogens: Studies Using Human Estrogen Receptor Overexpressed in Mammalian Cells. *Nucleic acids Research* **19,** 6595-6602.

276. Felgner, P.L. et al. (1987) Lipofectin: A highly Efficient, Lipid Mediated DNA/Transfection Procedure. *Proc. of Natl. Acad. of Sc.* **84,** 7413-7417

277. Puchalski, R.B. and Fahl, W.E. (1992) Gene Transfer by Electroporation, Lipofection, and DEAE-Dextran Transfection: Compatibility with Cell-Sorting by Flow cytometry. *Cytometry* **13,** 23-30

278. Bradford, M.A (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilising the Principle of Protein Dye Binding. *Anal. Biochem.* **72,** 248-254

279. Chu, G. et al. (1987) Electroporation for the Efficient Transfection of Mammalian Cells with DNA. *Nucleic Acids Res.* **15,** 1311-1326

280. Knutson, J.C. and Yee, D. (1987) Electroporation: Parameters Affecting Transfer of DNA into Mammalian Cells. *Anal. Biochem.* **164,** 44-52

281. Somayrac, L.M. (1981) Efficient Infection of Monkey Cells with DNA of Simian Virus 40. *Proc. of Natl. Acad. of Sc.* **78,** 7575-757

282. Lowry, O.H. et al. (1951) Protein Measurement with the Folin Phenol Reagent. *J. of Biol Chem.* **193,** 265-275

283. Laemli, U.K. (1970) Cleavage of Structural Proteins During the Assemble of the Head of Bacteriophage T4. *Nature* **277,** 680-685

284. Matsudaria, P.T. and Burgess, D.R. (1978) SDS Microslab Linear Gradient Polyacrylamide Gel Electrophoresis. Anal. Biochem. 87, 386-396

285. Towbin, H. et al. (1979) Electrophoretic Transfer of Proteins from Polyacrylamide Gels into Nitrocellulose Sheets: Procedure and Some Applications. *Proc. of Natl. Acad. of Sc.* **76,** 4350-4354

286. Burnette, W.N. (1981) Western Blotting: Electrophoretic Transfer of Proteins from SDS Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A. *Anal. Biochem.* **112,** 195-203

287. Gametchu, B. and Harrison, R.W. (1984) Characterization of Monoclonal Antibody to Rat Liver Glucocorticoid Receptor. *Endocrinology* **114,** 274-279

288. Giliespie, P.G. and Hudspeth, A.J. (1991) Chemiluminescence Detection of Proteins from Single Cells. *Proc. of Natl. Acad. of Sc.* **88,** 2563-2567

289. Bronstein, I. et al. (1992) Improved Chemiluminescent Western Blotting Procedure. *Biotechniques* **12,** 748-753

290. Clark, J.H. and Peck, E.J. (1978) Steroid Hormone Receptors: Basic Principles and Measurement In: Schradder, W.T. and O'Malley, B.W. (Eds) Lab. Method Manual for Hormone Action and *Molecular Endocrinology* **Chap 2,** p1-42.

291. Howell, G.M. et al. (1989) Identification of Dexamethasone Binding Sites on Male Rat Liver Plasma Membranes by Affinity Labelling. *Biolchemical J.* **260,** 435-441

292. Scatchard, G. (1949) The Attraction of Proteins for Small Molecules and Ions. *Annals of New York Academy of Sc.* **51,** 660

293. Rodbard, D. et al. (1980) Qualitative Characterization of Hormone Receptors. *Cancer* **46,** 2907-2918

294. Munson, D.J. and Rodbard, D. (1980) Ligand: A Versatile Computerized Approach for Characterization of Ligand Binding Systems. *Anal. Biochem.* **107,** 220-239

295. Pringle, J.R. et al. (1991) Immunofluorescence Method for Yeast. *Methods in Enzymology* **194,** 565-602

296. Polak, J.M. and Van Nordeen, S. (Eds) (1983) Immunocytochemistry: Practical Application in Pathology and Biology. John Wright PSG Littleton, Mass.

297. Hsu, S et al. (1992) Cell Cycle regulation of Glucocorticoid Receptor Function. *EMBO J.* **11,** 3457-3468

298. Mao.J. et al. (1992) Molecular Mechanism of RU486 Action: A Review. *Mol. and Cell. Biochemistry* **109,** 1-8

299. Bagchi, M.K. et al. (1988) Steroid Hormone Dependent Interaction of Human Progesterone Receptor with its Target Enhancer Element. *Mol. Endocrin.* **2,** 1221-1229

300. Nordeen, S.K. et al. (1993) Latent Agonist Activity of the Steroid Antagonist, RU486, is Unmasked in Cells Treated with Activators of PKA. *Mol. Endocrin.* **7,** 731-742

301. Guiochon-Mantel, G.A. et al. (1988) Receptors Bound to Antiprogestin Form Abortive Complexes With Hormone Response Elements. *Nature* **336,** 695-698

302. Johnson, J.A. et al. (1990) Identification of a Specific Domain in the β-Adrenergic Receptor Required for Phorbol Ester Induced Interaction of Catecholamine Stimulated Adenylate cyclase. *Mol. Pharm.* **38,** 289-293

303. Fleming, N. et al. (1992) Regulation of the cAMP Signal Transduction Pathways by PKC in Rat Submandibular Cells. *Eur. J. Physiol.* **421,** 82-89

304. Sibley, D. et al. (1984) Phorbol Esters Promote β-Adrenergic Receptor Phosphorylation and Adenylate Receptor Desensitization in Duck Erythrocytes. *Biochem. Biophys. Res. Commun.* **121,** 973-979

305. Yoshimasa, T. et al. (1987) Cross-Talk Between Cellular Signaling Pathways Suggested by Phorbol-Ester Induced Adenylate Cyclase Phosphorylation. *Nature* **327,** 67-70

306. Jakobs, K.H. et al. (1985) Modulation of Adenylate Cyclase of Human Platelets by Phorbol Esters. *Eur. J. of Biochem.* **151,** 425-430

307. Johnson, J.A. et al. (1986) Phorbol Ester Induced Augmentation and Inhibition of Epinephrine-Stimulated Adenylate Cyclase in S49

Lymphoma Cells. *J. of Cyclic Nucleotide Protein Phosphorylation Res.* **11,** 199-216.

308. Katada, J.A. et al. (1985) Protein Kinase C Phosphorylates the Inhibitory Guanine Nucleotide Binding Regulatory Component and Apparently Repress its Function in Normal Inhibition of Adenylate Cyclase. *Eur. J. of Biochem.* **151,** 431-437.

