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NUCLEAR IMPORT OF THE GLUCOCORTICOID RECEPTOR: FROM PLASMA MEMBRANE GLUCOCORTICOID BINDING SITES TO NUCLEAR LOCALIZATION SIGNAL BINDING PROTEINS AND IMPORT MODELS

by

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ABSTRACT

Relatively little is known about the molecular mechanisms involved in the import of proteins into the nucleus. This lack of knowledge extends to the mode of nuclear entry of steroid-receptor complexes and modulation of steroid hormone action during its passage across the nuclear envelope. The main goal of this thesis was to identify the molecular machinery that interacts with the nuclear localization signals located in steroid hormone receptors. Synthetic peptides corresponding to the glucocorticoid and thyroid hormone receptor nuclear localization signals were radioiodinated and incubated with cytosol, high salt- and detergent-extracted rat liver nuclei or nuclear envelopes in the presence of a crosslinking agent. Two specifically labeled polypeptides of 60 and 76 kDa were identified with both synthetic peptides in all fractions by autoradiography after SDS-PAGE. The two general conclusions drawn from my data are: first, that these two specifically labeled polypeptides may act as shuttling vectors in nuclear transport; and second, the binding sites may be part of a general mechanism for nuclear entry of most nuclear proteins. Nuclear import of proteins is comprised of two steps, the first step being the binding of the protein to the outer nuclear envelope and nuclear pore complex, and the second step being the translocation of the protein through the nuclear pore complex into the nucleus. I established an *in vitro* system, which consists of isolated rat liver nuclei, capable of the first step but not the second step, to study glucocorticoid receptor nuclear binding. I have also demonstrated that digitonin-permeabilized cells can be used to study both steps (binding and import) of the glucocorticoid receptor translocation into nuclei in an ATP-dependent manner similar to other nuclear proteins. These *in vitro* models will allow us to further define the mechanisms involved in the nuclear import of the glucocorticoid

receptor, particularly the hormone-induced nuclear translocation of the receptor, and to establish the role of the 60- and 76-kDa polypeptides in nuclear import.

The ligand-induced nuclear translocation of the glucocorticoid receptor is dependent on the intracellular hormone concentration which is influenced by membrane binding sites for the glucocorticoid ligand. With the affinity label, dexamethasone 21-mesylate, I have identified a 45-kDa microsomal membrane site that displays the characteristics of a low affinity glucocorticoid binder. This protein may limit the amount of free hormone for the receptor and therefore blunt the action of the steroid hormone in the nucleus.

This thesis is dedicated to my parents, Margaret and Yoland, for their love and support, and to Heather for making my life complete. A special dedication to my grandfather, F. Norman Hughes, is made for his teachings which instilled in me scientific curiosity and the need for truths in our lives.

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GENERAL INTRODUCTION

Preface to Parts I and II

The subject of this thesis is glucocorticoid hormone action, that is, the cellular actions of glucocorticoids and the intracellular consequences of their action. Unlike many hormones which bind to plasma membrane receptors, steroid hormones including glucocorticoids must traverse the cellular plasma membrane to bind to their intracellular receptors. The glucocorticoid-receptor complex undergoes a transformation process whereby it acquires the ability to translocate to the nucleus, and bind DNA to regulate gene expression. The major focus of this thesis is nuclear import of the cytoplasmic glucocorticoid receptor which is described in Part I. However, nuclear translocation of the glucocorticoid receptor is dependent on ligand binding [1]. Therefore, nuclear import of the glucocorticoid receptor is dependent on the bioavailability (intracellular free concentration) of hormone. The bioavailability of intracellular ligand is dependent on the diffusion or transport of the hormone across the plasma membrane, competition between the receptor and other intracellular ligand-binding proteins, and the inactivation of the ligand by metabolic enzymes. Hence, plasma membrane and microsomal ligand-binding sites may regulate the intracellular ligand concentration and affect the nuclear translocation of the glucocorticoid receptor. Thus, a second minor focus described in Part II of this thesis describes the identification of microsomal dexamethasone-binding sites.

PART I: Nuclear Import of the Glucocorticoid Receptor

I. INTRODUCTION

1. Steroid Hormone Receptors and the Nuclear Receptor Superfamily

During the period that started with the discovery of testosterone in 1889 and ended with the purification of 1,25-dihydroxyvitamin D_3 (vitamin D_3=cholecalciferol=calcitriol) in 1971, the classical steroid hormone molecules (androgens, estrogens, progestins, mineralocorticoids and glucocorticoids) and the secosteroids (vitamin D) were chemically characterized [2]. During this period, the physiological actions of steroid hormones and their biosynthetic and metabolic pathways became fairly well understood. In 1960, the observation that the insect molting steroid hormone, ecdysone, induced puffing in *Drosophila* larval salivary gland polytene chromosomes suggested that steroid hormones have an effect on gene expression in the cell nucleus (see [3]). In 1962 using newly available radiolabeled estradiol, an intracellular steroid receptor was discovered by Jensen and co-workers [4]. This receptor was thought to mediate the effects of the hormone in the cell nucleus. In 1963, Litwack observed the cytoplasmic to nuclear translocation of [^{14}C]-cortisol, a labeled glucocorticoid hormone [5].

A model based on the estrogen receptor, which was called the two-step model, was proposed to account for the action of all steroid hormones [6, 7]. The general mechanism of action of steroid hormones remains largely unchanged today [8]. The first step in this model is the binding of steroid after its entry into the cell to its cytoplasmic receptor followed by the second step in which the receptor-steroid complex translocates to the nucleus and binds to DNA. In the early days of steroid hormone receptor research two of the most studied questions were the intracellular localization of these receptors and the regulation of their intracellular

concentration. These studies continue today and controversy still surrounds the intracellular localization of unliganded receptors. Steroid receptors are transcription factors which ultimately act in the cell nucleus. They bind in the enhancer-promoter regions of hormone responsive genes to enhancer elements called hormone response elements (reviewed in [9-11]). Hormone response elements are conserved nucleotide sequences that form perfect or imperfect palindromes, inverted or direct repeats with specific spacings separating the repeats. Steroid receptors bind to these elements as dimers, each receptor binding to a repeat or half-site of six base pairs. Steroid receptors are thought to enhance or repress gene transcription by direct protein-protein interactions with other transcription factors, co-activators or adapters, which bridge the distance between the receptor and the basal transcription apparatus [12-15]. It is also thought that the steroid receptors alter chromatin structure thus modifying access to other transcription factors [11, 16-18].

Much progress in steroid receptor research was made with the development of a covalent affinity label, dexamethasone 21-mesylate, [16] which facilitated glucocorticoid receptor (GR) purification. This led to the generation of specific antibodies against this receptor [17] which in turn led in 1984 to the cloning of the first steroid receptor, the GR [18]. Subsequent to the cloning of the rat GR has come an avalanche of information, confirming earlier protein studies on the modular structure of GR [19-26]. These early studies identified in the receptor an N-terminal domain to which antibody bound, a central DNA-binding region and a C-terminal ligand-binding domain separated by a hinge region. The deduced cDNA amino acid sequence revealed a two 'zinc-finger' DNA-binding region of the C4-type similar but not identical to the C2H2-type 'zinc-fingers' of the *Xenopus* TFIIIA transcription factor [27, 28]. The ligand-binding region was very hydrophobic which was expected because of the hydrophobic nature of the steroidal ligands. Following the cloning of

GR came the cloning of all other steroid receptors either by similarity of their DNA-binding domains or by use of receptor-specific antibodies. A pattern of the modular structure for all steroid receptors emerged. The N-terminal domain is the least conserved and varies greatly in size [20]. The central DNA-binding domain is a highly conserved, roughly sixty-six amino acid domain, with twenty invariant amino acids including the eight zinc-coordinating cysteinyl residues. A lesser conserved large ligand-binding domain is at the C-terminus. The twenty invariant amino acids in the DNA-binding region are characteristic of steroid/nuclear receptors and differentiate these transcription factors from other 'zinc-finger' transcription factors. Another nomenclature often used for steroid receptor domains is the following; the N-terminal domain is called the A/B domain, the DNA-binding domain is called C and the C-terminal ligand-binding domain is designated E/F. The C and E/F domains are separated by the D hinge region. This domain nomenclature was based on sequence comparisons between the chick and human estrogen receptors which revealed these phylogenetically conserved regions [29].

Using the highly conserved steroid receptor DNA-binding region as a probe, two independent groups, to their surprise cloned the cellular counterpart to a viral oncogene, v-*erbA* [30, 31]. Moreover, they discovered that the ligand for the c-*erbA* receptor was thyroid hormone. This important discovery linked a non-steroidal ligand receptor with the family of steroid receptors. This extended family is now called the nuclear receptor superfamily. Thyroid hormone and steroid hormone are both small hydrophobic molecules and have similar modes of action because of the similarity of their receptors. The discovery of c-*erbA* also linked a known oncogene (v-*erbA*) with this class of transcription factors, which allowed for the possibility that other steroid receptors may have transforming activity. The expansion of the nuclear receptor superfamily continued, as the receptor for the secosteroid vitamin D_3 [32] and the receptor for the teratogenic morphogen, retinoic acid, were also

found to belong to this superfamily [33-36]. A similar protein to the retinoic acid receptor was cloned and called the retinoid-X receptor [37]. It had been previously cloned and called H-2RIIBP [38] because it bound the H-2 region II of a major histocompatibility class I gene. Unlike the retinoic acid receptor, the retinoid-X receptor did not bind all-*trans*-retinoic acid, the principal active metabolite of retinol (vitamin A). The retinoid-X receptor was called an orphan receptor, as were many other 'receptors' or transcription factors cloned in this way which had no known ligand [39, 40]. In 1992, a ligand, 9-*cis*-retinoic acid, was found for the retinoid-X receptor [41, 42]. Nine-*cis*-retinoic acid is also a metabolite of retinol and is the first new hormone to be discovered in many years [43]. Some of the orphan receptors may function without ligands but are nonetheless important transcription factors [39, 40].

A significant finding with the retinoid-X receptor was that it heterodimerized with certain members of the nuclear receptor superfamily [44-47]. While the classical steroid receptors bind their cognate hormone response elements as homodimers [9], the retinoid-X receptor preferentially heterodimerizes with the retinoic acid receptor, the thyroid hormone receptor, the vitamin D receptor, the peroxisome proliferator-activated receptor and the orphan receptor, COUP-TF1 (also known as ear3 [52, 53]) [48] . Some orphan receptors, such as NGFI-B (also known as *nur77*) and FTZ-F1, bind DNA as monomers [49]. The *Drosophila* homologue to the retinoid-X receptor, known as *Ultraspiracle* (*usp*), heterodimerizes with the ecdysone receptor, and *usp* is required for ecdysone mediated gene transcription [50, 51].

Evolutionary studies show that nuclear receptors share a common ancestor, and that the receptors can be divided into three subfamilies based on similarities of their amino acid sequences [52] (see Table 1). This classification agrees well with a previous classification based on nuclear receptor DNA-binding specificity which is dependent on the P box [53], a conserved sequence at the base of the first 'zinc-

Table 1. Nuclear Receptor Classification

A. Classification based on phylogeny of DNA-binding domain sequences (Laudet *et al.*, 1992, [52])

Subfamily	Group	Examples
I	TR	TRα, TRβ
	RAR	RARα, RARβ, RARγ
	ear1	ear1 (REV-*erbA*), E75, PPAR
II	COUP	COUP-TF1 (ear3), ARP-1 (COUP-TF2), *svp*, ear2, TR2
	RXR	RXR, H-2RIIBP, *usp*, NGFI-B (*nur77*)
	HNF-4/*tll*	HNF-4, *tll*
III	ER	ER, ERR1, ERR2
	GR	GR, PR, MR, AR
	FTZ-F1	FTZ-F1
	KNi/VDR	KNi, Knrl, *egon*, VDR, EcR

B. Classification based on DNA-binding specificity (Forman and Samuels, 1990, [53])

Class	P box*	Examples
I	cGSckV	AR, GR, MR, PR
II	cEGckG	E75, ear1 (REV-*erbA*), EcR, RXR, H-2RIIBP, *usp*, TR2, VDR, NGFI-B (*nur77*), PPAR, RARα, RARβ, RARγ, TRα, TRβ
III	cEGckS	ARP-1 (COUP-TF2), COUP-TF1 (ear3), ear2, *egon*, KNi, Knrl, *svp*, v-*erbA*
IV	cEGckA	ER, ERR1, ERR2

*P box amino acid residues are shown; conserved residues are shown in lower case.
Abbreviations: TR, thyroid hormone receptor; RAR, retinoic acid receptor; ear, *erbA* related; E75, *Drosophila* orphan receptor from 75B ecdysone-induced chromosomal puff; PPAR, peroxisome proliferator-activated receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; ARP, ApoA1 regulatory protein; *svp, seven-up*; TR2, orphan receptor from testis; RXR, retinoid-X receptor; H-2RIIBP, H-2 region II binding protein; *usp, ultraspiracle* (=2C1-3=CF1=XR2C); NGFI-B, nerve growth factor inducible factor B (=*nur77*=N10=TR3=TIS1=NAK1=ST-59); HNF-4, hepatocyte nuclear factor 4; *tll, tailless*; ER, estrogen receptor; ERR, estrogen receptor-related; GR, glucocorticoid receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; AR, androgen receptor; FTZ-F1, *fushi tarazu*-factor 1=SF-1 (steroidogenic factor 1)=ELP (embryonal long terminal repeat binding protein); KNi, *knirps*; Knrl, *knirps*-related; *egon*, embryonic gonad gene product; VDR, vitamin D receptor; EcR, ecdysone receptor.

finger'. P box amino acids make nucleotide base specific contacts and discriminates between the various hormone response elements by virtue of their binding affinity [54-57]. This was demonstrated in experiments for which residues of GR were changed for residues of the estrogen receptor that changed the DNA recognition from a GRE (glucocorticoid-response element) to an ERE (estrogen-response element) [58]. The protein-DNA contacts have been solved for the GR DNA-binding domain by X-ray diffraction studies of crystals [59, 60] and by solution nuclear magnetic resonance studies [61-63]. Amero *et al.* [64] also constructed an evolutionary tree of the nuclear receptor DNA-binding domain, this time based on parsimony analysis as opposed to the Fitch method of least squares used by Laudet *et al.* [52]. The results are very similar to those presented in Table 1 with the two following exceptions. The *tll* gene product appears as a sole member of its group in subfamily I (not in subfamily II as shown), and egon, KNi, and Knrl appear also as a group in subfamily I (not in subfamily III as shown). No one classification is better than the other without further information.

2. Steroid Receptor-associated Proteins and the Intracellular Localization of Steroid/Nuclear Receptors

Classical steroid receptors are known to exist in at least two different forms identified by their sedimentation at different positions on sucrose or glycerol gradients. This was first demonstrated for the estrogen receptor [65]. The larger and heavier sedimenting estrogen binding complex (8-10 S) is called the untransformed receptor since it can bind hormone but not DNA. The smaller and lighter (4 S) fraction is called the transformed receptor and results from the conversion of the untransformed receptor to a DNA-binding form (reviewed in [66, 67]). In 1979 it was found that molybdate, a transition metal oxyanion, stabilized the receptor in its untransformed state [68]. Addition of molybdate aided greatly in receptor

purification because it protected the receptor from degradation and inactivation. It was not until 1984 that the non-hormone binding components started to be identified (reviewed in [66, 69]). Now it is known that the 4 S form is a receptor monomer that can bind to its cognate hormone response element as a homo- or heterodimer, while the 8-10 S form is composed of one receptor molecule complexed with other proteins that include a dimer of the 90-kDa heat shock protein, hsp90 [70, 71], along with one molecule of p56/59 [72, 73], p40 [74, 75] and p23 [76, 77], and in some cases with hsp70 [78, 79]. There may be still other unidentified factors, and it has been suggested that the untransformed receptor is a core unit of a larger complex [80]. The p56/59 has been shown to be heat inducible and is called hsp56; it is distinct from the well-known hsp60 class [81]. Hsp56 is identical to FKBP-51/52/59 [72], an immunophilin that binds the immunosuppressant drug FK506. The p40 component has recently been shown also to be an immunophilin [74, 75], and p23 has recently been cloned [76, 77]. Hsp90 interacts with steroid receptors by making several contacts with the hydrophobic ligand-binding domain [82-89]. Hsp90 is an abundant cellular protein (1-2 % of the total cellular protein), in vast excess over steroid receptors and binds other proteins in the cell as well [90]. Although no clear function for hsp90 is known, it is thought to be a molecular chaperone. Evidence for this comes from the fact that hsp90 facilitates protein folding *in vitro* [91, 92] by preventing aggregation of proteins and preserving their activity. The role of hsp90 in steroid hormone action has been studied somewhat [66, 69, 93-97]. In the case of GR it maintains the receptor in a high affinity ligand-binding state [98], and represses dimerization, nuclear translocation (particular only to GR and the mineralocorticoid receptor), DNA binding and transactivation [97, 99]. Ligand binding relieves this repression for GR [100-105] and other steroid receptors [106-108]. Deletion of chicken hsp90 residues 221-290 destroyed hsp90's ability to bind human GR, while deletions of residues 530-581 or 392-419 still allowed hsp90 binding to GR but the receptor did not

bind hormone, nor did it accumulate in the nucleus [109]. This demonstrates the requirement for a wild-type hsp90 molecule to maintain the receptor in a hormone binding state *in vivo* and the requirement for hormone binding so that nuclear accumulation of the receptor can occur. Alternatively, distinct regions of hsp90 may regulate different functions of GR. Interestingly, rat epididymal sperm appear to lack hsp90 and contain GR that fails to bind hormone [110]. Not all nuclear receptors exist in a complex with hsp90. The thyroid hormone and retinoic acid receptors do not bind hsp90 and exist in the unliganded state in the nucleus tightly bound to chromatin [111, 112] while the classical steroid receptors exist in a complex with hsp90 and do not bind DNA in the unliganded state [113].

Pratt [113] (Table 2A) and Picard *et al.* [114] (Table 2B) classified nuclear receptors into three classes based on their association with hsp90 and intracellular localization. Pratt added a fourth class for the dioxin receptor. However, I have removed this class since I do not consider the dioxin receptor to be a nuclear receptor superfamily member because it has a different DNA-binding motif [115]. Both classifications subdivide the receptors into the same classes. Picard's class I and Pratt's class II are those receptors which exist in the cytoplasm in the unliganded state. Picard's class II and Pratt's class III receptors are in the nucleus when unliganded but require ligand for DNA binding. Picard's class III and Pratt's class I are those receptors which exist in the nucleus bound to DNA in the absence of ligand. The GR, mineralocorticoid receptor and possibly the vitamin D receptor exist in the cytoplasm in the unliganded state [116, 117] while the estrogen, androgen and progesterone receptors exist in the nucleus in the unliganded state [113]. All these receptors are complexed with hsp90 with the possible exception of the vitamin D receptor [8]. However, the thyroid hormone and retinoic acid receptor are not

Table 2. Nuclear Receptor Classification Based on Hsp90 Association and Receptor Intracellular Localization

A. Classification based on hsp90 association (Pratt, 1990 [113])

Class	Examples	Description
I	TR, RAR, (VDR?)	-Receptors do not form a complex with hsp90. -Unliganded receptors move directly to tight association with the nucleus.
II	GR, (MR?)	-Receptors form a complex with hsp90. -Hsp90 is required for a high affinity steroid binding conformation. -Unliganded receptor is retained in a cytoplasmic docking complex that is recovered in the cytosolic fraction after cell rupture. -Steroid promotes temperature-dependent dissociation of hsp90 from the receptor in intact cell and cytosol.
III	PR, ER, (AR?)	-Receptors form a complex with hsp90. -Hsp90 not required for high affinity ligand binding conformation. -Unliganded receptor is retained in a *nuclear* docking complex that is recovered in the cytosolic fraction. -Steroid promotes temperature-dependent dissociation of hsp90 from the receptor in intact cell and cytosol.

B. Classification based on differential efficacies of inactivation function of ligand-binding domain (Picard *et al.*, 1990 [114])

Class	Prototype	Description
I	GR	A receptor of the first class, defined by apo-GR, is cytoplasmic and requires hormone binding for virtually all of its actions-nuclear translocation, specific DNA binding and likely for transcriptional regulatory activity.
II	ER, PR	A second class, typified by the estrogen and progesterone receptors, is characterized by aporeceptors that are competent for nuclear translocation, but whose binding to cognate DNA sequences and subsequent transcriptional effects remain strongly hormone dependent.
III	TR	The third class, exemplified by the unliganded thyroid receptor (apo-TR), is localized to the nucleus, binds with high affinities to specific DNA sequences and represses transcription from a TRE-associated promoter; thus, hormone binding serves only to relieve this repression and perhaps to stimulate expression modestly from the same promoter.

associated with hsp90 and are tightly bound to DNA. The thyroid hormone and retinoic acid receptors do not redistribute from the nucleus during cellular fractionation as do the estrogen, progesterone and androgen receptors in the absence of ligand.

3. Protein Targeting to the Nucleus of the Eukaryotic Cell

All proteins are synthesized in the cytoplasm and contain the information necessary to be targeted to the appropriate location within a cell [118, 119], be it to a specific organelle, a specific membrane or even to be secreted into the extracellular space. This information is contained either in a targeting signal in the amino acid sequence or in an amino acid site for post-translational modification. Within the cell are binding proteins, translocation complexes and modifying enzymes that recognize the signals and that ultimately direct the proteins into the right pathway for their final destination. This series of events was formulated into the signal hypothesis and was first proposed by Blobel and Dobberstein in 1975 [120, 121].