309. Clark, R.B. et al. (1993) Okadaic Acid Induces Both Augmentation and Inhibition of β_2-Adrenergic Stimulation of cAMP Accumulation in S49 Lymphoma Cells. *J. of Biol. Chem.* **268,** 3245-3250

310. Brandon, D.D et al. (1989) Glucocorticoid Resistance in Humans and Non-Human Primates. *Cancer Res.* **49,** 2203-2213

311. Anemri, E.S. et al. (1991) Overexpression and Characterization of the Human Mineralocorticoid Receptor. *J. of Biol. Chem.* **266,** 18072-18081

312. Cadepond, F. et al. (1992) Hormonal Regulation of Nuclear Localization Signals of the Human Glucocorticoid Receptor. *Experimental Cell Res.* **201,** 99-108

313. Schmidt-Zachmann, M.S. and Nigg, E.A. (1993) Protein Localization to the Nucleolus: A Search for Targetting Domains in Nucleolin. *J. of Cell Science* **105,** 799-806

314. Lasky, R. and Dingwall, C. (1993) Nuclear Shuttling: The Default Pathway of Nuclear Proteins. *Cell* **74,** 585-586

315. de Groot, P.R. et al. (1993) Multiple and Cooperative Events Regulate the CREM Activator Function. *EMBO J.* **12,** 3903-3911

316. Nichols, M. et al. (1992) Phosphorylation of CREB Affects its Binding to High and Low Affinity Sites: Implications for cAMP Induced Gene Transcription. *EMBO J.* **11,** 3337-3346

317. Glineur, C. et al. (1990) Phosphorylation of the v-Erb A Protein is Required for its Function as an Oncogene. *Genes and Dev.* **4,** 1663-1676

318. De Paoli-Roach, A.A. (1984) Synergistic Phosphorylation and Activation of ATP-Mg-Dependent Phosphoprotein Phosphatase by F_A/GSK-3 and Casein Kinase II ($PC_{0.7}$). *J. of Biol. Chem.* **259,** 12144-12152

319. Norbury, C. and Nurse, P. (1992) Animal Cycles and their Control. *Ann. Rev. Biochem.* **61,** 441-470

321. Takimoto, G.S. et al. (1992) Hormone Induced Progesterone Receptor Phosphorylation Consist of Sequential DNA-Independent and DNA-Dependent Stages: Analysis of the Zinc Finger Mutants and the Progesterone Receptor Antagonist ZK98299. *Proc. of Natl. Acad. of Sc.* **89,** 3050-3054

322. Hsieh, J.C. et al. (1991) Human Vitamin D Receptor is Selectively Phosphorylated by PKC on Serine 51, a Residue Crucial to its Transactivation Function. *Proc. of Natl. Acad. of Sc.* **88,** 9315-9323

323. Jehn, B. et al. (1992) Overexpression of *Mos, Ras, Src* and *Fos* Inhibits Mouse mammary Epithelial Cells Differentiation. *Mol. and Cell. Biol.* **12,** 3890-3902

324. Jaggi, R. et al. (1986) v-mos and H-ras Expression Represses Glucocorticoid Hormone Dependent Transcription from the Mouse Mammary Tumor Virus Promoter. *EMBO J.* **5,** 2609-2616

325. Philibert, D. et al. (1984) In Adrenal Steroid Antagonism. (Ed) Agarval, M.R. (de Groyer, Berlin NY) pp 77-101

326. Dalman, F.C. et al. (1989) Direct Evidence that the Glucocorticoid Receptor Binds to hsp90 at or near the Termination of Receptor Translation *in vitro. J. of Biol. Chem.* **264,** 19815-19821

327. Pratt, W.B. (1987) Transformation of Glucocorticoid and Progesterone Receptors to a DNA Binding State. *J. Cell. Biochem.* **35,** 51-68

328. Cadepond, F. et al. (1991) Heat Shock Protein 90 as a Critical Factor in Maintaining Glucocorticoid Receptor in a Nonfunctional State. *J. of Biol. Chem.* **266,** 5834-5841

329. Pratt, W.B. (1990) Interaction of Heat Shock Protein 90 with Steroid Receptors: Organising Some Diverse Observations and Presenting the Newest Concepts. *Mol. Cell. Endocrinol.* **74,** c69-c76

330. Scherrer, L.C. et al. (1992) Evidence that the Hormone Binding Domain of the Steroid Receptors Confers Hormonal Control on Chimeric Proteins by Determining their Hormone-regulated Binding to Heat Shock Protein 90. *Biochemistry* **31,** 7325-7329

331. Schowalter, D.B. et al. (1991) Characterization of Progesterone Receptor Binding to Heat Shock Protein 90 and 70 kDa Heat Shock Protein. *J. of Biol. Chem.* **266,** 21165-21173

332. Chambraud, B. et al. (1990) Several Regions of Human Estrogen Receptor are Involved in the Formation of Receptor Heat Shock Protein 90 Complexes. *J. of Biol. Chem.* **265,** 20686-20691

333. Picard, D. et al. (1988) A Movable and Regulable Inactivation Function within the Steroid Binding Domain of the Glucocorticoid Receptor. *Cell* **54,** 1073-1080

334. Czar, M.J. et al. (1994) Characterization of the Protein-Protein Interactions Determining the hsp (hsp90, hsp70 and sp56) Heterocomplex. *J. of Biol. Chem.* **269,** 11155-11161

335. Scherrer, L.C. et al. (1990) Structural and Functional Reconstitution of the Glucocorticoid Receptor hsp90 Complex. *J. of Biol. Chem.* **265,** 21397-21400

336. Hutchison, K. (1992) Monovalent Cation Selectivity for ATP-Dependent Association of the Glucocorticoid Receptor hsp70 and 90. *J. of Biol. Chem.* **267,** 14047-14053

337. Yem, A.W. et al. (1992) The hsp56 Component of Steroid Receptor Complexes Binds to Immobilized FK506 and Shows Homology to FKBP-12 and FKBP-13. *J. of Biol. Chem.* **267,** 2868-2871

338. Lebeau, M-C et al. (1992) P59, and hsp90-Binding Protein. *J. of Biol. Chem.* **267,** 4281-4284

339. Tai, P.K. et al. (1992) Association of a 59 kDa Immunophilin with the Glucocorticoid Receptor-Complex. *Science* **256,** 1315-1318

340. Schreiber, S.L. (1991) Chemistry and Biology of Immunophilins and their Immunosupressive Ligands. *Science* **251,** 283-287

341. Walsh, C.T. et al. (1992) Cyclosporin A, the Cyclophilin Class of Peptidylprolyl Isomerases, and Blockage of T-Cell Signal Transduction. *J. of Biol. Chem.* **267,** 13115-13118