The nuclear envelope separates the nucleoplasm from the cytoplasm and is a double membrane system, perforated by nuclear pore complexes where the two membranes come together [122]. The nuclear envelope is lined on its inner face by the nuclear lamina, a meshwork that provides structural integrity to the nucleus and sites of attachment for the chromatin (reviewed in [123]). The nuclear envelope barrier blocks some viruses from replicating, and also limits the cell to one round of DNA replication per mitosis, as it now appears that a replication licensing factor can only have access to the nucleus when the nuclear envelope breaks down [124-126].

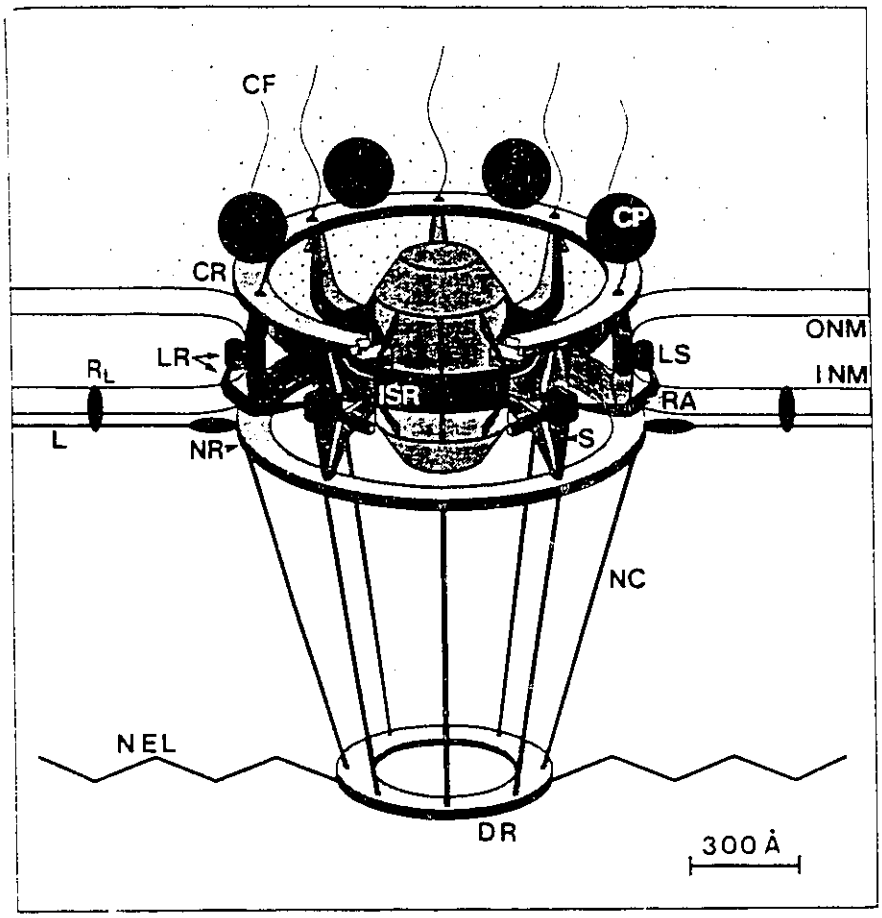
For decades, targeting of proteins to the cell nucleus was the least studied of targeting mechanisms. It was assumed that the nuclear pore complex allowed free diffusion of molecules into and out of the nucleus [127, 128]. Electron microscopic [129-132] and permeability studies [133-135] of the nuclear envelope showed that the

nuclear pore complexes (see Fig. 1) are large protein complexes comprising a grommet-like structure with 8-2-2 symmetry with an outer diameter of 100 nm and a diffusion pore of only 10 nm. Some electron microscopic studies reveal a central plug or transporter in the nuclear pore complex (see Fig. 1). Others have argued that the plug is an artifact of preparation or consists of material in the process of translocation. New evidence suggest that the plug may be a vault ribonucleoprotein particle [136]. The 100 Ångstrom nuclear pore did allow the free diffusion of small molecules and proteins up to 40 kDa [135]. However, larger proteins up to 67 kDa are limited in their diffusion rates while even larger proteins are excluded from the nucleus.

The remarkable 'elasticity' of the nuclear pore complex in allowing passage of large karyophiles (nuclear-loving or seeking molecules) has been shown by the nuclear import of non-deformable gold particles up to 23 nm in diameter coated with karyophilic proteins [137, 138]. This suggests that the pore complex physically expands to such a large diameter and allows passage of protein, RNA and DNA complexes without the need for unfolding the protein or disruption of the complex. This property of nuclear import distinguishes it from mitochondrial or endoplasmic reticulum import in which the protein must be unfolded to pass through the small translocation channel or pore [139-148]. Only extremely large complexes need to be unfolded to pass through the nuclear pore complex. Two examples of this are; first, the nuclear export of Balbiani ring hnRNP particles or granules of the dipteran *Chironomus tentans* [149, 150]. These ribonucleoprotein particles arise from giant RNA transcripts of chromosomal puffs in salivary gland cells. Unfolding and partial uncoating of the Balbiani ring granules changes their diameter from 50 to 25 nm. Second, transfer of the *Agrobacterium spp.* T-DNA complex into plant nuclei is mediated by the VirD2 and VirE2 proteins (reviewed in [151]). Transport of the T-DNA complex is similar to the Balbiani ring granule because translocation through the

Figure 1. A Three-dimensional Ribbon Representation of the Nuclear Pore Complex

The figure was reproduced from Akey and Radermacher [130]. For clarity, the dimensions of certain features have been altered; for example, the inner spoke ring (ISR) is disproportionately short and thin. Abbreviations: CR, cytoplasmic ring; NR, nucleoplasmic ring; LR, lumenal ring; S, spokes; LS, lumenal spoke domain; RA, radial arm dimers; T, transporter; CP, cytoplasmic particles; CF, cytoplasmic filaments; NC, nucleoplasmic cage; DR, distal ring; NEL, nuclear envelope lattice; ONM, outer nuclear membrane; INM, inner nuclear membrane; L, nuclear lamina; R_L, lamin receptor.


CF
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CR
ONM
LS
LR
R_L
INM
ISR
RA
L
NR
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NC
NEL
DR
300 Å

nuclear pore complex occurs in a polar fashion as an unfolded and extended RNP fiber.

The permeability and electron microscope findings led to the investigations of how larger proteins get into the nucleus. Two proteins, nucleoplasmin and the SV40 large T-antigen have been extensively studied in this regard. Nucleoplasmin is an abundant protein in *Xenopus* oocytes and is involved in the assembly of histones into chromatin. Nucleoplasmin is a 165-kDa pentamer whose five 16-kDa tails can be clipped with pepsin to leave an 85-kDa pentamer core [152]. When nucleoplasmin was microinjected into the oocyte cytoplasm it accumulated in the nucleus while microinjected nucleoplasmin cores could not [152]. However, if only one pepsin tail was left on the core, nucleoplasmin entered the nucleus. This suggested that a sequence in the tail was sufficient and necessary to target the core into the nucleus. The possibility remained that the tail promoted nuclear retention and not nuclear entry. This issue was addressed by direct microinjection of the nucleoplasmin core into the nucleus of the oocyte [152]. The core contained all the information necessary for its retention in the nucleus. Therefore the tail must provide additional targeting information. Attempts to define a smaller signal sequence in this large 12- to 16-kDa tail segment were originally unsuccessful (see next section).

The SV40 large T-antigen is a 92-kDa viral nuclear protein that is required for viral replication. A mutant hybrid SV40-adenovirus produces SV40 large T-antigen defective in replication. This mutant accumulates in the cytoplasm [153]. Analysis revealed a point mutation in a basic stretch of amino acids [153]. This finding allowed the identification of the first nuclear localization signal (NLS) (see next section) [154, 155].

Since the studies of nucleoplasmin and SV40 large T-antigen, NLSs in many nuclear proteins have been identified and characterized. The NLS is not proteolytically removed after nuclear entry. It is assumed that continued presence

of the NLS is required for nuclear reentry after the breakdown and reformation of the nuclear envelope at mitosis. NLSs are not limited to large nuclear proteins; they exist as well in small proteins like histone H1 [156]. A protein may contain more than one NLS (see Discussion, Tables 13 and 14). It is not clear why a protein contains more than one NLS, although from *in vitro* studies it is clear that multiple signals increase the efficiency of nuclear import [157]. It had never been shown until recently, that a physiologically relevant nuclear protein diffuses in to the nucleus. This would suggest that most nuclear proteins enter the nucleus via an NLS-mediated process. However, the C or catalytic subunit of protein kinase A is released from its membrane-bound R or regulatory subunit in to the cytoplasm by cyclic AMP binding and has been shown convincingly to enter the nucleus by diffusion [158].

Nuclear import of NLS-bearing proteins is a two-step process with a rapid, energy independent binding to the outer nuclear pore that is followed by a slower, energy dependent translocation through the pore [159-161]. Nuclear import appears to be a facilitated import process and not an active process [162]. Active transport is the movement against an electrochemical or chemical activity gradient (concentration gradient for diffusible species). Nuclear accumulation of a protein results from binding of protein to non-diffusible intranuclear components, subsequent to facilitated transport through the nuclear pore complex. Many studies have shown that nuclear import of proteins and nuclear export of RNA occurs through the nuclear pore complex [163, 164]. Permeability studies show that a particle 20-26 nm in diameter bearing an NLS can be transported across the nuclear pore complex [137]. It was taken for granted that small ions readily diffused in and out of the nucleus. However, the nucleus is not freely permeable to calcium (reviewed in [165, 166]). There appear to be specific channels at the nuclear envelope for the uptake of calcium as measured by patch-clamping [167, 168]. However, the exclusion of Ca^{++} from the nucleus is being challenged since a nuclear

Ca^{++} reporter dye shows rapid equilibrium of Ca^{++} between the cytoplasm and the nucleus [169]. Previous results may have been due to sequestration of the dye in the cytoplasm and/or the hydrolysis of dyes in the nucleus (see [169]). Furthermore, a recent report [170] demonstrates that small changes of cytosolic Ca^{++} cause similarly rapid changes in nuclear Ca^{++}, consistent with the free diffusion of Ca^{++} through nuclear pores. In contrast, large cytosolic Ca^{++} increases, above 300 nM, were attenuated in the nucleus. Therefore, the nuclear envelope shapes but does not block the passage of Ca^{++} from cytosol to nucleus [170].

Much less information is available on export of proteins from the nucleus but export seems to be the default pathway for proteins that are not retained in the nucleus by association with intranuclear components [171, 172]. Some proteins shuttle in and out of the nucleus, or recycle, as is the case of nucleolin [173], hsp70 [174, 175], steroid receptors [176-178] and other proteins [179]. Maybe all nuclear proteins shuttle continuously between the cytoplasm and the nucleus, with intranuclear binding determining the rate of the export component. However, this remains to be proven.

Chaperonins, or chaperone proteins, are proteins that help in protein folding and translocation across membranes by a transient association with a protein during folding or movement to a particular cellular location [180-183]. The role of hsp60 and hsp70 in import of proteins into the mitochondria [184, 185] and endoplasmic reticulum [186, 187] has been demonstrated clearly. A role for hsp70 has also been demonstrated for nuclear import [188] as hsp70 complements the location and functional defects of a cytoplasmic mutant SV40 large T-antigen. Antibodies to the peptide DDDED which recognize hsp70's cytosolic cognate, hsc70, can inhibit transport of nuclear proteins into the nucleus [189, 190]. Furthermore, hsc70 has been identified as an NLS-binding protein (discussed later) [191], and hsc70 colocalizes with karyophilic proteins during their transport into the nucleus *in vitro*

[192]. Shi and Thomas [193] demonstrated that the transport of proteins into the nucleus of permeabilized cells depleted of 70-kDa hsps requires hsp70 or hsc70.

The mechanism of nuclear import appears highly conserved in fungal, plant and metazoan eukaryotes. However, it is known to differ between two nuclei lying in the same cytoplasm of the protozoan *Tetrahymena thermophila* [194] in which differential nuclear uptake of proteins is observed. *Tetrahymena* and other ciliate protozoans possess two structurally and functionally distinct nuclei that contain different proteins. Therefore, certain proteins must be selectively accumulated in one of the two nuclei. Nuclear protein import is conserved in all eukaryotic cells. A yeast protein can be imported into the germinal vesicle of *Xenopus* oocyte [195] and a mammalian protein can be imported into the nucleus of a yeast cell [196], insect cell [197], or plant cell [198]. Perhaps this should be expected as nuclear import of proteins is such a fundamental and essential process in any eukaryotic cell.

Nuclear import of proteins has gained considerable interest (reviewed in [199-203]), as the regulation of distribution of transcription factors between the cytoplasm and nucleus is a potentially powerful means of controlling gene transcription [204]. Indeed, it has been shown that the activity of some transcription factors is controlled at least in part by their transport from the cytoplasm to the nucleus at a certain stage of the cell cycle [205-207], in response to developmental cues [208-212], growth factors [213-215] or heat shock [216-220].

4. Nuclear Localization Signal Sequence Classes

Two approaches have been taken to prove the functionality of an NLS. The negative (or subtractive) approach is the deletion from the protein sequence of a segment that is necessary for nuclear import. In the positive (or additive) approach, a minimal sequence sufficient for nuclear import has been fused or conjugated to a non-nuclear carrier and introduced by transfection of the mutated cDNA or by direct

microinjection of the protein. The localization is usually visualized by immunofluorescence. Both approaches are important as only one method is not sufficient proof of NLS function. The additive approach is limited because a sufficient import sequence may miss a second NLS in the protein, and a sequence that can confer nuclear import on a heterologous protein may not actually function in the host protein as the sequence may be masked by the structure of the protein. It has been clearly demonstrated that the context of a protein affects the function of an NLS, probably because of the protein conformation around the NLS [221, 222]. The negative approach is limited since it does not map precisely the position of an NLS because the deletion may disrupt upstream or downstream structures which will affect the nearby NLS.

Consensus sequences for NLSs have been difficult to establish. Most NLSs however share the property of being composed primarily of basic residues. Early work identified a basic heptamer of SV40 large T-antigen as the typical NLS motif [199-203, 223, 224]. Later, a bipartite basic structure in nucleoplasmin was put forward as the predominant NLS motif [225]. Although the majority of NLSs in nuclear proteins fall into one of these two classes, many reported NLSs do not conform to either of these types of signals. Dang and Lee [226] divided basic sequence NLSs into two classes based on sequence similarities of NLSs. Those with an N-terminal helix-breaking amino acid are grouped in class A, others with a C-terminal or internal prolyl residue are grouped in class B. I have sub-divided the NLSs into eight classes based on published examples. My classification is different, more extensive, and current. Recently, Boulikas has classified 107 transcription factors with putative core NLS hexapeptides into 13 classes [227]. This subdivision applies only to basic sequence NLSs and would fall mostly into my first class of SV40-like NLSs (see below).

(A) SV40-like

The first NLS identified was that of the SV40 large T-antigen, PKKKRKV [154, 155]. As similar sequences were found to function as NLSs in other proteins [199-203, 223, 224], this signal was thought to be the archetype signal. The SV40-like NLS is a small sequence of seven or eight amino acids containing a single cluster of basic residues often flanked by a prolyl or acidic residue. Chelsky *et al.* [228] further refined this sequence to a tetrameric consensus sequence, K(R/K)x(R/K), in which x is limited to a few preferred amino acids, K, R, P, V, or A, but not N. Two extensive reviews by Boulikas have presented a set of rules for what he calls a core NLS [227, 229]. First, a core NLS is proposed to consist of a hexapeptide with four R and K residues. Second, D and E residues as well as bulky amino acids (F, Y, W) are not frequently placed between K and R residues. Three, acidic residues and P or G residues that break the α-helix are frequently in the flanking region of the hexapeptide stretch. Four, hydrophobic residues ought not to be present in the core NLS flanking so as to allow for the exposure of the NLS on the protein surface. Boulikas [227] classifies core NLSs into three main categories which are further subdivided. The catagories are "highly basic NLS" which contains five or six (K/R) residues in a hexapeptide stretch; "typical NLS" containing four (K/R) residues in a stretch of six amino acids; and "non-typical NLS", containing two or three (K/R) residues within a hexapeptide. The "typical NLS" motifs were further subdivided into nine motifs: $(K/R)_4$, $(K/R)_3X(K/R)_3$, $(K/R)_3X(K/R)$, $(K/R)_2X(K/R)_2$, $(K/R)X(K/R)_2X(K/R)$, $(K/R)_2X(K/R)X(K/R)$, $(K/R)_3X(K/R)_2$, $(K/R)_2XX(K/R)_2$, and $(K/R)_3XX(K/R)$. The "non-typical NLS" motifs were subdivided into three motifs; $(R)_3$, $(R/K)_2X(R/K)$, and $(K/R)_2X_n(R/K)_2$. X represents any residue but is usually not D, E, F, Y, or W. $(K/R)_4$ is a stretch of four consecutive K or R residues and is the most predominant motif with a frequency of 25 %. The asymmetrical motifs are not dependent on the polarity of the polypeptide chain.

(B) Bipartite

It took many years to define the nucleoplasmin NLS because it was more complex than that of SV40 large T-antigen [230, 231]. The nucleoplasmin NLS consists of two basic clusters of amino acids separated by a spacer region of ten amino acids, KRPAATKKAGQAKKKK. The carboxy-terminal basic sequence is similar to the NLS of SV40 large T-antigen. However, in nucleoplasmin, both basic clusters are required for efficient nuclear import [231, 232]. This bipartite NLS may be the true archetype of an NLS as this consensus sequence (two adjacent basic residues separated by approximately ten amino acids from a second domain that contains at least three out of five basic amino acids) has been identified in sequence searches of the SWISS-PROT protein database in 56 % of the nuclear proteins and in only 4 % of non-nuclear proteins [225].

(C) Matα2-like

This type of NLS, found in the yeast mating-type switch factor, consists of a short hydrophobic region that contains one or more basic amino acids [233, 234]. Two signals actually exist in Matα2, NKIPIKD at amino acid residues 1 to 13 [233], and VRILESWFAKNI at residues 141 to 159 [234]. Each of these two NLSs is a competent nuclear targeting signal on its own. Similar sequences have been identified in a number of other nuclear proteins [199] and at least one other protein with a proven NLS similar to that of Matα2 is known, the maize R transcription factor [235].

(D) Cap Structure

The U snRNPs are uracil-rich small nuclear ribonucleoprotein particles that play a role in spliceosome function. After export from the nucleus, the U snRNAs are complexed with Sm proteins in the cytoplasm and the complex is then imported into the nucleus. The import is dependent on the trimethylguanosine cap structure

of the pol II U snRNAs (U1-U5) and on protein constituents [236, 237]. It has been proven by competition and interference experiments that the import pathways for these 'cap structure NLSs' are distinct from the pathway for the basic sequence NLSs [238, 239]. However, this cap structure 'NLS' is limited in its functionality, as the cap is essential for U1 and U2 snRNP nuclear import in *Xenopus* oocytes but not in somatic cells [240]. The differential cap dependence for nuclear import is mediated by soluble cytosolic factors [240].

(E) Hydrophobic

There is only one demonstrated example of a hydrophobic NLS sequence, $FV(X)_{7\text{-}20}MXSLXYM(X)_4MF$. This signal is noteworthy because of the lack of any charged residues. It targets the adenovirus E1a protein to the *Xenopus* oocyte nucleus in a developmentally regulated manner [211]. A similar but as yet unproven sequence exists in GR in a region that does contain a nuclear localization function [1, 211], but which has yet to be further delimited.

(F) Methylated Arginyl Residues or Consecutive Arginyl Residues

The role of methylated arginyl residues in nuclear import has been suggested from studies which show that the only difference between forms of basic fibroblast growth factor (bFGF) that get into the nucleus from those which do not is that the former have methylated arginyl residues [241, 242]. The different forms of bFGF result from alternate splicing and the use of alternate initiation codons [243]. Burgess *et al.* note that a common feature of methylated arginyl-containing proteins, such as histones, is their nuclear localization [241].

Although consecutive arginyl residues constitute a basic stretch of amino acids, these differ from the usual NLSs in that most NLSs of a basic nature appear to be a mix of arginyl and lysyl residues with a preference for lysyl residues. The maize

transcription factor R contains three functional NLSs [235], one of the SV40-type, another of the Matα2-type, and a third unusual NLS, containing three consecutive arginyl residues. Similar NLSs have been found in other viral proteins [235], such as RRNRRRR in HIV Rev [244], or RLPV$(R)_6$VP in the adenovirus preterminal protein [245].

(G) Sugar-dependent

A sugar dependence has been demonstrated for the nuclear import of glycoconjugates that show ATP-dependent nuclear import [246]. Glucose, fucose and mannose can target human serum albumin-conjugates to the nucleus while other sugars cannot. It is not known whether the sugar-dependent signal is physiologically relevant or whether the nuclear localization is mediated by some other protein, such as an NLS-bearing lectin.

(H) Others

There are other examples of NLSs that show no similarity to the above classes, such as the signal found at residues 2-59 of the HTLV-1 Tax transactivator [247], residues 1-29 (MK$(X)_{11}$CRLKKLKCSKEKPKCA) of the yeast Gal4 transactivator [222, 248] or AAFEDLRVLS of the influenza viral nucleoprotein [249]. This latter sequence may not be an NLS, but rather a sequence that specifies nuclear accumulation (retention) [249]. In addition, other signals show some similarity to one of the above classes but display some unusual features. For example, a serum-regulated pentapeptide NLS of adenovirus E1a, KRPRP, confers a serum-dependent nuclear entry to non-nuclear proteins [214], which suggests that in addition to this basic sequence a serum factor is required.