342. Pratt, W.B. et al. (1992) *Trends Endocinol. Metab.* **3,** 326-333

343. Smith, D.F. and Toft, D.O. (1993) Steroid Receptors and Their Associated Proteins. *Mol. Endocrinol.* **7,** 4-11

344. Smith, D.F. et al. (1992) Assembly of Progesterone Receptor in Heat Shock Proteins and Receptor Activation are ATP-Mediated Events. *J. of Biol. Chem.* **267,** 1350-1356

345. Hutchison, K.A. et al. (1994) Proof that Heat Shock Protein 70 is Required for the Assembly of the GR into a Heterocomplex with Heat Shock protein 90. *J. of Biol. Chem.* **269,** 5043-5049

346. Sanchez, E.R., (1990) The 56-59 kDa Protein Identified in Untransformed Steroid Receptor Complexes is a Unique Protein that Exits in Cytosol in a Complex with Both the 70 and 90 kDa Protein. *Biochemistry* **29,** 5145-5152

347. Perdew, G.H. and Whitelaw, M.L. (1991) Evidence that the 90 kDa Heat Shock Protein Exists in Cytosol in Heteromeric Complexes Containing hsp70 and Three Other Proteins with Mr of 63, 56 and 50 kDa. *J. of Biol. Chem.* **266,** 6708-6713

348. Pratt, W.B. (1993) The role of Heat Shock Proteins in Regulating the Function, Folding and Trafficking of the Glucocorticoid Receptor. *J. of Biol. Chem.* **268,** 21455-21458

349. Baulieu, E.E. et al. (1989) Contragestion and Other Clinical Applications of RU486, an Antiprogestin at the Receptor. *Science* **245,** 1351-1357

350. Beck, C. A. et al. (1993) The Steroid Antagonist, RU486 Exerts Different Effects on the Glucocorticoid and Progesterone Receptors. *Endocrinology.* **133,** 728-740

351. Jung-Testas, I. (1983) Inhibition of Glucocorticoid Action in Cultured L-929 Mouse Fibroblasts by RU486, a New Anti-Glucocorticoid Receptor. *Experimental Cell Res.* **147,** 177-182

352. William, T. and Beato, M. (1986) Steroid-Free Glucocorticoid Receptor Binds Specifically to Mouse Mammary Tumor Virus-DNA. *Nature* **324,** 688-691

353. Rusconi, S. and Yamamoto, K.R. (1987) Functional Dissection of the Hormone and DNA Binding Activities of the GR. *EMBO J.* **6,** 1309-1315

354. Mullick, A. and Katzenellenbogen, B.S. (1986) Antiprogestin Receptor Complexes: Differences in the Interaction of Antiprogestin RU38486 and the Progestin R5020 with the PR of Human Breast Cancer Cells. *Biochem Biophys. Res. Comm.* **135,** 90-97

355. Demarzo, M.A. et al. (1992) Effects of the Steroid Antagonist, RU486, on Dimerization of the Human Progesterone Receptor. *Biochemistry* **31,** 10491-10501

356. El Ashry, D. et al. (1989) Human Progesterone Receptor Complexed with the Antagonist RU486 Binds to Hormone Response Elements in a Structurally Altered Form. *Mol. Endocrin.* **3,** 1545-1558

357. Guiochon-Mantel, A.G. et al. (1988) Receptors Bound to Antiprogestin Form Abortive Complexes to Hormone Response Elements. *Nature* **336,** 695-698

358. Meyer, M.E. et al. (1990) Agonist Antagonist Activities of RU486 on the Functions of Human Progesterone Receptor. *EMBO J.* **9,** 3923-3932

359. Kleine-Hitpass, L. et al. (1991) Two Types of Antiprogestins Identified by their Differential Action in Transcriptionally Active Extracts from T47D Cells. *Nucleic Acids Res.* **19,** 12227-12234

360. Rauch, M. et al. (1985) Mechanisms of Antiprogestin RU486 in the Rabbit Endometruim: Effects of RU486 on Progesterone Receptor and on the Expression of the Uteroglobin Gene. *Eur. J. Biochem.* **148,** 213-218

361. Sheridan, P.L. et al (1988) Human Progesterone Receptor Phosphorylation and Nuclear Down Regulation are Independent of Phosphorylation. *Mol. Endocrin.* **2,** 1329-1342

362. Baulieu, E.E. (1989) Contraception and other Clinical Applications of RU486, an Anti-Progestin of the Receptor. *Science* **245,** 1351-1357

363. Horwitz, K.B. (1992) The Molecular Biology of RU 486. Is there a Role For Antiestrogens in the Treatment of Breast Cancer. *Endocrinology Reviews* **13,** 146-163

364. Mao, J. et al. (1992) Molecular Mechanism of RU486 Action: A Review. *Mol. Cell. Biochem.* **109,** 1-8

365. Gronemeyer, H. et al. (1991) Progestin Receptors: Isoforms and Anti-Hormone Action. *J. of Steroid Biochem. and Mol. Biol.* **40,** 271-278

366. Allan, G.F et al. (1992) Hormone and Antihormone Induce Distinct Conformational Changes which are Central to Steroid Receptor Action. *J. of Biol. Chem.* **267,** 19513-19520

367. Kido, H. et al. (1986) Diacylglycerol Amplifies the Induction *In Vitro* of tyrosine Aminotransferase and ornithine Decarboxylase by Glucocorticoids. *Biochem. Biophys. Res. Comm.* **138,** 275- 282

368. Kido, H. et al. (1987) Inhibition by 1-(5-isoquinolinesulphonyl) 2-Methylpiperazine, an Inhibitor of PKC Enzyme Induction by Glucocorticoid and of Nuclear Translocation of GR Complexes. *Biochem. Biophys. Res. Comm.* **144,** 152-159

369. Denner, L.A et al. (1990) Regulation of Progesterone Receptor Mediated Transcription by phosphorylation. *Science* **250,** 1740-1743

370. Hidaka, H. et al. (1984) Isoquinoline-Sulfonamides, Novel Potent Inhibitors of Cyclic Nucleotide Dependent Protein Kinase and Protein Kinase C. *Biochemistry* **23,** 5036-5041

371. Kleine, L.P. and Whitefield, J.F. (1987) Serum Activated T51B Rat Liver Cells Transiently Accumulate cAMP-Dependent Protein Kinases on their Surfaces During G1. *J. of Cell. Physiol.* **132,** 354-358.