It is not clear whether nuclear and nucleolar targeting sequences are different. As proteins destined for either the nucleus or nucleolus must first cross

the nuclear envelope, the subnuclear localization may be effected by an additional distinct nucleolar binding sequence. It appears that the nucleolar localization signal of HTLV-1 Rex [250] is due to an overlapping RNA-binding function that results in its accumulation in the nucleolus by binding RNA [251-254] or by binding the nucleolar protein B23 [255]. It has been clearly demonstrated that another nucleolar protein, nucleolin, does not contain a linear nucleolar signal. Instead, it uses a bipartite NLS to enter the nucleus and then accumulates within the nucleolus by virtue of its binding to other nucleolar components (RNA) via distinct domains [172, 256]. The same is true for the NLS-binding and RNA-binding protein, NSR1 [257], and for the ribosomal transcription factor UBF that accumulates in the nucleolus by binding to the RNA polymerase I promoter region of DNA [258].

On the basis of secondary structure prediction plots NLSs are thought to be mainly α-helical and may be flanked by β-turns [259]. Modifications of the NLS flanking sequences can dramatically affect the kinetics of nuclear import [260, 261]. These modifications are typically kinase-mediated phosphorylations of flanking serinyl or threonyl residues near the basic NLS sequence. Increased rate of nuclear import by phosphorylation of flanking NLS residues has been demonstrated elegantly by laser microphotolysis in which nuclei of cells loaded with a fluorescent nuclear substrate were individually photobleached and the kinetics of nuclear substrate uptake measured [133, 260]. Confocal laser scanning microscopy also has been used to measure nucleocytoplasmic fluorescence ratios by real time digital image analysis [262].

The cotransport of a molecule not carrying an NLS can occur by binding to or 'piggybacking' on a protein that does contain an NLS. There are examples of this mechanism occurring naturally in the cell [263], and in artificial situations in which an NLS of a nuclear protein has been deleted [264]. The first situation is exemplified by the arylhydrocarbon receptor, also known as the Ah, dioxin or TCDD receptor. The

Ah receptor is a basic helix-loop-helix (bHLH) transcription factor [115] with properties similar to steroid receptors [265-267]. It does not possess an NLS but instead binds to another bHLH protein known as Arnt, the arylhydrocarbon receptor nuclear translocator [263, 268], that does contain an NLS. The second situation is exemplified by a mutant progesterone receptor whose NLSs have been deleted. This mutant receptor can enter the nucleus when wild-type receptor is present. This suggests that dimerization with wild-type receptor is responsible for importing NLS-defective receptors [264].

Finally, proteins bearing an NLS may not immediately enter the nucleus after their synthesis in the cytoplasm. Their signal may be masked by the protein's tertiary or quarternary structure. Alternatively, a nuclear protein may be anchored to the cytoplasm by another protein [208, 269]. In both cases, signals are required so that the protein can enter the nucleus. Entry most likely results either from an allosteric change in conformation (unmasking) or by release from the cytoplasmic anchor protein which has been shown to occur by phosphorylation or proteolysis of an inhibitor or anchoring domain [208, 269].

5. Practical Applications and Future Perspectives

Steroid hormone agonists and antagonists are used routinely in the treatment and cure of many disorders; for example, glucocorticoids are used as cytolytic agents in treatment regimens for lymphomas and leukemias [270-272] and retinoids are used as differentiating agents and possibly as chemopreventive agents in many forms of cancer [273-276]. Of note is the use of steroid antagonists alone or in conjunction with other chemotherapeutic agents in the treatment of steroid-dependent cancers [277], primarily breast cancer and prostate cancer. Both cancers affect about 1 in 10 people and are major causes of death. The efficacy of the steroid treatments is hampered in part by the complexity of steroid hormone action. Antagonists can

block steroid hormone action at many steps; for example they can block DNA binding of steroid receptors or allow binding to DNA but block transactivation of specific hormone regulated genes [278]. It is becoming clear that for the treatment of breast carcinoma, antagonist bound estrogen or progesterone receptors, when bound to DNA, can still transactivate certain genes because of cross-coupling of signaling pathways [279-281]. Therefore it may be desirable to find antagonists that block DNA binding or, even better, that block a prior step, such as nuclear translocation of the receptor in the case of GR, so that no nuclear events can take place.

A new aspect of NLS research is the use of NLSs to target drugs to the cell nucleus to increase the efficacy of treatment for cancer and other diseases (K. Sheldon, D. Liu, J. Ferguson, and J. Gariépy, unpublished). Systems are being developed to deliver such drugs to cells. One possibility is that NLS conjugates could target radionuclides, laser-activated chromophores and chemotherapeutic agents to the cell nucleus, their primary site of action. This would increase their intranuclear concentration and effectiveness. Perhaps, this nuclear targeting would reduce their efflux from the cell by the plasma membrane *mdr* P-glycoprotein and/or reduce their metabolism by microsomal or lysosomal enzymes. Hence, this may reduce the required dosage of these toxic compounds and their associated side-effects.

An NLS in the *gag* matrix protein of the human immunodeficiency virus (HIV) has been shown to allow nuclear entry of the viral genome as a large preintegration complex [282]. This allows the retrovirus to replicate in non-dividing cells, which most retroviruses can not do since they require the breakdown of the nuclear envelope, at mitosis, to enter the nucleus. Two new interesting possible applications arise. First, the matrix protein NLS is now a target for drug and gene therapy to prevent replication of HIV in non-dividing cells. Second, the use of NLS-bearing proteins to efficiently target large pieces of DNA to the nucleus for transfection and

integration purposes, such as in gene therapy, and for creation of stable transfectants or transgenic animals.

6. Steroid and Nuclear Receptor NLSs

In the absence of ligand, cellular GR is predominantly cytoplasmic [1, 283-285]. Only upon binding of glucocorticoid does the receptor translocate to the nucleus [1, 286]. We have previously shown the association of GR with the rat liver nuclear envelope using immunocytochemistry [287], binding studies [288], immunoblotting and affinity labeling [289]. Significantly, the immunoblot analysis revealed immunoreactive GR in extracts from nuclear envelope isolated from intact, but not from adrenalectomized rats, even though GR is still present in the cytosol [290]. These data show that in the presence of glucocorticoid, part of the cellular GR is localized to the nuclear envelope. It has been our hypothesis that these receptors are in the process of translocation across the nuclear envelope. This hypothesis has been strengthened by electron microscopic studies in which the nuclear envelope GR has been localized to the nuclear pore complexes [291] and our identification of a sequence in the hinge region of the receptor that was similar to the SV40 large T-antigen NLS. We hypothesized that this sequence could target the receptor to the nucleus by regulated passage across the nuclear envelope. In 1987, Picard and Yamamoto [1] published their results identifying two NLS functions in GR that they called NL1 and NL2. They performed immunofluorescence studies of transiently transfected COS-7 or CV-1 cells expressing wild-type or deletion mutants of GR to identify regions important for nuclear localization. These regions were then confirmed to be true NLSs by their ability to target a non-nuclear protein, β-galactosidase, to the transfected cell nucleus. NL1 is a twenty-seven amino acid region (497-524 of rat GR) that overlaps the C-terminal end of the DNA-binding region. This stretch also contains the region of similarity to the SV40 large T-

antigen NLS (TKKKIKG, 512-518 of rat GR). It may be that NL1 is not an SV40-like NLS but is in fact a bipartite NLS, as a sequence $RK(X)_{10}RKTKKKIKG$ within residues 497-524 and overlapping the C-terminal end of the DNA-binding region exists [1]. This bipartite NLS is suggested by experiments with the androgen receptor [292, 293] but has not been confirmed in GR. NL2, on the other hand could not be mapped to such a small sequence. It consisted of the whole ligand-binding domain (540-795 of rat GR) and does not harbor any sequence similarities to the SV40 large T-antigen NLS. Interestingly, this signal targets β-galactosidase to the nucleus in a hormone-dependent manner, unlike NL1 which is a constitutive signal when taken out of context of the full-length receptor.

Studies using the human GR [294], rabbit and chicken progesterone receptors [264, 295], human estrogen receptor [114, 295], and human androgen receptor [292, 293, 296, 297] have identified an NLS function in the hinge region that is approximately ten amino acids away from the last conserved cysteinyl residue (which is not coordinated with the zinc atom) in the DNA-binding region. However, in the case of the estrogen receptor three clusters of basic residues in a forty amino acid stretch are needed for the NLS function as each cluster on its own is insufficient in targeting proteins to the nucleus [114, 295]. Synthetic peptides corresponding to the hinge NLS of GR, estrogen receptor, androgen receptor and retinoic acid receptor can direct human serum albumin into the nucleus [298]. A point mutation in the hinge NLS of the viral counterpart to thyroid hormone receptor, v-*erbA*, inhibited nuclear localization and DNA binding [299] and insertion mutants in this region of v-*erbA* demonstrated that NLS and DNA-binding regions overlap [300]. Mutational analysis of the thyroid hormone receptor hinge region shows that the basic region KRK is important in nuclear localization and transactivation [301], and the thyroid hormone receptor hinge region NLS peptide, SKRVAKRKL, can target pyruvate kinase to the nucleus [226].

There may be a second NLS in the ligand-binding domain of the other steroid receptors besides GR but this has yet to be proven convincingly. The observed ligand-induced nuclear translocation of the mineralocorticoid receptor makes this receptor a likely candidate to contain a second ligand-dependent NLS [94, 302, 303]. However, it is more likely that the ligand-binding domain represses the hinge region NLS in all or some of the steroid receptors when the receptor is unliganded. The progesterone receptor possesses a second NLS function which overlaps the second 'zinc-finger' of the DNA-binding domain. Both motifs are unmasked by hormone binding [264]. The glucocorticoid, estrogen, and possibly the androgen receptors contain apparently a hormone-dependent signal in their ligand-binding domain [1, 264, 294-296]. The results are convincing for the glucocorticoid receptor [1, 294]. However, the results are not as convincing for the estrogen receptor, as the ligand-binding domain does not function as an NLS on its own but must cooperate with another NLS [295]. An NLS function or more likely an inhibitory function in the ligand-binding domain is hinted at based on the hormone-induced nuclear translocation of the androgen receptor [296, 304] even when part of the hinge NLS is deleted [292]. On the other hand there is no evidence for a ligand-dependent NLS in the thyroid hormone receptor or retinoic acid receptor.

Chimeras of transcription factors and the ligand-binding domain of the estrogen receptor or GR have been constructed which result in the sequestration of the transcription factor in the cytoplasm of transfected cells and repression of DNA-binding activity. Both activities are relieved by binding of estrogen or glucocorticoid [305, 306]. This fusion scheme allows for hormone-inducibility of a transcription factor. The hormone-dependent nuclear translocation contrasts with earlier studies which failed to define an NLS function in the ligand-binding domain of the estrogen receptor [114]. However, the estrogen receptor ligand-binding domain may simply be repressing an NLS function in the fusion partner. Such an

NLS is known to exist in the Myc protein DNA-binding domain [228] or in the rel homology domain of v-*rel* [307, 308]. The derepression of the NLS by estradiol would explain the hormone-induced nuclear translocation of the chimeras. Why the estrogen receptor ligand-binding domain works like this for other transcription factors but not for itself is unclear.

An example of a 'natural' chimera is the fusion of the retinoic acid receptor (RAR) with another transcription factor, *Myl*/PML, identified in acute promyelocytic leukemia and caused by a chromosomal translocation [309]. Both *Myl*/PML and RAR are normally nuclear in the presence or absence of retinoic acid [310]. *Myl*/PML is normally concentrated in discrete subnuclear domains [311] or nuclear bodies that have been called PODs (PML oncogenic domains) by Dyck *et al.* [312]. The Myl/PML-RAR fusion protein is mainly cytoplasmic in the absence of retinoic acid and appears to be translocated to the nucleus in the presence of retinoic acid [310]. Furthermore, when *Myl*/PML-RAR chimeras and the retinoid-X receptor (a nuclear auxiliary factor required for efficient DNA binding of RAR) are overexpressed in COS cells, the chimera can retain the retinoid-X receptor in the cytoplasm [313]. These findings are unexpected since the RAR is not known to possess a ligand-dependent NLS, and is not known to be associated with hsp90. It is unclear how retinoic acid induces translocation of this chimera, but indicates some ligand-dependent process is occurring. It has recently been suggested that retinoic acid allows the unmasking of a dimerization surface on *Myl*/PML that allows it to dimerize with wild-type *Myl*/PML [312]. *Myl*/PML, and not RAR, may be responsible for nuclear localization of the chimera.

Another example of an unexpected translocation is that of the chicken progesterone receptor which is cytoplasmic in transfected cells. The reason for this cytoplasmic localization of a normally nuclear receptor is unclear. The progesterone receptor is normally nuclear in the absence or presence of hormone. The

overexpressed progesterone receptor can be induced to translocate to the nucleus by progesterone, and unexpectedly by dopamine alone [314]. Dopamine is a neurotransmitter that acts through a plasma membrane receptor. The fact that dopamine can activate the progesterone receptor is quite surprising. This cross-coupling of signal transduction pathways has profound implications for hormonal control of gene expression.

The hormone induced nuclear translocation of the vitamin D receptor has been demonstrated by indirect immunofluorescence of microwave-fixed cells [116, 117], and more recently with the use of a fluorescent calcitriol ligand (vitamin D_3 derivative) for receptor visualization (J. Barsony, unpublished).

7. Recycling of Steroid Receptors and Modulation of GR Localization

Munck and co-workers proposed in 1972 [315] that GR recycled from the nucleus back in to the cytoplasm where the receptor was re-activated so that it could again bind hormone and exert its effects once back in the nucleus. They showed that depletion of ATP from cells leads to the accumulation of a non-ligand binding form of GR in the nucleus, which they called the null receptor. However, it was the heterokaryon studies of Milgrom's group [178, 316] and DeFranco's group [176, 177] which demonstrated that the nuclear steroid receptor could end up in the nucleus of a fusion partner cell when protein synthesis is stopped. The most plausible explanation is that the receptor exits from the nucleus into the common cytoplasm where it is taken up into the second nucleus. Similar results were obtained with multinucleated myotubes [317], except that GR NL1 fusions which lack the ligand-binding domain (NL2) were taken up more efficiently by neighbouring nuclei than GR NL1-NL2 fusions which contained the ligand-binding domain and showed a more distal or diffuse nuclear labeling. A possible explanation for this observation is that the NL1 fusion which is constitutively active is readily taken up by the nearest

nuclei while the NL1-NL2 fusion is inactive in the cytoplasm in the absence of ligand and can diffuse before binding ligand which induces nuclear translocation. Other nuclear proteins have also been shown to shuttle back and forth between the cytoplasm and nucleus [173-175, 179].

Very little is known about protein export from the nucleus, and it is not known if signals are needed for this process. A recent report suggests that there are no export signals but rather that export of proteins from the nucleus is a default process whereby any protein not bound to an intranuclear constituent is exported [171, 172]. These conclusions were drawn from deletional analyses of nucleolin (a nucleolar protein with an NLS and RNA-binding motif) and lamin B [172] (a nuclear lamina protein with an NLS and CaaX box that aids in its membrane association through post-translational modifications)[318, 319], and studies of fusion of an NLS to a cytoplasmic enzyme, pyruvate kinase [172]. When proteins lacking their binding domain for nuclear components but containing an NLS are injected into the cytoplasm or nucleus, they are detected in the nucleus as the NLS provides an import signal. However when a second deletion destroys the NLS, proteins injected directly into the germinal vesicle (nucleus) of *Xenopus laevis* oocytes, they are exported to the cytoplasm and are not reimported into the nucleus. Therefore, nuclear retention is an important step in nuclear accumulation of proteins.

Apart from the well known effect of ligand binding to relieve hsp90 repression and allow nuclear import, several other conditions, treatments, and factors are known to affect the nuclear localization of GR (see Table 3). Effects such as v-*mos* transformation, phosphatase treatment, ATP depletion, okadaic acid treatment, H-7 treatment, RU486 binding, and the cell cycle are thought to be mediated by phosphorylation or dephosphorylation of the receptor or some accessory factor through the action of as yet unspecified kinase(s) or phosphatase(s). ATP may be required as an energy source or phosphate source. GR is a phosphoprotein whose

Table 3. Modulation of GR Subcellular Localization

Factor (Description)	Effect on Nucleocytoplasmic Distribution of GR	Reference
v-*mos* (oncogene)	V-*mos* transformed 6m2 cells result in the generation of a novel desensitized GR that is apparently trapped in the cytoplasm and incapable of being reutilized after nuclear entry and export. GR is not detectably phosphorylated by v-*mos*.	Qj *et al.*, 1989 [320]
protein phosphatase types 1/2A	Protein phosphatase types 1 (PP-1) and/or 2A (PP-2A) regulate nucleocytoplasmic shuttling of glucocorticoid receptors. A direct role for PP-1 and/or PP-2A in GR shuttling is suggested by site-specific hyper-phosphorylation of GRs *in vivo* during okadaic acid inhibition of recycling. These are the same sites that undergo *in vitro* site-specific dephosphorylation by PP-1 and PP-2A.	DeFranco *et al.*, 1991 [321]
heat shock (43 °C)	Unliganded GR of non-shocked L929 and WCL2 cells is localized primarily in the cytoplasmic fraction, whereas unliganded GR of heat-shocked cells is found almost exclusively in the nuclear fraction. Similar results were obtained when cells were subjected to chemical stress (sodium arsenite). However, Sanchez now reports that heat shock and chemical stress do not result in increased nuclear translocation or nuclear retention of dexamethasone-bound GR in L929 cells stably transfected with MMTV-CAT reporter plasmid.	Sanchez, 1992 [322] Sanchez *et al.*, 1994 [323]
over-expression (transient transfection)	Hormone-free mouse GR overexpressed in CHO cells are localized to the nucleus and are associated with both hsp70 and hsp90.	Sanchez *et al.*, 1990 [324] Martins *et al.*, 1991 [325]
ATP depletion (anaerobiosis)	GRs lacking hormone-binding activity (null receptor) are tightly bound in nuclei of ATP-depleted cells.	Mendel *et al.*, 1986 [326] Hu *et al.*, 1994 [327]
okadaic acid (phosphatase inhibitor)	Since okadaic acid does not affect the nuclear import of GR *per se*, the transcriptional enhancement mediated by unliganded GR in response to okadaic acid was most likely accounted for by the accumulation of some unliganded GRs within nuclei of transfected CV-1 and COS-1 cells.	Somers and DeFranco, 1992 [328] DeFranco *et al.*, 1991 [321]
H-7 (kinase inhibitor)	H-7 [1-(5-isoquinolinesulfonyl)-2-methyl piperazine] is an inhibitor of PKC and PKA. It also inhibits tyrosine amino transferase induction by glucocorticoids because it blocks nuclear translocation of GR complexes in hepatocytes.	Kido *et al.*, 1987 [329] *N.B.* Cont'd on next page.

Factor (Description)	Effect on Nucleocytoplasmic Distribution of GR	Reference
PHA (phytohemag-glutinin) (lectin, lymphocyte activator)	PHA-induced translocation of GR from the cytoplasm to the nucleus of peripheral lymphocytes is observed in the absence of steroid ligand. PHA stimulates lymphocytes by binding to cell surface carbohydrate groups on glycoproteins and activating second messenger signaling pathways.	Papamichail *et al.*, 1981 [330]
FK506 and rapamycin (immunosup-pressants)	Based on cell fractionation studies, it is postulated that FK506 and rapamycin potentiation of GR-mediated gene expression is the result of increased translocation of GR to the nucleus of L cells treated with low concentrations of dexamethasone.	Ning and Sanchez, 1993 [331]
phenol red (pH indicator, estrogen receptor agonist)	Under standard growth conditions [DMEM medium (which includes 45 μM phenol red) supplemented with 5 % FCS (which contains 2 nM cortisol)] GR is predominantly nuclear in about 2/3 of fluorescence-positive cells. In serum-free medium with 270 μM phenol red, GR was predominantly nuclear in about 1/2 of the stained cells. This effect is thought to be due to the weak binding activity of phenol red to GR.	Picard and Yamamoto, 1987 [1]
RU486, (RU38486, mifepristone) (GR antagonist)	1. RU486 binding produced a reduction in the amount of GR converted from 8S to 4S and stabilized the cytoplasmic GR-hsp90 complex. 2. In contrast to the redistribution of agonist-bound nuclear GR to the cytoplasm of v-*mos* transformed cells, RU486-bound GRs are efficiently retained within nuclei. Interestingly, withdrawal of RU486 does not lead to efficient depletion of nuclear GR in either nontransformed or v-*mos* transformed cells. GRs bound to RU486 were also efficiently retained within nuclei of G2 synchronized cells (see below) which suggests that a nuclear event which is blocked by RU486 is required for GR to exit.	1. Beck *et al.*, 1993 [332] 2. Hsu *et al.*, 1992 [333] Qi *et al.*, 1990 [334]
cell cycle (G_2 phase)	GRs that translocate to the nucleus of G_2 synchronized L cells in response to dexamethasone treatment were not efficiently retained there and redistributed to the cytoplasmic compartment.	Hsu *et al.*, 1992 [333]
molybdate (transition metal oxyanion, phosphatase inhibitor)	Concentrations of 10-20 mM molybdate ($MoO_4^=$) are known to stabilize the interaction between GR and hsp90. This stabilization impedes the transformation of GR into a form that can translocate to the nucleus and bind DNA.	Dahmer *et al.*, 1984 [335] Mendel *et al.*, 1986 [101] *N.B.* Cont'd on next page.