372. Klarlund, J.K. et al. (1985) Transformation of Cells by an Inhibitor of Phosphatases Acting on Phosphotyrosine in Proteins. *Cell* **41,** 707-717

373. Klarlund, et al. (1988) Numerous Proteins Phosphorylated on Tyrosine and Enhance Tyrosine Kinase Activities in Vanadate Treated NIH-3T3 Fibroblasts. *Biochimica et Biophysica Acta* **971,** 112-120

374. Brown, D.J. and Gordon, J.A. (1984) Stimulation of $pp60^{v\text{-}src}$ Kinase Activity by Vanadate in Intact Cells Accompanies a New phosphorylation State of the Enzyme. *J. of Biol. Chem.* **259,** 9580-9586

375. Swarup, G. et al. (1982) Phosphotyrosyl Protein Phosphatase of TCRC-2 Cells. *J. of Biol. Chem.* **257,** 7298-7301

376. Yonemoto, W. et al. (1987) Detection of Phosphotyrosine Containing Proteins in Polyoma-Virus Middle Tumor Antigen-Transformed Cells After Treatment with a phosphotyrosine Phosphatase inhibitor. *Mol. Cell. Biol.* **7,** 905-913

377. Aharoni, D. et al. (1993) Cross-Talk Between Adenylate cyclase Activation and Tyrosine Phosphorylation leads to modulation of the

Actin Cytoskeleton and to Acute Progesterone Secretion in Ovarian Granulosa Cells. *Endocrinology* **133,** 1426-1436

378. Smith, J.B. (1983) Vanadium Ions Stimulate DNA Synthesis in Swisss Mouse 3T3 and 3T6 Cells. *Proc. of Natl. Acad. of Sc.* **80,** 6162-6166

379. Carpenter, G. et al. (1981) Vanadate, Epidermal Growth Factor and the Stimulation of DNA Synthesis *Biochem Biophys. Res. Comm.* **102,** 1115-1121

380. Sanchez, E.R. et al. (1985) Evidence that the 90 kDa Phosphoprotein which Associates with Untransformed L-Cell GR is a Murine hsp. *J. of Biol. Chem.* **260,** 12398-12401

381. Sanchez, E.R. et al. (1987) Relationship of the 90 kDa Murine hsp with the Untransformed and Transformed State of the L-Cell Glucocorticoid Receptor. *J. of Biol. Chem.* **262,** 6986-6991

382. Sanchez, E.R. et al. (1986) The Molybdate Stabilized Glucocorticoid Binding Complex of L-Cells Contains 98-100 kDa Steroid Binding Phosphoprotein and a 90 kDa Non-Steroid Binding Phosphoprotein that is Part of the Murine heat-Shock Protein Complex. *J. of Steroid Biochemistry* **24,** 9-18

383. Dahmer, M.K. et al. (1984) Effects of Molybdate and Endogenous Inhibitors on Steroid Receptor Inactivation and Translocation. *Ann. Rev. Physiol.* **46,** 67-81

384. Raaka, B.M. et al. (1985) Effect of Molybdate on Steroid Receptors in Intact GH_1 Cells. *J. of Biol. Chem.* **260,** 14009-14015

385. Raaka, B.M. and Samuels, H.H. (1983) the GR in GH_1 Cells: Evidence from Dense Amino Acid Labelling and Whole Cell Studies for an Equilibrium Model Explaining the Influence of Hormone on the Intracellular Distribution of Receptor. *J. of Biol. Chem.* **258,** 417-425

386. Holbrook, N.J. et al. (1983) Characterization of Nonactivated and Activated GR-Complexes from Rat Liver Thymocytes. *J. of Biol. Chem.* **258,** 6477-6485

387. Wilson, E.M. et al. (1986) The Possible Role of Disulfide Bond Reduction in Transformation of 10S Androgen Receptor. *J. of Biol. Chem.* **261,** 6501-6508

388. Nishigori, H. and Toft, D.O. (1980) Inhibition of Progesterone Receptor Activation by sodium Molybdate. *Biochemistry* **19,** 77-83

389. Wheeler, R.H. et al. (1981) Pratt, W.B. (1987) Transformation of Glucocorticoid and Progesterone Receptors to the DNA Binding State. *J. of Biol. Chem.* **256,** 434-441

390. Meshinchi, S. et al. (1990) Elimination and Reconstitution of the Requirement for Hormone in Promoting Temperature Dependent Transformation of Cytosolic Glucocorticoid Receptor to the DNA-Binding Form. *J. of Biol. Chem.* **265,** 4863-4870

391. Meshinchi, S. et al. (1990) Selective Molybdate-Directed Covalent Modification of Sulfhydryl Groups in the Steroid Binding vs the DNA-Binding Domain of the Glucocorticoid Receptor. *J. of Biol. Chem.* **265,** 11643-11649

392. Pratt, W.B. et al. (1988) A region of the Steroid Binding Domain Determines Formation of the Non-DNA Binding 9S Glucocorticoid Receptor-Complex. *J. of Biol. Chem.* **263,** 267-273

393. Hollenberg, S.M. et al (1989) Identification of the Two Regions of the Human Glucocorticoid Receptor Hormone Binding Domain that Blocks Activation. *Cancer Res.* **49,** (Suppl) 22925-22945

394. Bresnick, E.H. et al. (1989) Evidence that the 90 kDa Heat Shock Protein is Necessary for the Steroid Binding Conformation of the L Cell Glucocorticoid Receptor. *J. of Biol. Chem.* **264,** 4992-4997

395. Sando, J.J. et al. (1979) Activation of Thymocytes Glucocorticoid Receptors to Steroid Binding Forms. *J. of Biol. Chem.* **254,** 4779-4789

396. Meshinchi, S. et al. (1988) Evidence that the Endogenous Heat Stable Glucocorticoid Receptor Stabilising Factor is a Metal Component of the Untransformed Receptor Complex. *J. of Biol. Chem.* **263,** 16809-16817

397. Leach, K.L. et al. (1982) Characterization of an Endogenous Glucocorticoid Receptor Stabilising Factor. *J. of Biol. Chem.* **257,** 381-388

398. Bailly, A. et al. (1977) A Low Molecular Weight Inhibitor of Steroid Receptor Action. *J. of Biol. Chem.* **252,** 858-863

399. Sato, B. et al. (1980) Mechanism of Activation of Steroid Receptors: Involvement of Low molecular Weight Inhibitor in the Activation of Androgen, Glucocorticoid, and estrogen Receptor Systems. *Endocrinology* **106,** 1142-1148

400. Meshinchi, S. et al (1991) Purification of the Endogenous Glucocorticoid Receptor Stabilising Factor. *Biochemistry* **30,** 8617-8622

401. Goidi, J.A. et al. (1977) Activation of Rat Liver GR Complex. *Biochemistry* **16,** 2125-2130

402. Bodine, P.V. and Litwark, G. (1988) Purification and Structural Analysis of the Modulator of the Glucocorticoid Receptor Complex. *J. of Biol. Chem.* **263,** 3501-3512.