Factor (Description)	Effect on Nucleocytoplasmic Distribution of GR	Reference
nuclear translocator (ASTP) (48-kDa protein, 93-kDa protein dimer)	The ATP-stimulated GR translocation promoter (ASTP) increases the binding of GR to isolated nuclei in the presence of ATP. ASTP is a histone binding protein which increases GR binding to nuclei or chromatin in the presence of ATP but does not affect GR binding to DNA-cellulose. This factor may piggyback with GR as its nuclear entry is hormone dependent. Its effect on nuclear occupancy may be due to increased nuclear retention.	Okamoto *et al.*, 1993 [336]
MTI-III (69-kDa protein)	A macromolecular-translocation inhibitor of transformed GR binding to isolated nuclei from rat liver. It is thought not to act through a nuclear acceptor site but to interact directly with GR. MTI-III can inhibit transformed GR binding to DNA-cellulose and to histone-agarose.	Liu *et al.*, 1993 [337]
modulators (ether aminophos-phoglycerides, PKC-stimulator)	Modulators are novel ether amino-phosphoglycerides that appear to be the 'endogenous molybdate factor'. First discovered as inhibitors of GR complex transformation, these acidic cytoplasmic biomolecules also stabilize the steroid-binding ability of the hormone-free untransformed GR.	Bodine and Litwack, 1990 (review, [338]) Hsu *et al.*, 1991 [339]

phosphorylation state changes upon ligand activation [340-342]. Although changes in GR phosphorylation do not correlate with changes in gene expression [343], DeFranco *et al.* [321] have correlated changes in GR phosphorylation with changes in its nucleocytoplasmic shuttling. Other effects are more easily explained such as the effect of molybdate which stabilizes GR in a large cytoplasmic complex [105]. Receptor overexpression may saturate some cytoplasmic component of the docking system and lead to artifactual localization in the nucleus [324]. Heat shock disrupts the untransformed receptor complex and leads to nuclear accumulation [322]. Phenol red acts as a receptor ligand and induces receptor nuclear translocation but not gene transactivation [1]. The stimulation of peripheral lymphocytes with phytohemagglutinin (PHA) may activate GR by a second messenger signaling pathway induced by the PHA-crosslinking of surface receptors. This is analogous to the dopamine activation of the progesterone receptor [314]. The effect of FK506 on increased nuclear translocation of GR may be explained alternatively by increased affinity of GR for nuclear structures (F. Sackey, T. Reich, R. Haché and Y. Lefebvre, unpublished). The point at which RU486, a GR antagonist, acts is controversial. RU486 may affect normal DNA-dependent phosphorylation of GR, similar to the DNA-dependent phosphorylation event observed for the progesterone receptor [344]. Other modulatory factors include a novel ether aminophosphoglyceride which is thought to act *in vivo* like molybdate does *in vitro*, and some poorly characterized proteins, nuclear translocator (ATSP) and MTI-III, which await further characterization to establish their *in vivo* role in GR action.

Other factors are known to block protein import into the nucleus but act in a general manner on all nuclear proteins and not solely on GR. These include the lectin, wheat germ agglutinin, that is thought to bind to O-linked glycoproteins of the nuclear pore complex and inhibit facilitated uptake of protein but not binding to the nuclear envelope [345]. Antibodies to nuclear pore complex proteins and

glycoproteins [346, 347] or antibodies to hsp70 [189] have a similar effect to wheat germ agglutinin. These antibodies and wheat germ agglutinin have been shown to interfere with facilitated nuclear import but not to hinder diffusion of small molecules through the nuclear pore. Chilling the cell to 4 ^{0}C or treating with metabolic inhibitors blocks the energy-dependent nuclear translocation process of proteins but not their nuclear envelope binding consistent with the energy requirement for the translocation step but not the binding step [159-161]. Treatment with N-ethylmaleimide, a sulfhydryl alkylating reagent, also inhibits specific protein uptake into nuclei in *in vitro* experiments with egg extracts [348] and in permeabilized cells [349, 350] probably by inactivating some protein component(s) of the nuclear import pathway.

8. Project Goals

As regulators of gene expression such as steroid receptors must traverse the nuclear envelope and enter the nucleus to effect their action, the elucidation of the mechanism by which they are transported into the nucleus is critical to our understanding of cellular processes. The two main research goals of this project that form Part I of the thesis were: first, to identify NLS-binding proteins of the NL1 and NL2 signals of GR; and second, to establish an *in vitro* nuclear import assay to study the nuclear import of GR, and eventually to prove the function of identified and purified NLS-binding proteins in a reconstitution assay. During the course of the research work I noted that NLSs were often close to DNA- or RNA-binding domains of relevant nuclear proteins. I have included this data demonstrating the association of the two functions (nuclear localization and nucleic acid binding) in nuclear proteins and have presented some models for how and why this has occurred in the Discussion.

II. METHODS AND MATERIALS

1. Animals

Male Sprague-Dawley rats weighing 200-250 g were obtained from Charles River Canada Inc. (Montreal, QC) and maintained on a diet of Purina LabChow and tap water *ad libitum*. Animals were housed at the Loeb Medical Research Institute (12 h light/dark cycle) and treated according to the guidelines of the Medical Research Council of Canada. The rats were killed by decapitation and the livers were quickly removed and placed in ice-cold homogenization buffer (0.32 M sucrose, 3 mM $MgCl_2$ and 1 mM dithiothreitol). The livers were rapidly stripped of connective tissue, weighed and minced. Adrenalectomy was performed at Charles River Canada under ether anesthesia and rats were subsequently maintained with 0.9 % saline drinking solution. Adrenalectomized animals were used for experiments 3 or 9 days post-surgery depending on the purpose, *i.e.* high or low concentrations of cytosolic GR respectively.

2. Materials

Unlabeled steroids were purchased from Steraloids (Wilton, NH) or Sigma (St. Louis, MO). TNT^{TM} coupled transcription-translation kits with SP6 or T7 RNA polymerase were purchased from Promega (Fisher Scientific, Ottawa, ON). RNasin, a ribonuclease inhibitor, was purchased from Promega. Translation-grade [^{35}S]-methionine (SA, 1142-1200 Ci/mmol) was from NEN-Dupont (Boston, MA) or Amersham (Oakville, ON). [^{125}I]-NaI (SA, 17 mCi/μg) was from NEN-Dupont, ICN (Mississauga, ON), or Amersham (SA, 15 mCi/μg). NCS tissue solubiliser was obtained from Amersham. Ready Protein as purchased from Beckman (Palo Alto, CA). Deoxyribonuclease I (DNAase I, RNAase-free, and shipped on dry ice), ribonuclease A (RNAase A), trypsin, protein A-sepharose, buffers, dithiothreitol (DTT), Coomassie

Brilliant Blue R-250, β-mercaptoethanol, N,N,N',N'-tetramethylethylene-diamine (TEMED), protease inhibitors, protein standards, and BSA were purchased from Sigma. Digitonin and octyl-β-D-thioglucopyranoside were purchased from Calbiochem (La Jolla, CA). ATP was from Pharmacia (Bromma, Sweden). Electrophoresis-grade acrylamide, N,N'-methylene bisacrylamide, SDS, Tris, glycine were purchased from Bio-Rad (Mississauga, ON). Prestained protein standards (Rainbow Markers) were from Amersham. Crosslinker bis(sulfosuccinimidyl)suberate (BS^3) was from Pierce (Rockford, IL), other crosslinking reagents were either from Pierce or Sigma. The BuGR antibody was purchased from Affinity BioReagents (Neshanic Station, NJ) and horseradish peroxidase-labeled antibodies were from Bio-Rad. ECL Western detection kits were purchased from Amersham. Durapore and HA filters were purchased from Millipore (Bedford, MA). All other reagents were reagent grade or better and were purchased from Sigma, Fisher Scientific, or BDH (Toronto, ON).

3. Cell Culture

S49.1 murine lymphomas (American Type Culture Collection, Rockville, MD; TIB 28) [351] and SV40 transformed African Green monkey kidney cells, COS-7 (ATCC #CRL 1651) [352] were grown at 37 °C in Dulbecco's modified Eagle's medium (Gibco-Bethesda Research Laboratories, Burlington, ON) with 10 % heat-inactivated fetal calf serum (Gibco-Bethesda Research Laboratories) in a humidified atmosphere containing 5 % CO_2. COS cells were removed from plastic flasks by trypsinization and replated on 18 x 18 mm glass coverslips (grade appropriate for immunofluorescence) 24-48 h before *in vitro* import (see below).

4. Peptide Synthesis

Nuclear localization signals and other synthetic peptides used in this study are listed in Table 4. The GR NLS, SV40 large T-antigen NLS (SV40 NLS) and the GR NLS harboring a single amino acid substitution (GR NLS-T) were commercially synthesized by IAF BioChem International Inc. (Baie d'Urfé, QC). A long form GR NLS peptide (GR NLS-L) with an acetylated N-terminus and the thyroid hormone receptor (TR) NLS peptide were synthesized by AminoTech (Nepean, ON). The purity of the peptides was judged by HPLC (all peptides were greater than 80 % pure) and composition was confirmed by amino acid analysis. The other synthetic peptides used in the competition assay were from Sigma except for the rat B-50 fragment which was a gift from Dr. H. Zwiers, University of Calgary (Calgary, AB).

5. Radio-iodination of Synthetic Peptides

The peptides were radiolabelled using IODO-GEN^{TM} (Pierce Chemical Co., Rockford, IL) [353]. Briefly, 30 μg of peptide was added to a glass tube coated with 3 μg of IODO-GEN that contained 150 μCi of Na^{125}I in NaOH which had been neutralized with an equivalent amount of HCl and buffered at pH 7.4 in 50 mM sodium phosphate. After 10 min at room temperature, the mixture was decanted to a clean tube to stop the reaction. The mixture volume was brought up to 1 ml with crosslinking buffer which contained unlabeled NaI (see below), and was divided between four Sephadex columns. Unincorporated Na^{125}I was removed by spin filtration at 1600 x *g* for 4 min through a 1-ml column of Sephadex G-10 (Pharmacia) equilibrated in crosslinking buffer. Iodinations typically yielded specific activities of 1-2 x 10^6 c.p.m./μg GR NLS peptide, 3.6 x 10^5 c.p.m./μg TR NLS peptide, 7 x 10^5 to 1.3 x 10^6 c.p.m./μg GR NLS-L and 2.4 x 10^6 c.p.m./μg for SV40 NLS, assuming 100 % recovery of the peptides. Counting efficiency of the ^{129}I standard was 76-82 % in the ^{125}I window.

Table 4. Signal and Competitor Peptide Sequences

Synthetic peptides	Peptide sequences	M_r
Signals		
GR NLS-L	Ac-^{505}GMNLEARK*TKKKIKG*YGC-OH[a]	2067
GR NLS	H-CGYGTKKKIKG^{518}-OH[b]	1182
TR NLS	H-CGYGLAKRKLI^{187}-OH[b]	1221
SV40 NLS	H-CGYGPKKKRKV^{132}-OH[b]	1264
GR NLS-T	H-CGYGTKTKIKG^{518}-OH[b]	1155
Competitors		
CD4 FRAGMENT 37-53	H-^{37}KILGNQGSFLTKGPSKL-OH	1788
RAT B-50 FRAGMENT	H-^{38}IQASFRGHITRKKL-OH	1655
EGFR PKC-SUBSTRATE	H-^{650}VRKRTLRRL-OH	1198
CALMODULIN ANTAGONIST	H-^{290}LKKFNARRKLKGAILTTMLA-OH	2274

[a]Receptor sequences are underlined, and signal sequence in italics.
[b]Signal sequences are underlined for rat GR, rat TRβ, and SV40 large T-antigen.

6. Cell fractionation

A. Nuclei

The preparations of rat liver nuclei and nuclear envelopes have been described previously [288] and are based on the methods of Widnell and Tata [354], and Kay *et al.* [355], respectively, with slight modifications. All procedures were carried out at 0-4 °C. Tissue (70-80 g) in 250 ml of 0.32 M sucrose containing 3 mM $MgCl_2$ and 1 mM DTT was homogenized with a Polytron (Beckman Instruments, Rexdale, ON) at setting 3-4 with 30 sec bursts. The homogenate was then filtered twice through four layers of cheesecloth. The filtrate was then diluted to 1 liter and centrifuged at 4000 x g for 20 min. The pellet was resuspended in 2.4 M sucrose containing 1 mM $MgCl_2$ and 1 mM DTT, adjusted to pH 7.4 with $NaHCO_3$, and centrifuged at 50 000 x g for 60 min. For nuclei import assays (see below), nuclei were frozen in import buffer with 50 % (v/v) glycerol at -20 °C.

B. Nuclear Envelope

Nuclear envelopes were then prepared from the purified nuclear pellet by washing in freshly prepared 0.25 M sucrose containing 1 mM $MgCl_2$ and 1 mM DTT, adjusted to pH 7.4 with $NaHCO_3$, and centrifuged at 750 x g for 5 min. The nuclei were resuspended in digestion buffer (10 mM Tris, 0.30 M sucrose, 0.1 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH 8.5) at 4 °C to which a freshly made solution of DNAase I was added to give a final concentration of 10 μg/ml. The DNAase I digestion was performed on the bench for 2-3 min and carefully monitored by phase contrast microscopy. When digestion was judged complete, the crude nuclear envelope suspension was immediately centrifuged at 12 000 x g for 6 min. The crude nuclear envelopes were washed with 10 mM Tris containing 1 mM DTT, pH 7.4, spun at 33 000 x g for 10 min and the pellets again resuspended in a minimum volume of 10 mM Tris buffer containing 1 mM DTT, pH 7.4. This suspension was layered onto six

discontinuous sucrose gradients consisting of 4.5 ml of 0.25 M sucrose and 9.5 ml each of 1.5, 1.8, and 2.0 M sucrose (all sucrose solutions made up in 10 mM Tris containing 1 mM DTT, pH 7.4). The major band of purified nuclear envelope was removed from the 1.5/1.8 M sucrose interface with a Pasteur pipette, although some material (fractured nuclear envelope) was recovered from the 0.25/1.5 M interface. The nuclear envelopes were then washed by resuspension in 25 mM TAPS containing 1 mM DTT, pH 8.6 (brought to the required pH with ammonium hydroxide) or 10 mM Tris, pH 7.4, containing 1 mM DTT and centrifuged at 33 000 x g for 10 min. Purified nuclear envelopes were then resuspended in a small volume of 25 mM TAPS, pH 8.6, containing 1 mM DTT or 10 mM Tris, pH 7.4, containing 1 mM DTT and immediately frozen in liquid nitrogen.

Nuclei and nuclear envelopes used in crosslinking studies were resuspended in crosslinking buffer (0.25 M sucrose, 10 mM HEPES-KOH pH 7.4, 25 mM KCl, 3 mM $MgCl_2$, 5 mM NaI) and used fresh or stored at -80 °C after rapid freezing in liquid nitrogen, with no difference in the crosslinking results (data not shown). Note that buffers containing free amines such as Tris are not compatible with the crosslinking agents used in this study.

C. Cytosol

S49 lymphoma cells from 1 liter of confluent culture (approximately 10^9 cells) were washed in three changes of PBS (10 mM sodium phosphate buffer pH 7.4, 130 mM NaCl) and lysed in two cell pellet volumes of hypotonic buffer (10 mM HEPES-KOH pH 7.4, 25 mM KCl, 3 mM $MgCl_2$, 5 mM NaI) by freeze-thaw. The lysate was cleared by spinning for 15 min at 10 000 x *g* at 4 °C. The supernatant is referred to as the low speed cytosol which was further centrifuged at 100 000 x *g* for 1 h at 4 °C to obtain a high speed cytosol devoid of any particulate matter.

D. Mitochondria

Mitochondria from rat liver were prepared by differential centrifugation exactly as described by Fleischer and Kervina [356].

7. Crosslinking

Crosslinking conditions were modifications of previously reported methods [358]. In preliminary experiments we confirmed the findings of Adam *et al.* [357] that detergent- and high salt-extraction of nuclei and nuclear envelopes enhanced crosslinking of the GR NLS to these subfractions, whereas extraction did not affect crosslinking to cytosolic fractions (see Figure 2A). Therefore, in typical experiments, nuclei or nuclear envelopes (50-100 μg protein) in as small a volume of crosslinking buffer as possible were incubated with an equal volume of 2 % octyl-β-D-thioglucopyranoside (Calbiochem, La Jolla, CA) and 600 mM KCl for 1 h on ice. To nuclear and nuclear envelope extracts diluted six-fold in crosslinking buffer, or to cytosol (50-100 μg) in crosslinking buffer, was added approximately 1 x 10^6 c.p.m. of ^{125}I-labeled NLS peptide (approximately 1.0 μg protein) with or without a 10- to 100-fold excess of unlabeled competing peptide, and incubated for 30 min at room temperature or 33 °C in the presence or absence of 2 mM $CaCl_2$, 3 mM $NaHCO_3$ with or without 1 mM ATP (Pharmacia). The samples were then cooled on ice for 5 min. In most experiments, the crosslinker BS^3, bis(sulfosuccinimidyl)suberate, (Pierce Chemical Co.) was added to a final concentration of 0.50 mM. Experiments in which it was necessary to reduce volumes, crosslinking at room temperature was terminated after 15 min by incubation with 10 % trichloroacetic acid on ice for at least 5 min to precipitate proteins. Recovery of precipitates did not appear to differ if samples were on ice for 5 min or 120 min (data not shown). The precipitates were pelleted by microcentrifugation for 3 min, then washed twice with 95 % acetone, dried before resuspension in sample buffer and heat denatured for 3 min at 100 °C. Alternatively,

the crosslinking reaction was stopped by quenching with SDS-PAGE sample buffer which was either 2x Laemmli buffer (0.12 M Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 10 % β-mercaptoethanol, 0.002 % bromophenol blue) [358] or 10x Phorcast^{TM} sample buffer (Amersham; 0.5 M ammediol-0.5 M glycine, pH 9.5, 10 % β-mercaptoethanol, 10 % SDS, 0.1 % bromophenol blue, 30 % glycerol) and then samples were heat-denatured and applied to the gels. The two stopping procedures yielded identical amounts of crosslinked products. Samples were run on 7.5 % SDS-PAGE [358] or on 11-23 % gradient gels (Amersham) according to the manufacturer's instructions. Gels were stained with Coomassie blue and dried before autoradiography with an intensifying screen (Dupont Cronex, Lightning-Plus^{TM}) at -80 ^{o}C using pre-flashed film [359]. Autoradiographs were quantified by scanning with a laser densitometer (LKB UltroScan XL, Pharmacia).

8. DNA Preparation

Plasmid DNA was prepared by a standard alkaline-lysis maxi-preparation protocol [360, 361], by first transforming competent *E.coli* DH5α cells [362]. The DNA was purified by twice banding on cesium chloride gradients [363]. This procedure yielded DNA that was greater than 95 % supercoiled as judged by ethidium bromide staining of an agarose gel electrophoresis of the DNA (data not shown).

9. Coupled *In Vitro* Transcription-translation of GR

GR protein was produced according to the manufacturer's specifications from a commercial kit (TNT, Promega) for one-step coupled *in vitro* transcription and translation programmed with plasmid DNA (kindly provided by Dr. Keith Yamamoto, San Francisco, CA), coding for GR and its derivatives under the control of the SP6 or T7 RNA polymerase promoter. The DNA substrate (1 μg, circular plasmid) was added to a tube containing 25 μl of TNT rabbit reticulocyte lysate, 2 μl of 20x TNT reaction

buffer, 1 µl of SP6 or T7 RNA polymerase, 1 µl of 1 mM amino acid mixture minus methionine, 4 µl of [^{35}S]-methionine (at 10 mCi/ml), 1 µl of RNasin (40 U/µl) in a final volume of 50 µl. The mixture was incubated for 2 h at 30 °C, after which 5 mM unlabeled methionine, 8-10 % glycerol, and 2 mM DTT, were added with or without 20 mM Na_2MoO_4 (final concentrations). The samples were centrifuged for 15-20 min at 354 000 x g (100 000 rev./min) in a TLA100 rotor on a tabletop ultracentrifuge (Beckman) at 4 °C to remove ribosome-bound counts and the supernatant was stored at -80 °C. Incorporation of label was assessed by either gel electrophoresis and fluorography, or by trichloroacetic acid precipitation of the labeled products and measurement of the radioactivity in a scintillation counter. The trichloroacetic acid precipitation protocol followed was that outlined in the TNT coupled transcription-translation kit. Incorporation of [^{35}S]-methionine was typically 10-15 % as measured by the trichloroacetic acid precipitation method. The full-length GR (amino acids 1-795) has twenty-four methionyl residues, while NL2 (GR amino acids 547-793 plus BuGR epitope 407-423 at C-terminus) has fifteen, and luciferase has fourteen. Efficiency of ^{35}S counting was 69-72 %.

10. Transformation of GR

Transformation of translated GR and its derivatives was by ligand binding with heat treatment [104], or heat alone [364], or high salt [365] as follows. For transformation purposes, translation products without added molybdate were used. Translation product mixtures were either incubated with 100-1000 nM dexamethasone for 2 h at 4 °C, then heated to 25 °C for 30 min (ligand transformation), or heated to 25 °C for 30 min (heat transformation), or receptor was treated with 0.3 M NaCl (salt transformation).

11. Sucrose Density Gradient Measure of GR Sedimentation (Transformation)

Sucrose density gradients were prepared by layering four concentrations of sucrose solution in 5-ml tubes and allowing a linear gradient to form by diffusion (see [366, 367]). Stock solutions of sucrose (10, 15, 20, 25 % (w/v)) were prepared in homogenization buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 10 % glycerol) with or without 20 mM sodium molybdate (Na_2MoO_4), and with or without 0.3 M NaCl. When required, the molybdate was added to maintain the receptor in an untransformed state, while the NaCl was added to transform the receptor. It is important to use NaCl and not KCl for the specific assay used here because K^+ causes the SDS to precipitate during gel electrophoresis. To prepare the gradient, 1.1 ml of each of the four different sucrose solutions were layered in a centrifuge tube. Linearization of the gradient occurred after 16-24 h at 4 °C. The protein sample (100 µl) was then layered on the top of the gradient and the gradients were centrifuged for a minimum of 16 h at 4 °C at 55 000 rev./min in a SW55Ti rotor (Beckman). After the centrifugation the tubes were fractionated by collecting two-drop fractions from the bottom of the tube in a sealed chamber under positive pressure supplied by a peristaltic pump pumping air. The linearity of the gradient was verified by checking the refractive index of the sucrose in the fractions on a refractometer (Fisher Scientific). External and internal sedimentation standards labeled by reductive methylation [368], were also run in parallel. ^{35}S-labeled GR derivatives (6 µl in 100 µl import buffer) were layered on to the gradient either in the presence or absence of molybdate or in the presence or absence of high-salt concentration to determine the state of the receptor and the effects of transformation. After centrifugation and fractionation, the fractions were run on SDS-PAGE and the gels fluorographed by incorporation of PPO into the gel. The position (fraction number) of the hemoglobin internal standard was observed by eye as it was the only pigmented protein detectable.

12. Gel Fluorography

The detection of some low-energy emitting radionuclides such as ^{35}S is enhanced by incorporation of fluor directly into the gel [369]. The gel was soaked in glacial acetic acid for 15 min, and then placed in 20 % (w/v) PPO in glacial acetic acid for another 15 min. The gel was then washed extensively with water to precipitate the PPO in the gel and to remove the glacial acetic acid prior to drying the gel under vacuum (no or low heat preferred). Exposures were done at -80 °C.

13. Nuclear Binding Assay

The method was adapted from an assay for the uptake of $^{45}Ca^{++}$ into rat liver nuclei [166]. Lysate (3-25 µl) containing the translated receptor in transformed or untransformed states was incubated for 30 min under various conditions (4 or 30 °C, with or without 4 mM ATP) with purified rat liver nuclei (50 µg) in import buffer (0.25 M sucrose, 50 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 25 mM KCl with 2 mM DTT freshly added), in a final volume of 50 µl. One ml of ice-cold buffer was added to stop the reaction and unbound protein was separated from the bound fraction by filtration through a 13-mm 0.45 µm pore hydrophilic PVDF-membrane (Durapore, from Millipore) in a 13-mm Swinnex disk filter holder. The filter with the retained nuclei was then washed with another 4 ml of cold buffer. The nuclear-bound radioactivity was quantified by scintillation counting of the filters.

14. Bacterial Expression of GR DNA-binding Domain with NL1

The method for overexpression of the GR sequence, encoding amino acids 407-556, with a linker at the C-terminus encoding 14 additional nonreceptor residues, under the control of a T7 RNA polymerase promoter (plasmid T7X556) was as described by Yamamoto and co-workers [370, 371] with minor modifications. Plasmid T7X556 was used to transform BL21(DE3)/pLysS, an *E. coli* stain carrying a stable integrant of T7

gene 1 (RNA polymerase) under the control of the *lac* UV5 promoter. T7 RNA polymerase was induced at mid-logarithmic growth (A_{600}=0.6) by addition of IPTG to 0.5 mM, resulting in expression of T7X556. Three hours after induction, cells were collected by centrifugation. After freezing and thawing, cell pellets were resuspended in three volumes of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 % glycerol, 5 mM DTT, 500 mM NaCl). Lysozyme was added to a concentration of 100 µg/ml, then Triton X-100 was added to a final concentration of 0.1 % and incubated for 30 min on ice. The lysate was sonicated on ice with a microtip to shear the DNA, then centrifuged at 12 000 x g for 20 min at 4 °C. Ammonium sulphate was added to the supernatant to 15 % saturation, and the precipitate was removed by centrifugation at 12 000 x g for 10 min at 4 °C. The supernatant was brought to 30 % saturation with ammonium sulphate and the precipitate collected by centrifugation, dissolved in TEGDZ50 buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 10 % glycerol, 5 mM DTT, 50 µM $ZnCl_2$, 50 mM NaCl), and dialysed against the same buffer. Extracted protein concentrations were approx. 8-10 mg/ml, with the receptor derivative comprising approx. 10 % of the total protein. The extract was stored at -80 °C.

15. Digitonin-permeabilized Cell Nuclear Import Assay

Digitonin permeabilization of COS cells on coverslips and *in vitro* import reactions were carried out according to Adam *et al.* [372-374]. Rabbit reticulocyte lysate (Promega) was extensively dialyzed against import buffer (20 mM HEPES pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM DTT, and 1 µg/ml each aprotinin, leupeptin, and pepstatin) and the diffusate was centrifuged at 100 000 x g prior to freezing for storage. COS cells on coverslips in six-well plastic tissue culture dishes were rinsed in cold import buffer followed by immersion in buffer containing 40 µg/ml digitonin (Calbiochem; diluted from a 20 mg/ml stock solution in DMSO). The cells were allowed to permeabilize for 5 min at 4 °C after

which the digitonin containing buffer was aspirated and replaced with cold import buffer and the coverslip inverted onto a 50-µl drop of complete transport mixture (import buffer, cytosol, ATP-regenerating system, and nuclear substrate) on a sheet of parafilm in a humidified box, and incubated for 5-30 min at 30 °C. The complete transport mixture contained 100 nM allophycocyanin-NLS conjugate (kindly provided by Dr. Stephen Adam, Chicago, [372]) or GR import substrate (6 µl crude *E. coli* extract preincubated for 30 min at room temperature with 1 µg of BuGR anti-GR antibody) in 50 % dialyzed reticulocyte lysate diluted with import buffer in the presence or absence of 0.5 mM ATP and an ATP-regenerating system (5 mM creatine phosphate, 20 U/ml creatine phosphokinase). At the completion of the incubation, the coverslips were rinsed in import buffer and fixed with 3 % formaldehyde in import buffer for 10 min. Rinsing the samples before fixation removes debris that may have accumulated during the incubation and does not affect the nuclear fluorescence signal. Each coverslip was mounted on a glass microscope slide in a small amount of 50 % glycerol in phosphate-buffered saline and the coverslip edges sealed with nail polish.

16. Immunofluorescence Microscopy

Samples were observed at 400x magnification by phase contrast and epifluorescence with a Zeiss IM-35 or Axiophot microscope equipped with a 40x planapochromat objective. For photography, fields were chosen at random, and photographed with Kodak T-Max film (ASA 1600).

17. Western Analysis with ECL (Enhanced Chemiluminescence) Detection

Samples were electroblotted to PVDF membranes based on the procedure of Towbin *et al.* [375]. Prior to electroblotting the gels were soaked in transblot buffer (192 mM glycine, 25 mM Tris pH 8.3, containing 20 % methanol and 0.1 % SDS), for 20-

30 min. The PVDF membrane is wetted for 1 sec in methanol, rinsed in water, and soaked in transblot buffer for 20-30 min. Whatman 3MM filter paper and Scotch-Brite foam pads were soaked in buffer and a sandwich made with a foam pad, 3-4 filter papers, the gel, the PVDF membrane, 3-4 filter papers and another foam pad. Care was taken to avoid trapping air bubbles between the layers. The sandwich was placed into the Bio-Rad transblot cell with the gel closest to the cathode (-) and the PVDF membrane closest to the anode (+). Transblotting was done overnight in a cold room at 30-35 V and then for 30 min at 70-100 V. The PVDF membrane was removed from the sandwich for further processing. The PVDF membrane must not be air-dried because it would require wetting in 100 % methanol which may denature some epitopes. The ECL detection system (Amersham) is highly sensitive and prone to problems with non-specific binding of the antibodies. This is resolved by using low concentrations of primary and secondary antibodies, by blocking the membrane with 10 % powdered skim-milk, and by using detergent and high-salt to disrupt non-specific binding. After transfer, the non-specific binding sites were blocked by immersing the membrane in 10 % dried milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris-HCl pH 7.6, 500 mM NaCl, 0.05 % Tween) for 1 h at room temperature on a slow rocking platform. The membrane was briefly rinsed using two changes of TBS-T, then washed once for 15 min and twice for 5 min with fresh changes of TBS-T at room temperature. The membrane was incubated with diluted primary antibody for 1-2 h at room temperature (1:2000 dilution of BuGR). The membrane was washed as above, and then incubated with the diluted horseradish peroxidase-labeled secondary antibody for 1 h at room temperature (1:50 000 dilution of sheep anti-mouse immunoglobulin). The membrane was again washed as above. The excess buffer was drained from the blot and placed in a fresh container. The detection reagent (1:1 mix of reagents provided with kit; contains luminol, the light emitting compound once it is attacked by the free radicals generated by horseradish peroxidase from H_2O_2) was

added directly to the blot on the surface with the protein. The blot is incubated for precisely 1 min. The excess detection reagent was drained off and the blot placed between two acetate sheets in an autoradiography cassette with X-ray film (Kodak, X-RP) for a brief exposure.

18. Protein Determination and Gel Electrophoresis

Protein content was determined by the method of Lowry *et al.* [376], using BSA as a standard. Linearity with respect to protein concentration was obtained between 0-100 μg BSA (fraction V) in 500 μl of ddH_2O. Color development was for 30-35 min. Absorbance of tubes was read at 660 nm.

SDS-PAGE was performed using the discontinuous buffer system of Laemmli [358] or samples were run on pre-cast gradient gels (11-23 %) using the Phorcast™ gel system from Amersham according to the manufacturers specifications. Samples in SDS-sample buffer (63 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS (w/v), 0.05 % β-mercaptoethanol (v/v) and 0.0013 % bromophenol blue (w/v)) or in Phorcast sample buffer (50 mM ammediol- 50 mM glycine pH 9.5, 1 % β-mercaptoethanol, 1 % SDS, 0.01 % bromophenol blue, 3 % glycerol) were denatured by heating for 5 min at 96 °C. Samples were run at 20 mA/gel to stack for 20-60 min, and then at 30 mA/gel to separate for 4-8 h.

Gels were stained for 1 h in a solution of 0.2 % Coomassie Brilliant Blue R-250, 10 % acetic acid and 45 % methanol and destained by three or more changes of a solution of 10 % acetic acid and 25 % methanol with gentle shaking.

III. RESULTS

(i) Identification of NL1-binding Proteins by Chemical Crosslinking

1. Protocol

In the experiments described below, cellular NLS-binding proteins were identified by covalent binding of radiolabeled synthetic NLS peptide with a chemical crosslinking agent after incubation of the NLS peptide with subcellular fractions.

The GR and thyroid hormone receptor (TR) NLS peptides were chosen by sequence similarity to the NLS of SV40 large T-antigen [158, 159]. The sequence similarity search using the Microgenie program (Beckman) was set to detect a minimum of three identical amino acids out of seven of the SV40 NLS while maintaining at least 50 % of the charge identity. The results of this search are shown in Table 5 for the various human nuclear receptors (for simplicity). Similar or identical sequences exist in the receptor homologues from other species. For example, the GR NLS sequence is identical in rat, mouse and human receptors. A sequence similar to the NLS of the SV40 large T-antigen can be found in the hinge region for all the nuclear hormone receptors shown. The corresponding synthetic peptides used here are listed in Table 4 (in Materials and Methods section).

2. Preliminary Experiments

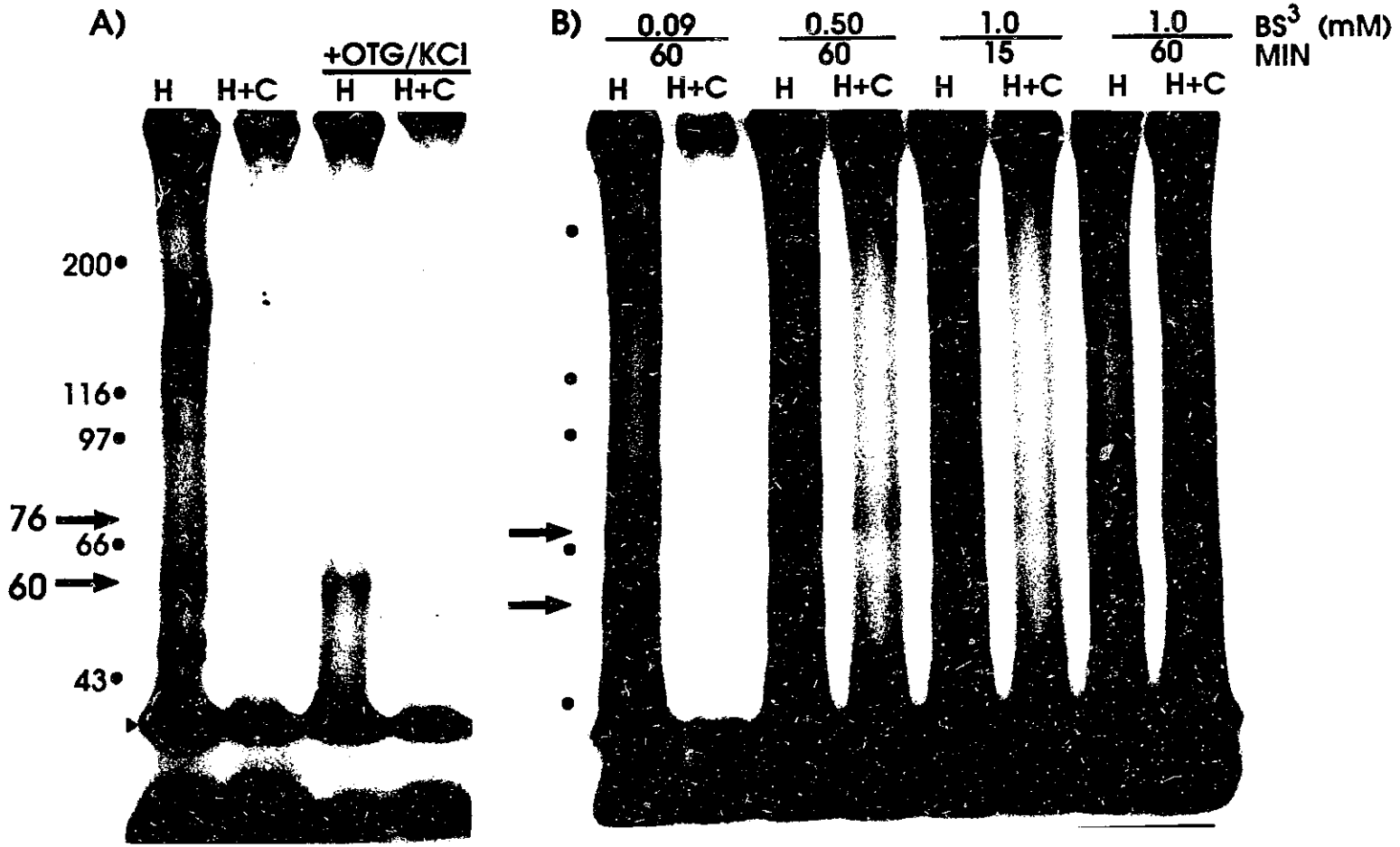
In preliminary work we assessed crosslinking at various concentrations from 0.09 to 1.0 mM BS^3 crosslinker and found that the only difference in results was a slight improvement in the signal to noise ratio at 0.5 mM compared to lower and higher concentrations of the crosslinker as determined by densitometric scanning of the autoradiogram (Fig. 2B). The addition of a detergent- and high salt-extraction improved the resolution of crosslinked nuclear products on SDS-PAGE (Fig. 2A)

Table 5. Potential Minimal NLS in Steroid and Nuclear Hormone Receptors

	Sequence	References
SV40 large T-antigen	^{126}PKKKRKV^{132}	[377]
RECEPTOR		
Human glucocorticoid	^{493}TKKKIKG^{499}	[378]
Human mineralocorticoid	^{675}SKKLGKL^{681}	[379]
Human progesterone	^{639}FKKFNKV^{645}	[380]
Human estrogen	^{263}RMLKHKR^{269}	[381]
	^{298}IKRSKKN^{304}	
Human androgen	$^{627/628}$ARKLKKL$^{633/634}$	[382, 383]
Human thyroid hormone		
Type α	^{132}VAKRKLI^{138}	[384, 385]
Type β	^{181}LAKRKLI^{187}	[30]
Human vitamin D	^{106}MILKRKE^{112}	[32]
Human retinoic acid		
Type α	^{162}RNKKKKE^{168}	[34]
Type β (hap)	^{155}RNKKKKE^{161}	[33, 36]

Figure. 2. Crosslinking of GR NL1 to Rat Liver Nuclei

A. Nuclei (50 μg) were extracted or not with 1 % octyl-β-D-thioglucopyranoside and 0.3 M KCl (OTG/KCl) for 1 h on ice. Samples were incubated with 2.4 μM [^{125}I]-GR NLS in the presence (H+C) or absence (H) of 25 μM unlabeled GR NLS peptide, and then crosslinked for 30 min at room temperature with 0.09 mM BS^3. B. Nuclei (extracted as above, 50 μg) were incubated with 2.5 μM [^{125}I]-GR NLS with (H+C) or without (H) 264 μM unlabeled GR NLS and crosslinked with increasing concentrations of BS^3 (0.09, 0.5, and 1.0 mM) for 15 or 60 min at room temperature.


A)
+OTG/KCl
H
H+C
H
H+C
200
116
97
76
66
60
43
B)
0.09
60
0.50
60
1.0
15
1.0
60
BS3 (mM)
MIN
H
H+C
H
H+C
H
H+C
H
H+C

In preliminary experiments, crosslinked products were quantified after incubation of the GR NLS-L with S49 lymphoma cytosol for 30 and 60 min and it was demonstrated that by 30 min, equilibrium binding had occurred as there was no increase in the intensity of the crosslinked products after 60 min of binding (data not shown). The addition of a detergent- and high salt-extraction step did not improve the resolution for crosslinked cytosolic polypeptides (data not shown).

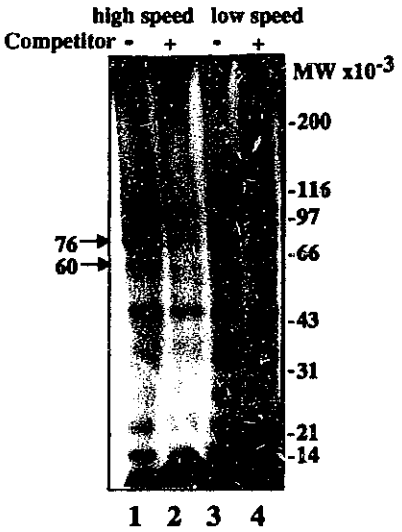
3. Crosslinking of the GR NLS to S49 Lymphoma Cytosol

We investigated crosslinking to cytosol prepared from the mouse lymphoma cell line S49, a well characterized cell line with respect to its responsiveness to glucocorticoids [351, 386]. Figure 3A shows the results of crosslinking of GR NLS to cytosolic fractions obtained after high and low speed centrifugations. In both cases, labeled crosslinked products were identified at 100, 76, 60, 45, 35, 21, and 14 kDa. The specificity of the binding was assessed by competition with excess unlabeled peptide. Based on this criterion, the 60- and 76-kDa polypeptides in the cytosolic fraction were judged to be specifically labeled. As specificity was difficult to establish in the fraction obtained after low speed centrifugation further experiments were performed on high speed fractions. Despite the fact that yields of binding proteins are low and detection is difficult, in all crosslinking experiments (n=13) carried out, labeling of the 76- and 60-kDa entities was found to demonstrate specificity by this criterion. The molecular masses of these two crosslinked polypeptides calculated from these gels were 78 $\pm$ 3 kDa (mean $\pm$ SD, n=13) and 61 $\pm$ 4 kDa (n=12), which includes the mass of the NLS peptide. The specificity of labeling of the other crosslinked products was inconsistent. Thus, although identification of other NLS-binding proteins may be confirmed in the future, in this thesis I focus on the further characterization of the 76- and 60-kDa NLS-binding proteins.

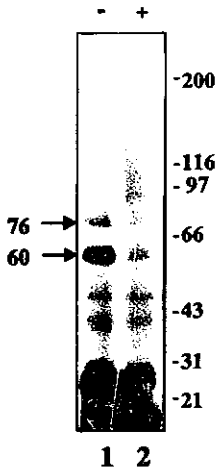
Figure 3. Crosslinking of the GR NLS to S49 Lymphoma Cytosol

Subcellular fractions were incubated with [^{125}I]-GR NLS in the presence or absence of unlabeled GR NLS in crosslinking buffer with 2 mM $CaCl_2$, 3 mM $NaHCO_3$ and 1 mM ATP for 30 min at 33 °C. Subfractions were subsequently incubated with 0.5 mM BS^3 for 15 min at room temperature. Samples were then run on SDS-PAGE before autoradiography, as described in the *Materials and Methods* section. A, Low and high speed cytosols were isolated from mouse S49 lymphoma cells, as described in *Materials and Methods*. Low speed cytosol (100 μg) and high speed cytosol (82 μg) were incubated with 0.7 μM [^{125}I]-GR NLS in the presence (+) or absence (-) of 58 μM GR NLS (15-day exposure). B, Effect of a longer form of GR NLS (GR NLS-L) on crosslinking to S49 cytosol. High speed cytosol (50 μg) was incubated with 10.6 μM [^{125}I]-GR NLS-L in the presence (+) or absence (-) of 106 μM GR NLS-L (3-day exposure). C, Coomassie blue-stained gel of S49 cytosol (50 μg protein) after crosslinking with [^{125}I]-GR NLS-L with (+) or without (-) competitor GR NLS-L peptide. Std, molecular weight (MW x 10^{-3}) standards (Bio-Rad) approximately 2 μg protein per band, which correspond to myosin (200 000), β-galactosidase (116 250), phosphorylase b (97 400), BSA (66 200), ovalbumin (42 699), carbonic anhydrase (31 000), trypsin inhibitor (21 500), and lysozyme (14 400).