403. Bodine, P.V. and Litwark, G. (1988) Evidence that the Modulator of the Glucocorticoid Receptor-Complex is the Endogenous Molybdate Factor. *Proc. of Natl. Acad. of Sc.* **85,** 1462-1466

404. Hutchison, K. et al. (1992) Reconstitution of the Multiprotein Complex of $pp60^{v\text{-}src}$, hsp90 and p50 in a Cell-Free System. *J. of Biol. Chem.* **267,** 2909-2908

405. Stancato, L.F. et al. (1993) Raf Exists in a Native Heterocomplex with hsp90 and p50 that can be Reconstituted in a Cell-Free System. *J. of Biol. Chem.* **268,** 21711-21716

406. Hutchison, et al. (1992) The Protein-Protein Complex Between $pp60^{v\text{-}src}$ and hsp90 is Stabilized by Molybdate, Vanadate and Tungstate , and an Endogenous Cytosolic Metal. *J. of Biol. Chem.* **267,** 13952-13957

407. Paneytou, G. and Waterfield, M. (1993) The Assembly of Signaling Complexes by Receptor Tyrosine Kinases. *Bioessays* **15,** 171-177

408. Wilks, A.F. (1993) Protein Tyrosine Kinase Growth Factor Receptors and their Ligands in Development, Differentiation and in Cancer. *Adv. Cancer Res.* **60,** 43-73

409. Bradshaw, R.A. and Prentis, S.T. (1987) Oncogenes and Growth Factors, Elsvier, Amsterdam

410. Cantley, L.C. et al. (1991) Oncogenes and Signal transduction. *Cell* **64,** 281-302

411. Aronica, S.M. and Katzenellenbogen, B.S. (1991) Progesterone Receptor Regulation in Uterine Cells: Stimulation by estradiol, cAMP and I-GF-1 and Suppression by Anti-Estrogen and Protein Kinase. *Endocrinology* **128,** 2045-2052

412. Yarden, Y. and Ullrich, A. (1988) Growth Factor Tyrosine Kinases. *Ann. Rev. Biochem.* **57,** 443-478

413. Ullrich, A. and Schlessinger, J. (1990) Signal Transduction by Receptors with Tyrosine Kinase Activity. *Cell* **61,** 203-212

414. Herrera, R. and Rosen, O.M. (1986) Autophosphorylation of the Insulin Receptor *In Vitro*. *J. of Biol. Chem.* **261,** 11980-11985

415. Cohen, S. et al. (1980) Epidermal Growth Factor-Protein kinase Interactions. *J. of Biol. Chem.* **255,** 4834-4842

416. Ushiro, H. and Cohen, S. (1980) Identification of Phosphotyrosine as a Product of EGF-Activated Protein Kinase in A431 cell Membranes. *J. of Biol. Chem.* **255,** 8363-8365

417. Pearson, T. and Gish, G.D. (1992) SH2 and SH3 Domains: From Structure to Function. *Cell* **71,** 359-362

418. Mayer, B.J. and Hanafusa, H. (1990) Association of the V-*crk* Oncogene Product with Phosphotyrosine Containing Proteins and Protein Kinase Activity. *Proc. of Natl. Acad. of Sc.* **87,** 2638-2642

419. Moran, M.F. et al. (1991) *Src* Homology Region 2 Domains Direct Protein-Protein Interaction in Signal transduction. *Proc. of Natl. Acad. of Sc.* **87,** 8622-8626

420. Vogel, U.S. et al. (1988) Cloning of Bovine GAP and its Interaction with the Oncogene *Ras* p^{21}*Nature* **335,** 90-93

421. Escobedo, J.A. et al. (1991) cDNA Cloning of a Nord 85 kDa Protein that has SH2 Domains and Regulates Binding of PI3-Kinase to the Platelet Derived Growth Factor β-Receptor. *Cell* **65,** 75-82

422. Skolnik, E.Y. et al. (1991) Cloning of PI3-Kinase-Associated p85 Utilising a Novel Method for Expression/Cloning of Target Proteins for Receptor Tyrosine Kinases. *Cell* **65,** 83-90

423. Otsu, M. et al. (1991) Characterization of Two 85 kd Proteins that Associate with Receptor Tyrosine Kinases. *Cell* **65,** 91-94

424. Stahl, M.L et al. (1988) Sequence Similarities of Phospholipase C with the Non-Catalytic Region of *src*. *Nature* **332,** 269-272

425. Suh, P.-G et al (1988) Inositol Phospholipid-Specific Phospholipase C: Complete cDNA and Protein Sequences and Sequence Homology to Tyrosine Kinase-Related Oncogene Products. *Proc. of Natl. Acad. of Sc.* **85,** 5419-5423

426. Molley, C.J. et al. (1989) PDGF Induction of Tyrosine Phosphorylation of GTPase Activating Protein. *Nature* **342,** 711-713

427. Kaplan, D.R. et al. (1990) Platelet Derived Growth Factor β-Receptor Stimulates Tyrosine Phosphorylation of GAP and Association of GAP with a Signaling Complex. *Cell* **61,** 125-133

428. Lui, XQ and Pawson, T. (1991) The EGF Phosphorylates GTPase Activating Protein (GAP) at Tyr460, Adjacent to the GAP SH2 Domains. *Mol. Cell. Biol.* **11,** 2511-2576

429. Trahey, M. and McCormic, F. (1987) A Cytoplasmic Protein Stimulates Normal N-Ras p21 GTPase, but does not Affect Oncogenic Mutants. *Science* **238,** 542-545

430. Bjorge, J.D et al. (1990) Activated Type I Phophatidylinositol Kinase is Associated with Epidermal Growth Factor Receptor Following EGF Stimulation. *Proc. of Natl. Acad. of Sc.* **87,** 3816-3820

431. Wahl, M.I. et al. (1989) Epidermal Growth Factor Induces Tyrosine Phosphorylation of PLC-II Independently of Receptor Internalization and Extracellular Calcium. *Proc. of Natl. Acad. of Sc.* **86,** 1568-1572

432. Margolis, B. et al. (1989) Epidermal Growth Factor (EGF) Induces Tyrosine Phosphorylation of PLC-II: A Potential Mechanism for EGF Signaling. *Cell* **57,** 1101-1107

433. Meisenhelder, J. et al (1989) Phospholipase C-γ is a Substrate for the Platelet Derived Growth Factor and EGF Receptor Protein Tyrosine Kinases *In Vivo* and *In Vitro*. *Cell* **57,** 1109-1122