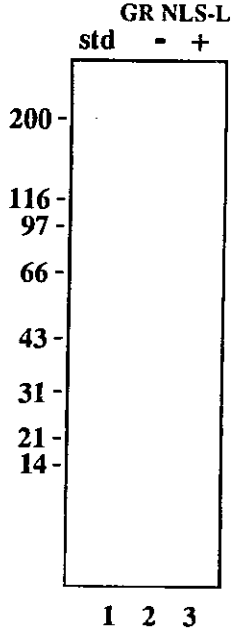
A) GR NLS



B) GR NLS-L



C) COOMASSIE BLUE



A minimal GR NLS similar to the NLS of SV40 large T-antigen was used for most experiments. A longer GR NLS, containing NLS-flanking amino acids of the receptor, was also used to identify NLS-binding polypeptides because of the increasing body of evidence that structural requirements outside of the basic stretch of amino acids play a role in nuclear transport [260, 261, 387] and to attempt to ensure that the peptide was in the correct conformation. A peptide, referred to as GR NLS-L, with the following characteristics was synthesized: it contained an additional eight amino acids at the N-terminus of the NLS (these eight amino acids demonstrate a high propensity for α-helical formation based on secondary structure prediction plots); its N-terminal group was acetylated to more closely resemble the *in vivo* situation; although the charge density has been altered, the net charge is identical to that of the original NLS; and, spacer glycines and other additional residues were moved from the amino- to the carboxy-terminus. The pattern of crosslinking obtained with this peptide is shown in Figure 3B and is identical to that obtained with the shorter NLS, confirming that at least this flanking region did not alter the interaction with the binding proteins. Further studies will address whether other parameters of the interaction are altered by the presence of these flanking amino acids.

Figure 3C shows the Coomassie blue staining pattern of an SDS-PAGE gel of the S49 lymphoma cytosol after crosslinking with the GR NLS-L. The stained gel demonstrates two points: first, the 60- and 76-kDa polypeptides do not correspond to major cytosolic proteins, which are at 93 and 44 kDa; and second, equal amounts of protein have been loaded to each lane of the gel. Thus, the specific binding, as judged by competition with excess unlabeled peptide, is not due to either labeling of major proteins or a loading artifact. The lessening of the intensity of the material that barely enters the gel in the presence of excess unlabeled peptide was often observed (Fig. 3B). On those occasions when this effect was not observed, less crosslinked product was obtained. Therefore, we interpret the lessening of the

intensity of the material that barely enters the gel by added peptide in this way; crosslinking also promotes the formation of higher molecular weight entities containing the specifically labeled products. Thus, excess peptide that specifically competes for NLS crosslinking interferes with this labeling as well.

4. Dose Dependence of GR NLS Binding to S49 Cytosol

Varying concentrations of [^{125}I]-GR NLS-L (from 48 nM to 2.4 μM), were incubated with S49 cytosol for 30 min, then crosslinked, run on SDS-PAGE and autoradiographed. The films were scanned and quantified by laser densitometry. A duplicate gel was sliced and counted in a γ-counter to confirm the densitometry readings. Figure 4 is the binding curve for the 76- and 60-kDa crosslinked products. The 60- and 76-kDa sites demonstrated saturable binding between 1.0-1.5 μM for [^{125}I]-GR NLS-L. These results also show that in the case of the S49 lymphoma cytosol the quantities of crosslinked 60-kDa products were greater than those of the 76-kDa products.

5. Binding Specificity of GR NLS to S49 Cytosol

Analysis of competition for the binding of iodinated GR NLS-L to the S49 lymphoma cytosolic 60- and 76-kDa polypeptides was then conducted by crosslinking in the presence of ten- to twenty-fold excess unlabeled peptide. Densitometric scanning was used to determine the order of potency of the competitors. The abilities of two other similar NLSs, the thyroid hormone receptor NLS (TR NLS) and the SV40 large T-antigen NLS (SV40 NLS), were compared to the ability of unlabeled GR NLS and GR NLS-L to compete for the binding of the radiolabeled GR NLS-L to the 76- and 60-kDa entities. Figure 5 shows the results of competition for the binding of the GR NLS-L to the 76- and 60-kDa sites. The pattern of competition for both sites was similar. GR NLS is the best competitor for the binding of [^{125}I]-GR NLS-L to the 76-

Figure 4. Dose Dependence of [^{125}I]-GR NLS Binding to S49 Lymphoma Cytosol

Concentrations of [^{125}I]-GR NLS-L from 0.048-2.4 μM were incubated for 30 min with high speed cytosol (100 μg) from S49 lymphoma, and then crosslinking was carried out with 0.5 mM BS^3 for 15 min at room temperature. The reactions were quenched with sample buffer. The gel was autoradiographed and then scanned on a laser densitometer. Peak heights were measured. Data from two experiments are shown (■ and ●) and were confirmed by counting gel slices from a third experiment.

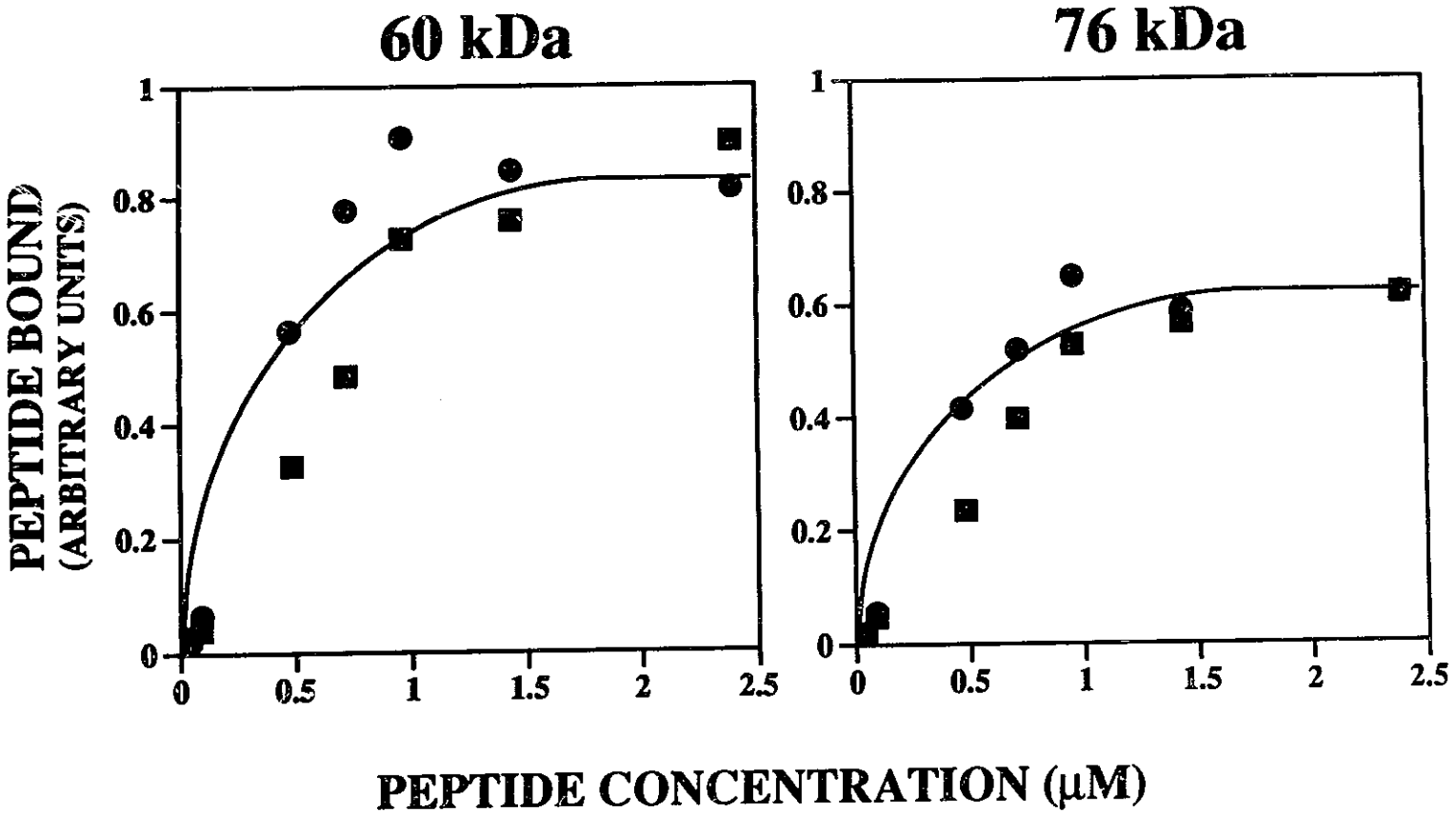
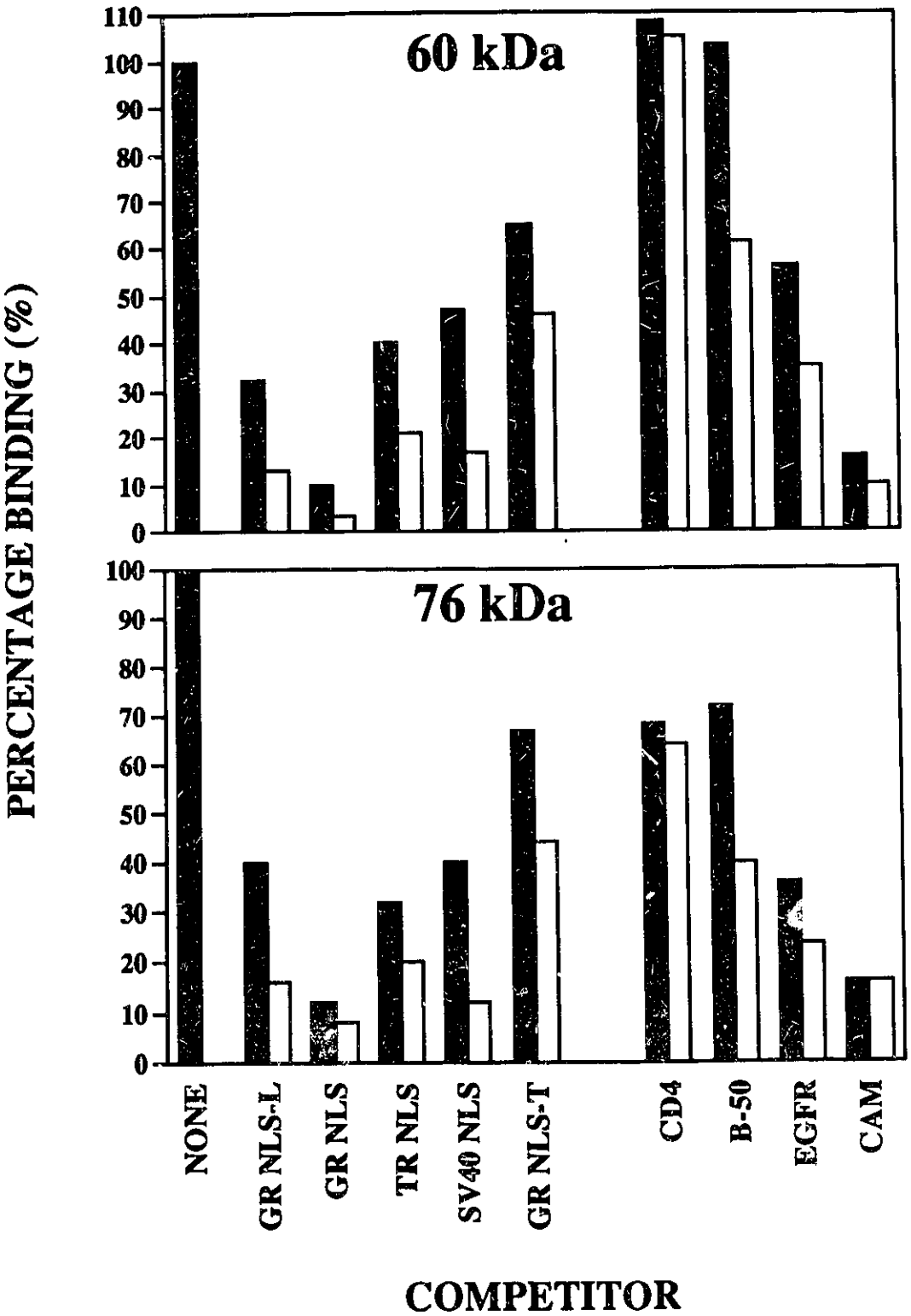

60 kDa
76 kDa
PEPTIDE BOUND
(ARBITRARY UNITS)
0
0.2
0.4
0.6
0.8
1
0
0.5
1
1.5
2
2.5
PEPTIDE CONCENTRATION (μM)

Figure 5. Specificity of Binding of the GR NLS to the NLS-binding Proteins

High speed cytosol (50 μg) isolated from S49 lymphoma was incubated with 10.6 μM [^{125}I]-GR NLS-L (specific activity, 1.3 x 10^5 c.p.m./μg) in the presence of 106 μM (10-fold, black bars) or 213 μM (20-fold, white bars) unlabeled competitor peptide. Total binding in the absence of unlabeled competitor peptide was set at 100 % binding (single black bar). The competitors used were the NLS peptides (GR NLS-L, GR NLS, TR NLS, SV40 NLS, and GR NLS-T), a fragment of the CD4 coreceptor (CD4), a fragment of the rat neuromodulin protein (B-50), a fragment of the EGF receptor (EGFR) and a calmodulin antagonist (CAM). After crosslinking, radiolabeled products were identified by autoradiography after application of samples to SDS-PAGE and then scanned on a laser densitometer. Peak heights were measured and used to plot each band separately.


60 kDa
76 kDa
PERCENTAGE BINDING (%)
110
100
90
80
70
60
50
40
30
20
10
0
NONE
GR NLS-L
GR NLS
TR NLS
SV40 NLS
GR NLS-T
CD4
B-50
EGFR
CAM
COMPETITOR

and 60-kDa polypeptides, and it appears to be an even better competitor than unlabeled GR NLS-L. Unlabeled TR and SV40 NLSs are also effective competitors for the binding to both the 76- and 60-kDa entities.

The specificity of the binding was also investigated (Fig. 5) with a mutant GR NLS (GR NLS-T), which would be expected, on the basis of altered charge and/or structure, not to be as efficient a mediator of nuclear import. Although not proven for GR, this mutation in the SV40 NLS has been shown to dramatically reduce nuclear import [153-155]. Consistent with this, the GR NLS-T was not an effective competitor for the binding of the GR NLS-L to either polypeptide.

The specificity of the binding was further investigated with CD4, a seventeen-amino acid fragment of the CD4 molecule, chosen because, like GR NLS-L, it has a positive charge, but, unlike NLSs, the lysyl residues are dispersed within the fragment. Other peptides that contain clusters of basic residues but are not known NLSs were also used. The similarity of the NLSs to protein kinase substrates led to the inclusion in this latter category of two protein kinase C substrates, a fragment of the epidermal growth factor receptor (EGFR) and a fragment of neuromodulin (B-50) [388] as well as a calmodulin-dependent kinase substrate (CAM). The results in Figure 5 show that the CD4 peptide does not compete at all for the binding of NLS to the 60-kDa polypeptide and that the EGFR and B-50 peptides do not compete well for the binding of [^{125}I]-GR NLS-L to this NLS-binding protein. However, there is competition for the binding of the NLS peptide to the 76-kDa binding polypeptide by CD4, B-50, and EGFR. Although the competition by a 20-fold excess of competitor CD4, B-50, and EGFR peptide was in no instance as effective as that by the receptor NLSs, it is clear that the amino acid sequence specifying the interaction of the NLS to the 76-kDa binding protein was not as stringent as that to the 60-kDa polypeptide. Interestingly, the calmodulin-dependent kinase substrate is a fairly potent

competitor for the binding of [^{125}I]-GR NLS-L to the 76- and 60-kDa NLS-binding proteins.

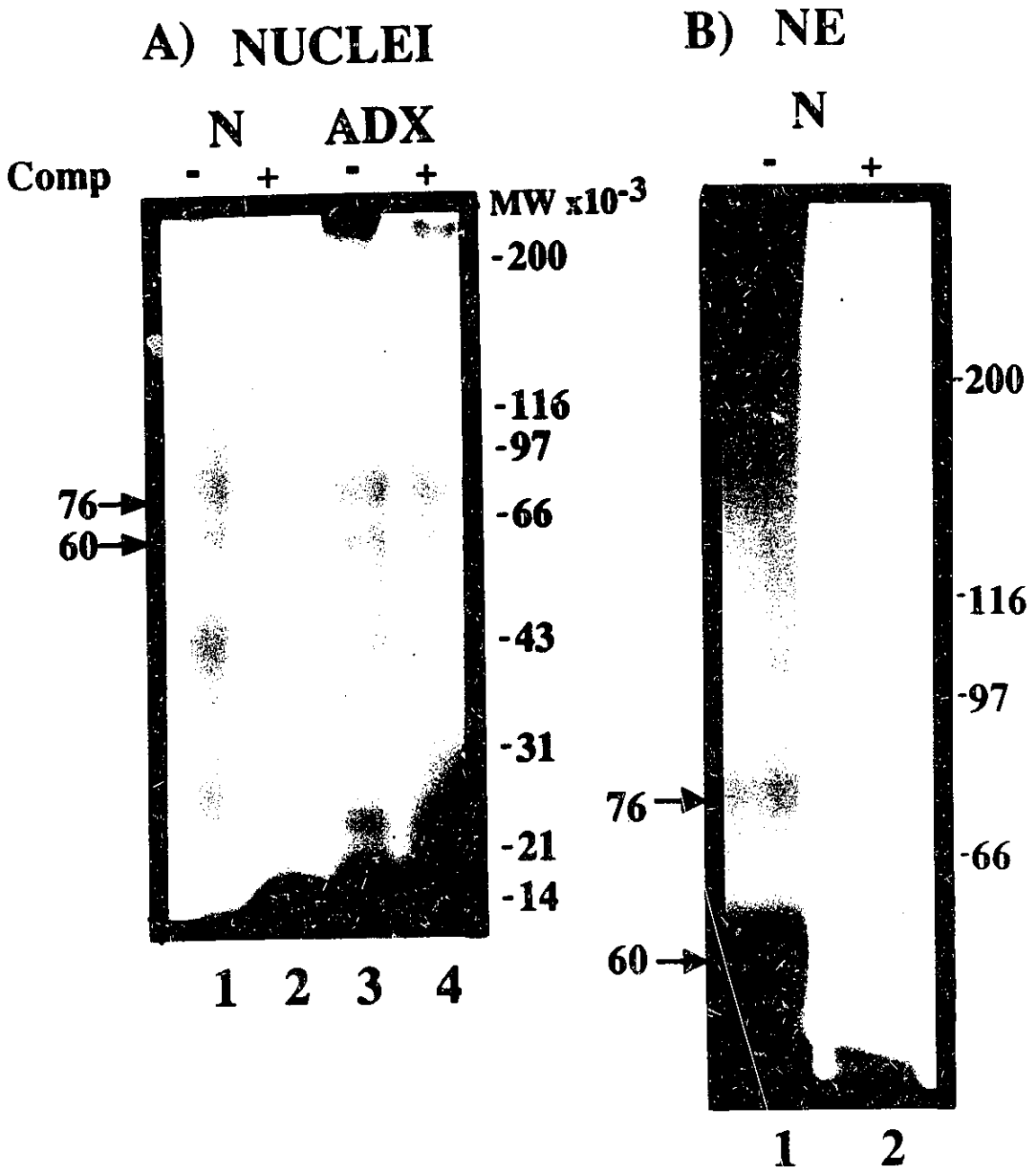
6. Crosslinking of the GR NLS Peptide to Rat Liver Subfractions

Figure 6A compares crosslinked products in male rat liver nuclei prepared from normal (intact) and adrenalectomized animals. The resolution of crosslinked products in liver nuclear subfractions was not as good as that obtained with the S49 lymphoma cytosol, probably because of generally low yields of binding protein. Furthermore, resolution of all bands was not as good in the rat liver cytosol as in the nucleus (data not shown), probably because of serum and connective tissue contamination in rat liver cytosol, as noted by Yamasaki and colleagues [389]. However, it was possible to identify crosslinked products migrating at 21, 45, 60 and 76 kDa in liver nuclear fractions. Again, as observed above, the 76- and 60-kDa entities were the only crosslinked products that were consistently specifically labeled, as assessed by the ability of excess unlabeled NLS to compete for the binding. Mixing of cytosolic and nuclear fractions before binding and crosslinking resulted in the same number of radiolabeled bands as were observed in the two fractions alone, supporting the conclusion that these products were identical in each fraction (data not shown). Thus these studies suggest that the polypeptides forming the 76- and 60-kDa specifically labeled crosslinked products are present in both nuclei and cytosol. Furthermore, crosslinked products migrating at 76 and 60 kDa were also identified on nuclear envelopes prepared from rat livers (Fig. 6B). There was much more 60-kDa crosslinked product observable in this fraction.

Adrenalectomy results in the loss of association of GR with nuclei and nuclear envelopes [289]. If GR association was mediated by a GR NLS-binding protein, a possible cause of the loss of GR association upon adrenalectomy would be the loss of a glucocorticoid-responsive GR NLS-binding protein. It was, thus, of interest to

Figure 6. Crosslinking of the GR NLS to Rat Liver Subfractions

A, Crosslinking of GR NLS to nuclei from livers of intact and adrenalectomized male rats. Nuclei (100 μg) isolated from livers of intact male rats (N) or from male rats nine days post-adrenalectomy (ADX) were extracted with 1 % octyl-β-D-thioglucopyranoside and 0.3 M KCl for 1 h on ice before incubation with 2.1 μM [^{125}I]-GR NLS with (+) or without (-) 67 μM unlabeled GR NLS (Comp) followed by crosslinking with 0.09 mM BS^3 (2-day exposure). B, Crosslinking of the GR NLS to rat liver nuclear envelopes. Nuclear envelopes (NE, 50 μg) isolated from male rat liver and extracted by detergent and high salt, as described in *Materials and Methods* were crosslinked with 0.5 mM BS^3 after binding with 2.1 μM [^{125}I]-GR NLS with (+) or without (-) 214 μM unlabeled GR NLS. Crosslinked products were identified by autoradiography after application of samples to SDS-PAGE (4-day exposure).