434. Ball, R.L. et al. (1990) Epidermal Growth Factor Potentiates cAMP Accumulation in A431 Cells. *J. of Biol. Chem.* **265,** 12836-12845

435. Bushman, W.A. et al. (1990) Overexpression of *c-src* Enhances β-Adrenergic-Induced cAMP Accummulation. *Proc. of Natl. Acad. of Sc.* **87,** 7462-7466

436. Emmapaola, S. et al. (1988) Kinetics and Regulation of Tyrosine Phosphosrylation of the Epidermal Growth Factor Receptor in Intact A431 Cells. *Mol. Cell. Biol.* 1345-1351

437. Schlessinger, J. et al. (1988) Epidermal Growth Factor as a Multifunctional Allosteric Protein. *Biochemistry* **27,** 3119-3123

438. Pyne, N.J. et al. (1989) Treatment of Intact Hepatocytes with Either the Phorbol Ester TPA or Glucagon Elicits the Phosphorylation and Functional Inactivation of the Inhibitory Guanine Nucleotide Regulatory Protein G_i. *FEBS Lett.* **243,** 77-82

439. Carlson, K.E. et al. (1989) Thrombin and Phorbol Esters Cause the Selective Phosphorylation of a G-protein in Human platelets. *J. of Biol. Chem.* **264,** 13298-13305

440. Houslay, M.D. (1991) Cross-Talk: A Pivotal Role for PKC in Modulating Relationships Between Signal Transduction Pathways. *Eur. J. Biochem.* **195,** 9-27

441. Nitsizuka, Y. et al. (1984) The Role of PKC in Cell Surface Signal Transduction and Tumor Promotion. *Nature* **308,** 693-698

442. Berridge, M. (1987) Inositol Triphosphate and Diacylglycerol: Two Interacting Second Messengers. *Ann. Rev. Biochem.* **56,** 159-193

443. Gilman, A.G (1984) G-Proteins and Dual Control of Adenylate Cyclase. *Cell* **36,** 577-579

444. Gilman, A.G (1987) G-Proteins: Transducers of Receptor Generated Signals. *Ann. Rev. Biochem.* **56,** 615-649

445. Birnbaumer, L. et al. (1990) Receptor-Effector Coupling by G-Proteins. *Biochem. et Biophysica Acta* **1031,** 163-224

446. Majerus, P.W. et al. (1988) Inositol Phosphates: Synthesis and Degradation. *J. of Biol. Chem.* **263,** 3051-3054

447. Berridge, M. (1984) Inositol Triphosphate and Diacylglycerol as Second Messengers. *Biochem. J.* **220,** 345-360

448. Berridge, M.J. and Irvine, R.F. (1984) Inositol Triphosphate, a Novel Second Messenger in Cellular Signal Transduction. Nature **312,** 315-321

449. Nitsizuka, Y. et al. (1989) The Family of Protein Kinase C for Signal Transduction. *JAMA* **262,** 1826-1833

450. Irvine, R.F. et al. (1989) How do Inositol 1,4,5-Trisphosphate and Inositol 1,3,4,5-Tetrakisphosphate Regulate Intracellular Ca^{2+} *Biochem. Society Transactions* **17,** 6-9

451. Bourne, H.R. et al. (1990) GTP-ase Superfamily: A Conserved Switch for for Diverse Cell Functions. *Nature* **348,** 125-132

452. Helper, J.R. and Gilman, A.G. (1992) G-Proteins. *Trends in Biochem Sc.* **17,** 383-387

453. Taylor, S.J. et al. (1990) Purification From Bovine Liver Membranes of Guanine Nucleotide-Dependent Activation of Phosphoinositide-Specific Phospholipase-C. *J. of Biol. Chem.* **265,** 17150-17156

454. Williamson, J.R. and Monck, J.R. (1989) Hormone Effect on Cellular Ca^{2+} Fluxes. *Ann Rev. of Physiol.* **51,** 107-124

455. Volpe, P. et al. (1988) "Calciosome" a Cytoplasmic Organelle: The Inositol 1,4,5-Triphosphate Sensitive Ca^{2+} Store of Non-Muscle Cells. *Proc. of Natl. Acad. of Sc.* **85,** 1091-1095

456. Seamon, K.B. and Daly, J.W. (1991) Forskolin: Its Biological and Chemical Properties. *Cyclic Nucleotide and Protein Phosphorylation Res.* **20,** 1-150

457. Iizuka, H. et al. (1989) Effects of the Tumor Promoter, Phorbol-12-Myristate-13-Acetate on the Epidermal Adenylate Cyclase System: Evidence for Adenylate Cyclase Regulation by PKC. *J. Invest. Dermatol.* **93,** 387-391

458. Johnson, J.A. et al. (1990) PKC Activators Sensitize cAMP Accumulation in Intact 132IN1 Human Astrocytoma Cells. *Mol. Pharmacol.* **37,** 296-303

459. Hui, K.K. and Yu, Y.L. (1989) Effects of Protein Kinase Inhibitor, 1-(5-isoquinolinylsulphonyl)-2-Methylpiperazine, on β-2-Adrenergic Receptor Activation and Desensitization in Intact Human Lymphocytes. *J. Pharmacol. Experimental Therapeutics* **249**, 492-498

460. Shoyab, M. et al. (1979) Biological Active Phorbol Esters Specifically Alters the Affinity of EGF membrane Receptors. *Nature* **279,** 387-391

461. Cohet, C et al. (1984) C-Kinase Phosphorylates the Epidermal Growth Factor Receptor and Reduces its EGF-Stimulated Tyrosine Protein Kinase Activity. *J. of Biol. Chem.* **259,** 2553-2558

462. Mechta, F. et al. (1989) Stimulation of PKC or PKA Mediated Signal Transduction Pathways Show Three Modes of Response Among Serum Inducible Genes. *New Biologist* **1,** 297-304

466. Hoeck, W. et al. (1992) Overexpression of the Glucocorticoid Receptor Represses Transcription from Hormone Responsive and Non-Responsive Promoters. *J. of Steroid Biochem. and Mol. Biol.* **41,** 283-289

467. Burnstein, K.L. et al. (1990) Human Glucocorticoid Receptor cDNA Contains Sequences Sufficient for Receptor Down Regulation. *J. of Biol. Chem.* **265,** 7284-7291

468. Bellingham, D.L. et al. (1992) Ligand Dependent Down Regulation of Stably Transfected Human GRs is Associated with Human Functional Glucocorticoid Responsiveness. *J. of Biol. Chem.* **266,** 10078-10085