A) NUCLEI
N
ADX
Comp
-
+
-
+
MW x10-3
-200
-116
-97
-66
-43
-31
-21
-14
76
60
1
2
3
4
B) NE
N
-
+
-200
-116
-97
-66
76
60
1
2

investigate whether the GR NLS-binding proteins were glucocorticoid responsive. However, adrenalectomy which eliminates glucocorticoid and mineralocorticoid production did not alter either the crosslinking pattern or the amount of any crosslinked product (Fig. 6A).

7. Effect of ATP and Temperature on Crosslinking

Imamoto-Sonobe *et al.* [390] have shown that the efficient association of nuclear proteins with isolated rat liver nuclei requires ATP and that the association occurred at 33 °C but not at 4 °C. Other groups have shown nuclear import to be a two-step process [160, 161]. The first step, binding to the nuclear envelope, is shown to be independent of ATP, while the second step, import into the nucleus, requires ATP. We investigated the energy requirements in the crosslinking studies of the nuclear fraction (Fig. 7). At 4 °C, in the absence of ATP, a faint band was observed at 60 kDa. After addition of ATP at this temperature, the intensity of this band increased and a distinct crosslinked product at 76 kDa was observed. At 33 °C, in the absence of ATP both bands were visible, but the intensity of each was increased after incubation in ATP.

8. Crosslinking of the TR NLS to Cellular Fractions

Figure 8 illustrates that the crosslinking of the TR NLS to rat liver nuclei, rat liver nuclear envelopes and S49 cytosol results in labeling of bands at 60 and 76 kDa. Thus, the same crosslinked products were identified as had been identified after crosslinking of the GR NLS.

9. Crosslinking of GR NLS to Rabbit Reticulocyte Lysate and hsp56 Immunoblot Analysis

We attempted to further identify the NLS-binding proteins by testing for some

Figure 7. The Effect of Temperature and ATP on the Crosslinking of Nuclear Polypeptides to the NLS of GR

Nuclei (100 μg protein) were preincubated for 2 h at 33 °C in 10 mM HEPES (pH 7.8), 60 mM KCl, 2 mM $CaCl_2$, 3 mM $NaHCO_3$, with or without 0.4 mM ATP. Nuclei were then extracted with 1 % octyl-β-D-thioglucopyranoside and 300 mM KCl for 1 h at 4 °C and incubated with 1.1 mM [^{125}I]-GR NLS in the presence or absence of 1 mM ATP either at 4 °C or 33 °C and in the presence (+) or absence (-) of 100-fold excess unlabeled peptide (cold peptide). Crosslinking was performed with 0.09 mM BS^3 crosslinker for 60 min at room temperature, before running on SDS-PAGE and autoradiography (30-day exposure).

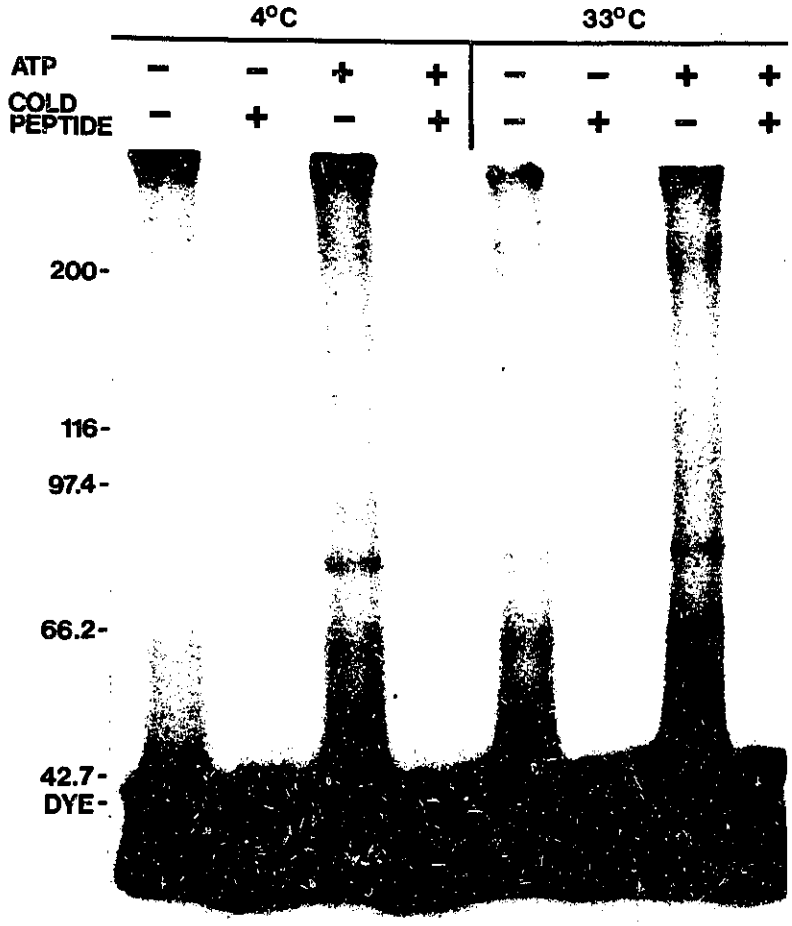
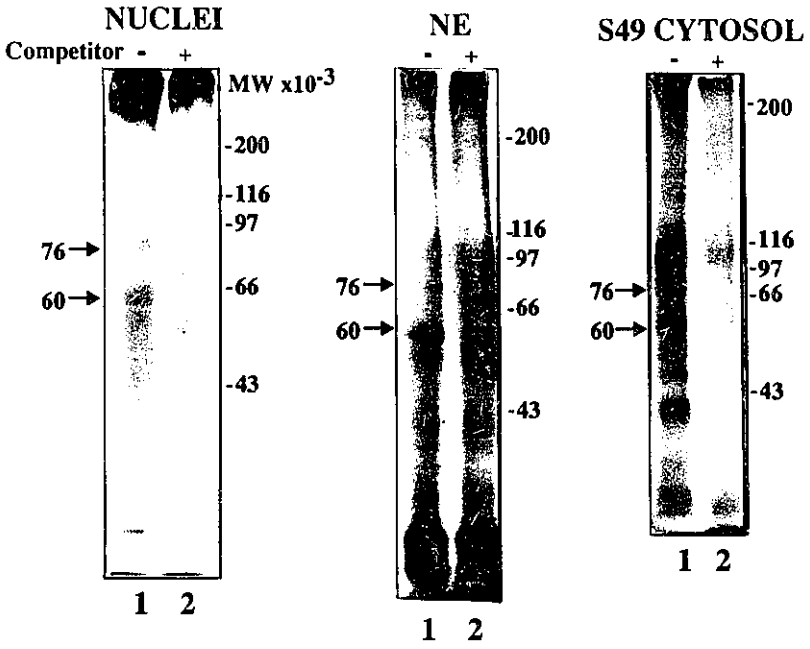

4°C
33°C
ATP
COLD PEPTIDE
200-
116-
97.4-
66.2-
42.7-
DYE-

Figure 8. Crosslinking of the TR NLS to Subcellular Fractions

Left panel, Crosslinking of the TR NLS to nuclei. Nuclei (100 μg) isolated from male rat livers were incubated with 2.1 μM [^{125}I]-TR NLS in the presence (+) or absence (-) of 50 μM TR NLS and crosslinked with 0.5 mM BS^3 for 15 min at room temperature. Center panel, Crosslinking of the TR NLS to nuclear envelopes. Nuclear envelopes (NE, 100 μg) isolated from male rat livers were incubated with 3.4 μM [^{125}I]-TR NLS in the presence (+) or absence (-) of 67 μM unlabeled TR NLS before crosslinking (13-day exposure). Right panel, Crosslinking of the TR NLS to cytosol. Low speed cytosol (100 μg) isolated from S49 cells was incubated with 3.4 μM [^{125}I]-TR NLS in the presence (+) or absence (-) of 101 μM unlabeled TR NLS before crosslinking (6-day exposure).

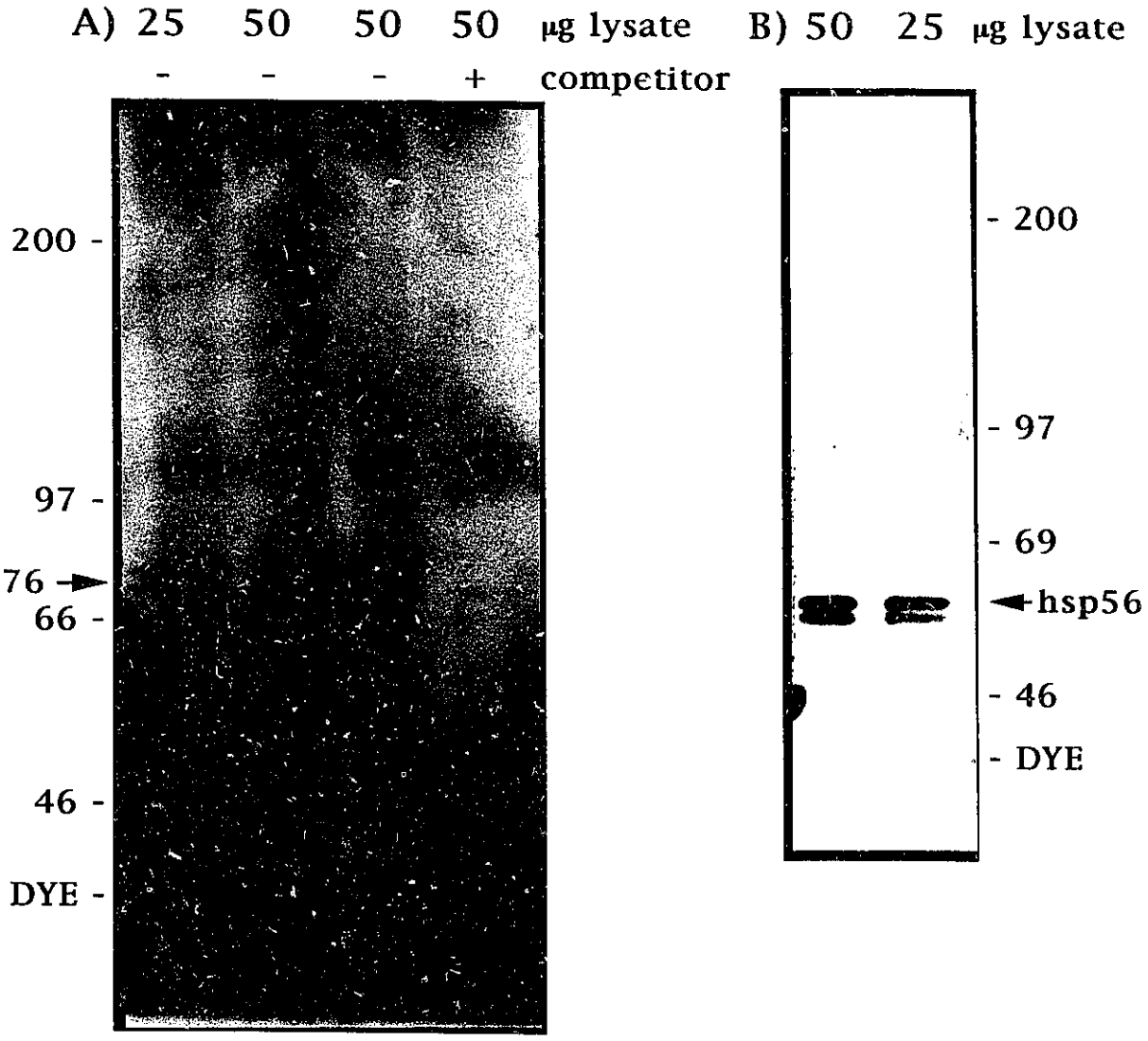

NUCLEI
Competitor - +
MW x10-3
-200
-116
-97
76
60
-66
-43
1 2
NE
- +
-200
-116
-97
76
60
-66
-43
1 2
S49 CYTOSOL
- +
-200
-116
-97
76
60
-66
-43
1 2

likely candidates. It is hypothesized by Pratt *et al.* [391] that the GR associated protein hsp56 is the 60-kDa NLS-binding protein because: the protein is associated with the untransformed receptor; the protein is thought to have a chaperone function; and, it also contains a stretch of acidic residues that could possibly interact with the basic NLS through electrostatic interactions. As hsp56 is a newly identified protein and recently cloned gene [391A], limited tools are available to study it at the protein level. We investigated whether we could immunoprecipitate the [^{125}I]-GR NLS crosslinked product of 60 kDa from rat or murine cells with anti-hsp56 antibodies and protein A-sepharose. The EC1 antibody [392, 393], which was raised against rabbit progesterone receptor but reacted with rabbit hsp56, shows poor crossreactivity to rodent hsp56 and does not immunoprecipitate (L. Faber, personal communication). A newly available commercial antibody raised against a peptide sequence of rabbit hsp56 (a gift from Affinity Bioreagents, [391A]) demonstrates species crossreactivity on immunoblots but it was not known if it could immunoprecipitate hsp56. Direct detection of hsp56 by Western blots of the immunoprecipitates is hampered by the presence of the immunoglobulin heavy chain at 55 kDa. Therefore, an indirect approach was taken to see if anti-hsp56 antibody could co-immunoprecipitate ^{35}S-labeled *in vitro* translated GR in rabbit reticulocyte lysate by washing the immunopellet under gentle conditions in TEG buffer (10 mM TES pH 7.6, 1 mM EDTA, 10 % glycerol, 50 mM NaCl with or without 20 mM Na_2MoO_4) [80, 81]. Attempts at co-immunoprecipitation with hsp56 with this antibody were unsuccessful. This was not surprising as the antibody is raised against a linear epitope of the protein and may not recognize the native protein. Other groups have been unsuccessful at immunoprecipitating with this antibody (James Stiehr, President, Affinity BioReagents, personal communication).

The anti-hsp56 antibody EC1 (a gift from Dr. Lee Faber, [392]) worked well on Western blots of rabbit reticulocyte lysate as can be seen in Figure 9B. To my

Figure 9. Crosslinking of GR NLS to Rabbit Reticulocyte Lysate and Hsp56 Immunoblot Analysis

A. ^{125}I-labeled GR NLS (0.8 μg) was crosslinked with BS^3 in crosslinking buffer with 1 mM ATP, 3 mM $NaHCO_3$ and 2 mM $CaCl_2$ containing 25 or 50 μg lysate in the presence (+) or absence (-) of 10-fold excess of unlabeled GR NLS (competitor). B. Immunoblot of rabbit reticulocyte lysate (25 and 50 μg protein assuming a 100 μg/μl concentration) with anti-hsp56 antibody, EC1, at 1:1000 dilution incubated overnight at 4 °C. The protein was detected after incubation with horseradish peroxidase-labeled sheep anti-mouse antibody at 1:100 000 dilution for 3 h at room temperature with ECL. Exposure was for 7 min.


A) 25 50 50 50 µg lysate
- - - + competitor
200 -
97 -
76 →
66 -
46 -
DYE -
B) 50 25 µg lysate
- 200
- 97
- 69
← hsp56
- 46
- DYE

surprise, crosslinking of the GR NLS peptide to rabbit reticulocyte lysate yielded a crosslinked band at 76 kDa but not at 60 kDa (Fig. 9A). This suggests that even though hsp56 is present it may be distinct from the 60-kDa crosslinked product since I do not observe it here.

I also tested to see if I could immunoprecipitate the 76-kDa crosslinked product with anti-protein kinase C antibodies, including antisera provided by Dr. Max Hincke (University of Ottawa) [394], antisera purchased from Gibco-BRL [395], or a monoclonal antibody purchased from Amersham (clone MC5), and protein A-sepharose. These experiments were also negative (data not shown).

(ii) *In Vitro* Nuclear Protein Import Models

A. *In Vitro* Binding of GR to Purified Isolated Rat Liver Nuclei

1. Preliminary Experiments

I first confirmed that the *in vitro* coupled transcription/translation kit programmed with GR cDNA under the control of SP6 promoter yielded the correct molecular weight products for the full-length receptor (pRdN93, contains both NL1 and NL2) and the ligand-binding domain (547EBU, contains only NL2) as detected by fluorography of an SDS-PAGE gel of the translation products and by Western blotting with an anti-GR antibody (Fig. 10). The transformation of GR from a non-DNA-binding state to a DNA-binding state was verified by the change in sedimentation from 8 S to 4 S on a sucrose gradient [66, 67]. Figure 11 shows the sedimentation profiles of GR and NL2, the hormone-dependent NLS and ligand-binding domain of GR, in the untransformed state. The translated products of the full-length GR sedimented on the gradients similar to [^{3}H]-dexamethasone-labeled cytosolic receptor (data not shown). GR is completely untransformed as is shown by its sedimentation at a higher sedimentation coefficient than BSA. It is always best to have two sedimentation standards to interpolate the value of the unknown and not extrapolate. However, in these experiments the heavier sedimenting standard was not detected because too little has been used or it had degraded. The majority of NL2 was untransformed with a small amount (13 % of the signal observed on the fluorogram) that was transformed even in the presence of molybdate. Ligand and heat treatment yielded a more efficient transformation of GR than heat alone (Fig. 11). In the transformation by ligand, 100 nM dexamethasone was used for the full-length rat GR while 1000 nM was used for the ligand-binding domain (NL2) because it is known to have a 300-fold reduction in ligand binding affinity [23]. Heat treatment of GR

Figure 10. Coupled *In Vitro* Transcription/Translation of GR Derivatives

The cDNAs for the full-length (minus nineteen Q residues) rat GR (pRdN93, simply called GR), the ligand-binding domain (NL2) alone fused to the BuGR epitope (p547EBU, simply called NL2), and firefly luciferase (LUC, from Promega) were transcribed and translated in rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. Samples (3 μl) of translation mix were run on SDS-PAGE and fluorographed (A) or transblotted and subjected to immunoblot analysis with BuGR (anti-GR) antibody (1:2000 dilution). The antibody was detected by enhanced chemiluminescence and autoradiography (B).