469. Lohse, M.J. et al. (1993) Molecular Mechanisms of Membrane Receptor Desensitization. *Biochimica et Biophysica Acta* **1179,** 171-188

470. Bouvier, M. et al (1989) Two Distinct Pathways for cAMP-mediated Down-Regulation of the β-Adrenergic Receptor. *J. of Biol. Chem.* **264,** 16786-16792

471. Shear, M. et al. (1976) Agonist-Specific Refractoryness Induced by Isoproterenol. *J. of Biol. Chem.* **251,** 7572-7576

472. Su, Y.F. et al. (1980) Catecholamine Specific Desensitization of Adenylate Cyclase. *J. of Biol. Chem.* **255,** 7410-7419

473. Allen, J.M. (1989) β-2-Adrenergic Receptor Regulation after Transfection into a Cell Line Deficient in the cAMP-Dependent Protein Kinase. *Mol. Pharmcol.* **36,** 248-255

474. Leach, K.L. and Raben, D.M. (1993) Nuclear Localization of PKC. *Biochem. Soc. Transactions* **21,** 879-887

475. Brewis, N.D. et al. (1993) PPX, a Novel Protein Ser/Thr Phosphatase Located to Centrosomes. *EMBO J.* **12,** 987-996

476. Leach, K.L. et al. (1989) Type 3 PKC Localization to the Nuclear Envelope of Phorbol Ester-Treated NIH 3T3 Cells. *J. Cell Biol.* 109, 685-695

477. Niedel, J.E. and Blachshear, P.J. (1986) Protein Kinase C In : Phosphoinositide and Receptor Mechanisms. J.W. Putney, Jr (Ed)

478. Irvine, R.F. and Devecha, N. (1992) Phospholipids in the Nucleus: Metabolism and Possible Functions. *Semin. Cell Biol.* **3,** 225

479. Devecha, N. et al. (1991) The Phosphoinoside Cycle Exists in the Nuclei of Swiss 3T3 Cells Under the Control of a Receptor (for EGF-1) in the Plasma Membrane, and Stimulation of the Cycle Increases Nuclear Diacylglycerol and Apparently Induces Translocation of PKC to the Nucleus. *EMBO J.* **10,** 3207

480. Cocco, L. et al. (1989) Changes in Nuclear Inositol Phospholipids Induced in Intact Cells by Insulin-Like Growth Factor-1. *Biochem. Biophys. Res. Comm.* **159,** 720

481. Martelli, A.M et al. (1992) Nuclear Localization and Signaling Activity of Phosphoinositidase Cβ in Swiss 3T3 Cells. *Nature* **358,** 242

482. Rubins, J.R. et al. (1990) GTP-Binding Proteins in Rat Liver Nuclear Envelopes. *Proc. of Natl. Acad. of Sc.* **87,** 7080

483. Seydel, U and Gerace, L. (1992) A 28 kDa GDP/GTP-Binding Proteins in Rat Liver Nuclear Envelopes. *J. of Biol. Chem.* **266,** 7602

484. Jaken, S.K. et al. (1987) Purification and Characterization of 3-Types of PKC from Rabbit Brain Cytosol. *Proc. of Natl. Acad. of Sc.* **84,** 4418-4422

485. Nishizuka, (1988) The Molecular Heterogeneity of PKC: Its Implications in Cellular Regulation. *Nature* **334,** 661-666

486. Kemp, B.E. and Pearson, R.B. (1990) Protein Kinase Sequence Recognition Motifs. *Trends in Biochem. Sc.* **15,** 342-346

487. Cavanaugh, A.H. and Simmons Jr., S.S. (1990) Glucocorticoid Receptor Binding to Calf Thymus DNA: Identification and Characterization of a Macromolecular Factor Involved in Receptor-Steroid Complex Binding to DNA. *Biochemistry* **29,** 989-996

488. Hargrave, P.A. and McDowell, J.H. (1992) Rhodopson and Phototransduction: A Model System for G-Protein Linked Receptors. *FASEB J.* **6,** 2323-2331

489. Khorana, H.G. (1992) Rhodopson, Photoreceptor of the Cell. *J. of Biol. Chem.* **267,** 1-4

490. Dohlman, H.G. et al. (1991) Model Systems for the Study of 7-Transmembrane-Segment Receptors. *Ann. Rev. Biochem.* **60,** 653-658

491. Wiley, H.S. et al. (1989) Global Modulation of the Epidermal Growth Factor Receptor is Triggered by Occupancy of only a Few Receptors. *J. of Biol. Chem.* **264,** 18912-18920

492. Miller, M. et al. (1991) Nuclear Pore Complex: Structure, Function and Regulation. *Physiol. Rev.* **71,** 909-949

493. Zhao, L.J. (1988) Nuclear Transport of Adenovirus DNA Polymerase is Facilitated by Interaction with Preterminal Protein. *Cell* **55,** 1005-1015

494. Mattaj, J.W. (1988) UsnRNp Assembly and Transport In: Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Mirnstiel, M.L. (Eds) Springer-Verlag, NY 100-114

495. Berridge, M.J. (1990) Calcium Oscillations. *J. of Biol. Chem.* **265,** 9583-9586

496. Kruffer, W. (1984) Down Regulation of Glucocorticoid Receptor After Hormone Withdrawal. *Nature* **312,** 711-716

497. Weigel, M.L. (1992) Chicken Progesterone Receptor is Phosphorylated by DNA-Protein Kinase During In Vitro Transcription Assays. *Mol. Endocrin.* **6,** 8-14

498. Truss, M. et al. (1992) Interplay of Steroid Hormone Receptors and Transcription Factors on MMTV Promoter. *Journal of Steroid Biochem and Mol. Biol.* **43,** 365-378

499. Rosini, G.R. (1984) RNA containing Nuclear Binding Sites for GR Complexes. *Biochemical and Biophysical Res. Comm.* **123,** 78-83

500. Mason, S.A. and Houslay, P.R. (1987) Site-Directed Mutagenesis of the Phosphorylation Site in the Mouse Glucocorticoid Receptor. *J. of Biol. Chem.* **268,** 21501-21504

501. Krebs, E.G. and Beavo, J.A. (1979) Phosphorylation-Dephosphorylation of Enzymes. *Ann. Rev. of Biochem.* **48,** 923-959

502. Daniel, V.G. et al. (1973) Induction of Cytolysis in Cultured Lymphoma Cells by cAMP and the Isolation of Resistant Variants. *Proc. of Natl. Acad. of Sc.* **70,** 76-79

501. Krebs, E.G. and Beavo, J.A. (1979) Phosphorylation-Dephosphorylation of Enzymes. *Ann. Rev. of Biochem.* **48,** 923-959

502. Daniel, V.G. et al. (1973) Induction of Cytolysis in Cultured Lymphoma Cells by cAMP and the Isolation of Resistant Variants. *Proc. of Natl. Acad. of Sc.* **70,** 76-79

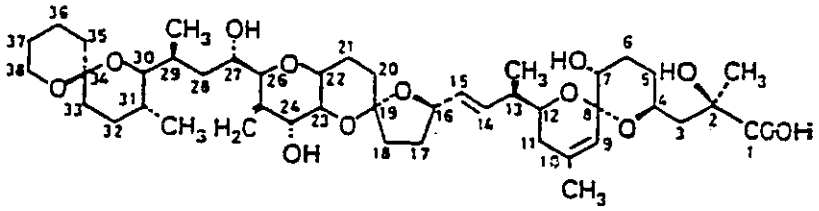
503. Gruol, D.J. and Dalton, D.K. (1984) Phenothiazines Cause a Shift in the cAMP Dose-Response: Selection of Resistant Variants in a Murine Thymoma Line. J. Cell. Physiol. **119,** 107-118

504. Vedeckies, W.V and Bradshaw, H.D. Jr. (1983) DNA Fragmentaion in S49 Lymphoma Cells Killed with Glucocorticoids and other Agents. *Mol. and Cell. Endocrinol.* **30,** 215-227

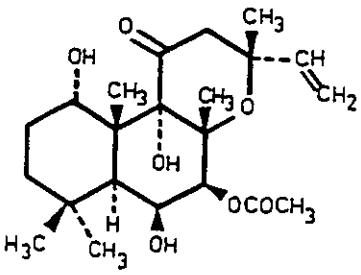
505. Friedrich, U. and Coffino, P. (1977) Mutagenesis in S49 Mouse Lymphoma Cells: Induction of Resistance to Ouabain, 6-Thioguanine and Dibutyryl cAMP. *Proc. of Natl. Acad. of Sc.* **74,** 679-683

506. Lamaire, I. and Coffino, P. (1977) cAMP-Induced Cytolysis in S49 Cells. *Cell* **11,** 149-155

STRUCTURES OF COMPOUNDS

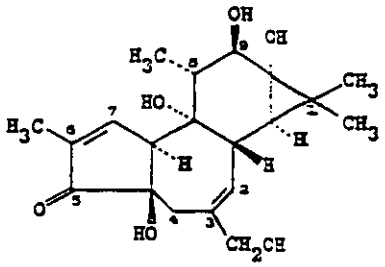


OKADAIC ACID (OA)
polyether C_{38} fatty acid



FORSKOLIN

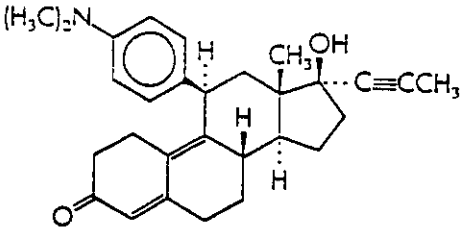
7β-acetoxy-8,13-epoxy-1α, 6β,9α-trihydroxyllabd-14 -en-11-one



TPA

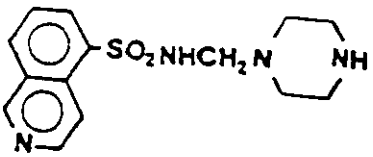
12-O-tetradecanoylphorbol -13 acetate

STRUCTURES OF COMPOUNDS



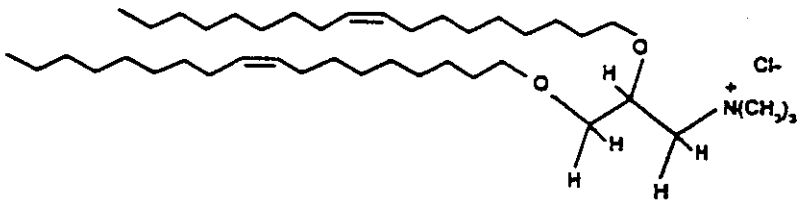
RU486 (MIFEPRISTONE)

17β-hydroxy-11β-(4-dimethyl aminophenyl)17α-1-propynyl-estra-4,9-dien-3-one



H-7

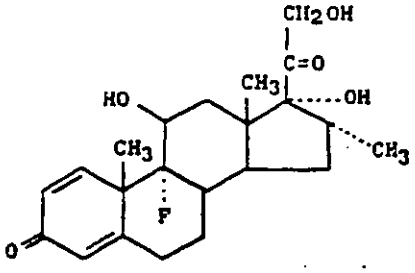
1-(5-Isoquinolinylsulphonyl)-2 -methylpiperazine



LIPOFECTIN (DOTMA)

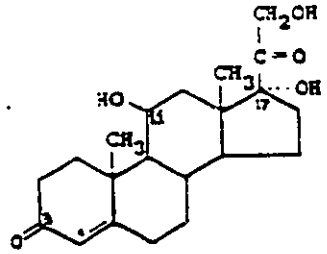
(N [1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride)

STRUCTURES OF COMPOUNDS



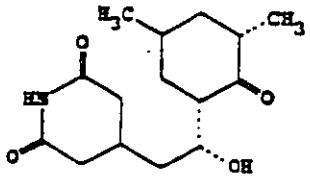
DEXAMETHASONE

9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione



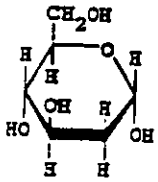
CORTISOL

4-Pregnene-11β,17α,21-triol 3,20-dione



CYCLOHEXIMIDE

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione



2-DEOXYGLUCOSE

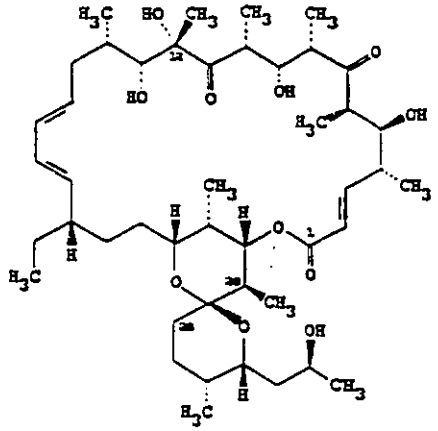
2-Deoxy-D-arabino-hexose

STRUCTURES OF COMPOUNDS

NaO_3V

SODIUM VANADATE

Sodium metavanadate



OLIGOMYCIN A

OLIGOMYCIN B (Oxooligomycin)

OLIGOMYCIN C (12-Deoxyoligomycin))