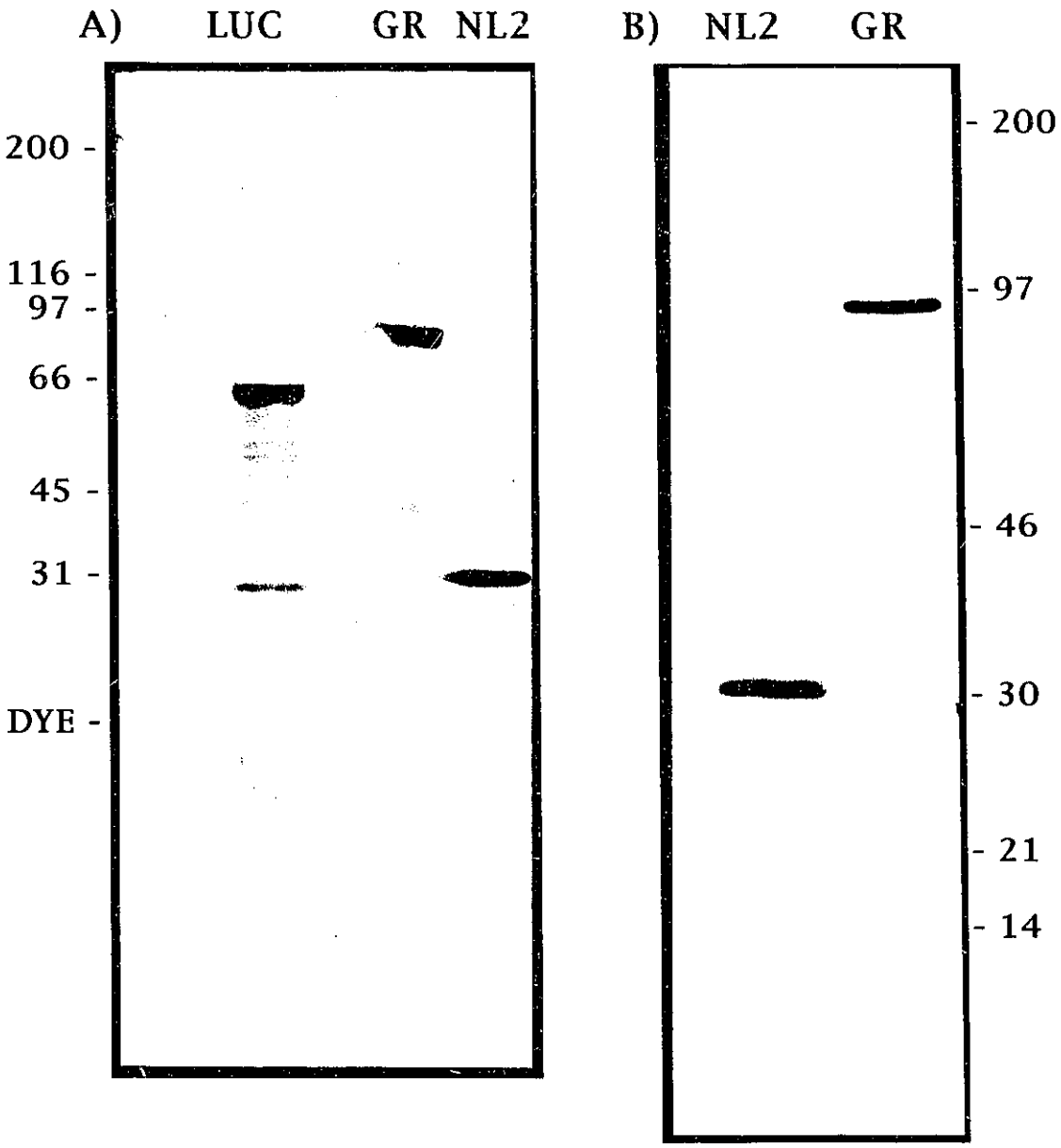
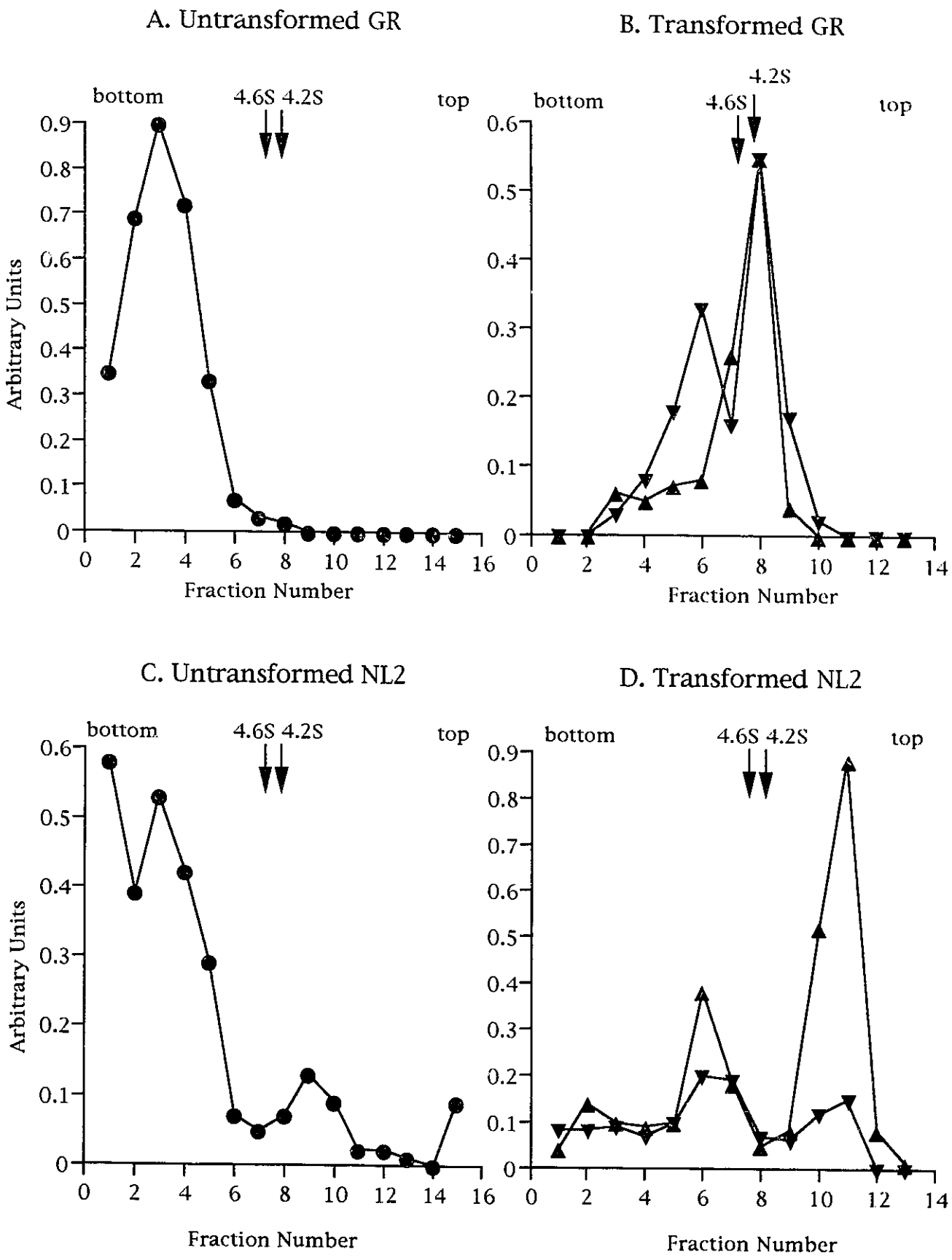

A)
LUC
GR
NL2
200 -
116 -
97 -
66 -
45 -
31 -
DYE -
B)
NL2
GR
- 200
- 97
- 46
- 30
- 21
- 14

Figure 11. Sucrose Gradient Analysis of Transformed and Untransformed Translation Products

Panels A and C, Untransformed ^{35}S-labeled GR and ligand-binding domain (NL2) were fractionated on 10-25 % sucrose gradients containing 20 mM molybdate. Fractions were run on SDS-PAGE, fluorographed and the results of densitometric scans are plotted here. Panels B and D, Translation products stored without molybdate were either heat transformed, 25 °C for 30 min (▼), or ligand transformed with 100-1000 nM dexamethasone for 2 h at 4 °C and then heat treated as above (▲). The transformed products were fractionated on 15-30 % sucrose gradients under low salt conditions in the absence of molybdate. [^{14}C]-BSA (4.6 S) served as an external standard and rabbit hemoglobin (4.2 S) served as an internal standard. Bottom and top refer to bottom (highest density) and top (lowest density) portions of the sucrose gradient.


A. Untransformed GR
B. Transformed GR
C. Untransformed NL2
D. Transformed NL2
bottom
top
4.6S
4.2S
Arbitrary Units
Fraction Number

transformed 59 % of the receptor while ligand and heat transformed 84 % of the receptor. The smaller ligand-binding domain was poorly transformed by heat alone (27 %) while ligand and heat transformed 59 %. None of the methods of transformation resulted in as high yields of the transformed ligand-binding domain (NL2) as the full-length GR.

2. Nuclear Binding Assay

I adapted a filtration assay for the measurement of $^{45}Ca^{++}$ binding to nuclei [166] for use in measurement of translated products binding to nuclei. I first identified a 0.22-0.45 μm pore membrane that retained nuclei (4-6 μm diameter) but had a low binding affinity for free protein. As the results in Table 6 demonstrate, a hydrophilic PVDF-membrane from Millipore showed the least binding to translated GR products.

Ligand-transformed GR bound to isolated rat liver nuclei about three times better than untransformed GR and was not affected by BSA in the import assay (Fig. 12). The biggest difference between quantities of the transformed and untransformed GR binding to nuclei was seen at a concentration of 50 μg/50 μl nuclear protein. Therefore, import assays were performed as follows; lysate (3-25 μl) containing transformed or untransformed translated receptors was incubated for 30 min under various conditions with purified rat liver nuclei (50 μg) in a final volume of 50 μl. One ml of ice-cold buffer was added to stop the reaction and unbound protein was separated from the bound fraction by filtration through a 13-mm 0.45 μm pore hydrophilic PVDF-membrane with the use of a syringe. The filter with the retained nuclei was then washed with another 4 ml of cold buffer. The filter with the nuclear-bound counts were quantified by scintillation counting. Three μl of GR-containing lysate typically contained 400 000 d.p.m. of trichloroacetic acid-precipitable ^{35}S-counts; thus 40 000 nuclear-bound counts represented 10 % binding.

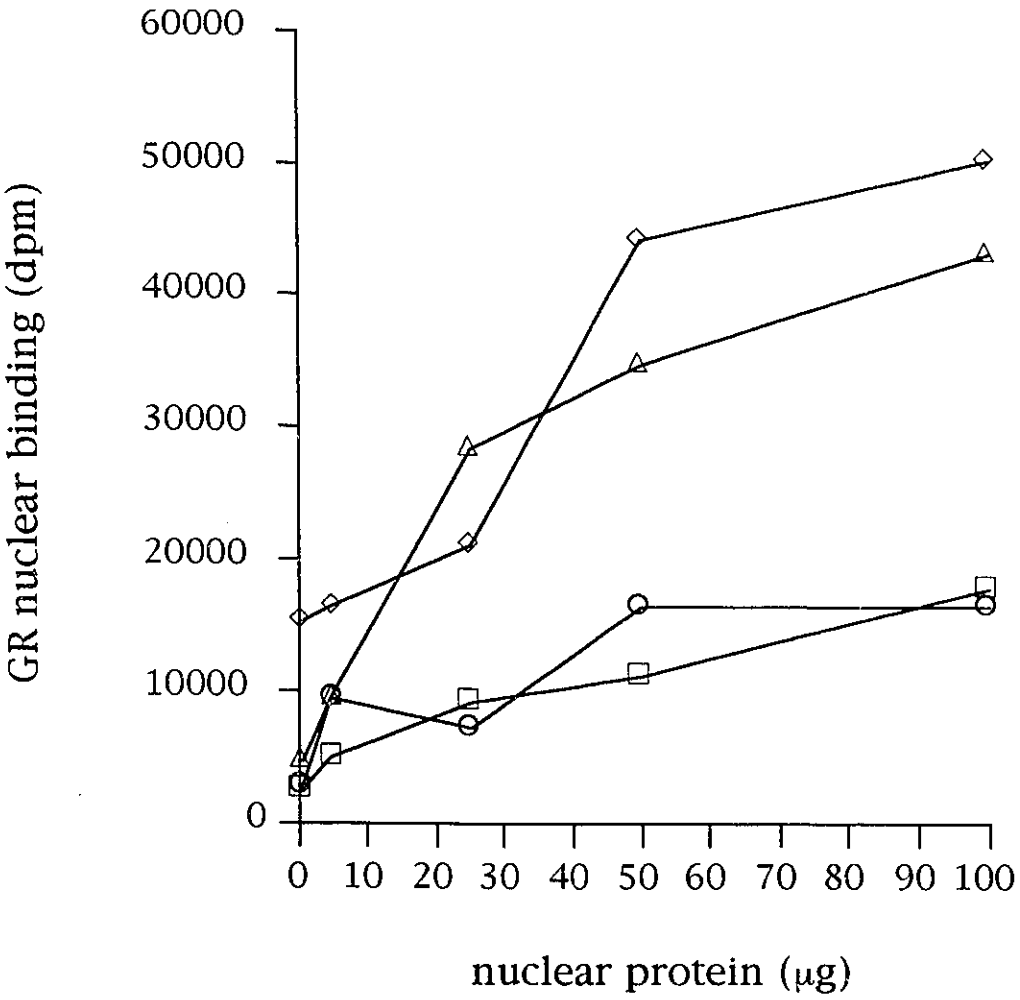
Table 6. Protein Binding to Filters

	Bound counts* (% of total)
Durapore (hydrophilic PVDF)	1.2 (0.04 %)
HA, Triton-free (nitrocellulose)	63.9 (2.3 %)
HA (nitrocellulose)	66.2 (2.4 %)
direct spotting of 3 µl lysate to dry filter (total counts)	2 730.7 (100 %)

*Values are expressed as d.p.m. x 10^{-3}. Three µl of untransformed GR-containing lysate in 50 µl import buffer with 4 mM ATP was diluted with 1 ml of cold import buffer, filtered and washed wth 10 ml of cold import buffer. All the filters were from Millipore.

Figure 12. Transformed and Untransformed GR Binding to Isolated Nuclei

Lysate (3 μl) containing either ligand-transformed GR (◇, △) or untransformed GR (□, ○) was incubated with increasing amounts of nuclear protein and the binding measured after 30 min by filtration assay. Binding was performed in the presence (◇, ○) or absence of 0.2 % BSA (△, □). Bound counts were quantified by scintillation counting.


60000
50000
40000
30000
20000
10000
0
GR nuclear binding (dpm)
0 10 20 30 40 50 60 70 80 90 100
nuclear protein (μg)

The concentration of translated GR in the lysate was calculated to be approx. 2 nM (similar to its intracellular concentration), and the total protein concentration of the lysate is approx. 100-200 mg/ml (roughly 30 % of which is hemoglobin) as specified by the manufacturer.

A dose-response relationship was seen between the concentration of GR and binding to rat liver nuclei (Table 7) with a six-fold difference between transformed and untransformed GR binding to nuclei at 25 µl of GR-containing lysate (50 % of import volume). There was no difference in the binding at 4 °C in the presence or absence of 4 mM ATP. However, a decrease was observed in binding at 33 °C in the presence of ATP compared to binding in the absence of ATP. In general, the binding results were similar at 4 °C and at 33 °C. The addition of supplemental lysate to GR-containing lysate had a small inhibitory effect on binding to nuciei. The cDNA for a 62-kDa peroxisomal (cytoplasmic microbody) protein, luciferase, was translated and added to nuclear import assays as a control for non-specific binding. As expected it bound poorly, less than the value obtained for untransformed GR.

The binding of NL2, the ligand-binding domain of GR that also comprises a ligand-dependent NLS [1], was tested in our nuclear binding assay and found to bind 6- to 10-fold better at 33 °C than at 4 °C, compared to GR which showed a 2- to 6-fold difference in binding at the two temperatures (see Table 8 and Discussion). There were no differences in the levels of nuclear binding of heat-transformed GR and NL2 versus ligand- and heat-transformed GR and NL2.

Finally, we tested the specificity of GR binding to membranes by using purified rat liver mitochondria (1 µm diameter) in our import assay. The translated products bound even better to purified mitochondria than to purified nuclei (Table 9).

Table 7. Binding of GR to Rat Liver Nuclei*

	import substrate	4 °C				33 °C			
		- ligand		+ ligand		- ligand		+ ligand	
		-ATP	+ATP	-ATP	+ATP	-ATP	+ATP	-ATP	+ATP
GR	3 µl	15.8	11.4	43.1	29.2	20.6	16.8	45.6	36.6
	12 µl	29.5	22.0	133.6	106.3	45.9	25.3	211.9	69.5
	25 µl	42.6	45.8	264.6	263.2	56.7	nd	213.2	101.8
GR + lysate	3 + 12 µl	14.0	6.6	20.2	25.0	6.1	6.1	29.8	10.5
	12 + 13 µl	16.4	16.2	71.8	71.0	16.1	15.0	90.3	30.2
luciferase + lysate	3 + 22 µl	5.8	6.2	nd	nd	7.5	8.9	nd	nd
	12 + 13 µl	nd	nd	18.1	nd	nd	nd	24.3	nd

*Data are expressed as d.p.m. x 10^{-3}. Import is at 4 or 33 °C in the presence or absence of 4 mM ATP with various amounts of GR that has been ligand- and heat-transformed (+ ligand) or not transformed (- ligand). GR containing lysate was in some cases supplemented with lysate containing no exogenously translated proteins (lysate). Lysate containing *in vitro* translated luciferase, a 62-kDa peroxisomal protein, was used as a control for non-specific binding to nuclei. nd=not determined.

Table 8. NL2 Binding to Isolated Rat Liver Nuclei

	GR		NL2	
	ligand	heat	ligand	heat
4 °C	14.5	6.4	10.9	6.4
33 °C	34.4	41.4	64.8	65.1

Values are expressed as d.p.m. x 10^{-3} of filter-bound counts. Import assays were performed at 4 or 33 °C with 3 µl of GR- or NL2-containing lysate that had been transformed with ligand (100 and 1000 nM dexamethasone, respectively) and heat, or heat alone (25 °C, 30 min).

Table 9. GR Binding to Isolated Rat Liver Mitochondria*

		Mitochondria	Nuclei
GR (-ligand)	3 µl	12.6	nd
	6 µl	29.6	22.0
GR (+ligand)	3 µl	20.9	nd
	6 µl	36.3	28.9
NL2 (-ligand)	3 µl	156.0	nd
	6 µl	409.6	295.9
NL2 (+ligand)	3 µl	158.5	nd
	6 µl	320.1	174.9

*Values are expressed as d.p.m. x 10^{-3} of ^{35}S bound to filter. Receptors (GR and NL2) were ligand- and heat-transformed (+ligand) or not (-ligand) and 3-6 µl of lysate was incubated for 30 min at 30 °C with either 50 µg purified rat liver mitochondria or 50 µg purified rat liver nuclei in import buffer with or without 20 mM Na_2MoO_4. Binding was stopped by flooding with buffer and filtration through hydrophilic PVDF-membranes. Filters were washed and counted. nd=not determined.

B. *In Vitro* Import of GR in to Permeabilized COS Cells

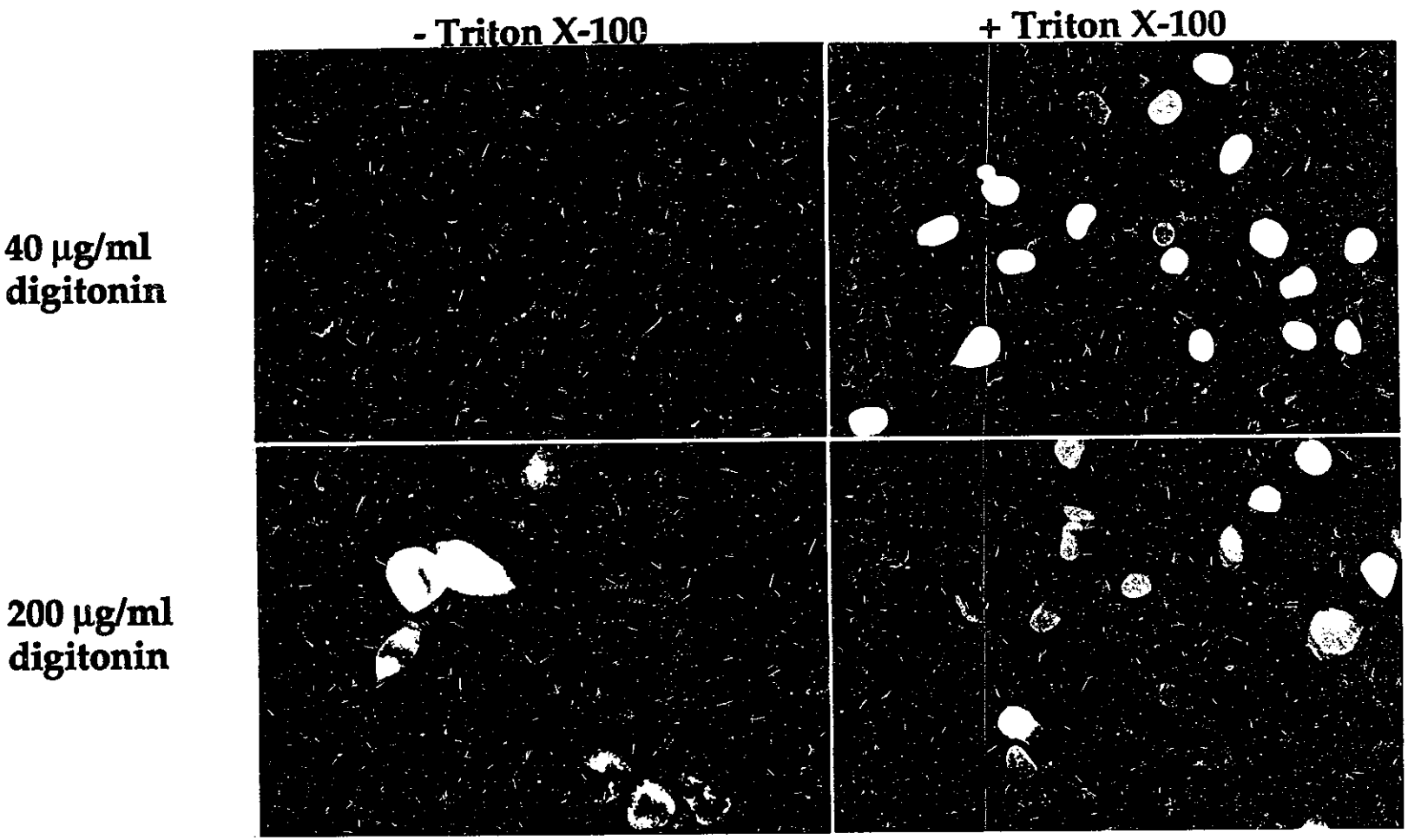
1. Preliminary Experiments

We (E. LaCasse and J. Kwast-Welfeld) set out to establish an *in vitro* nuclear import model for GR based on a recently described permeabilization system [372-374], as described in the Methods and Materials section. The permeability of the plasma membrane and the integrity of the nuclear envelope caused by detergent-permeabilization of COS cells grown on coverslips was verified. After incubation of COS cells which had been permeabilized with 40 or 200 μg/ml digitonin for 5 min at 4 ^{o}C with anti-lamin antibodies, we assessed the localization of anti-lamin antibodies by indirect immunofluorescence. The IgG antibodies are 150-kDa proteins which are too large to diffuse through the intact nuclear pore, and which recognize an intranuclear structure, the lamina, lining the inner nuclear envelope. The results in Figure 13 clearly show that permeabilization with 40 μg/ml of digitonin resulted in the anti-lamin antibodies not having access to the nucleus and being detected only in the cytoplasm. However, at 200 μg/ml digitonin, the antibodies stained intranuclear components which demonstrated that the higher detergent concentration has resulted in permeabilization of the nuclear envelope. To confirm that the antibodies were functional under these conditions we permeabilized fixed cells with 0.5 % Triton X-100 and observed staining of the lamina after antibody incubation. Therefore, further studies were performed with 40 μg/ml digitonin.

The permeabilization of the nuclear envelope by 0.02 % (200 μg/ml) digitonin is not as complete as permeabilization by 0.5 % Triton-X-100. This is evident in the photograph of cells permeabilized with 200 μg/ml digitonin which show a decreased nuclear fluorescence compared either to cells in the same field which were undergoing mitosis and show a strong cytoplasmic (redistributed) lamin fluorescence, or the Triton-permeabilized cells.

Figure 13. Plasma Membrane and Nuclear Envelope Permeability

COS cells grown on coverslips were permeabilized with either 40 or 200 μg/ml digitonin to assess the permeabilization of the plasma membrane and to verify the integrity of the nuclear envelope by exclusion of anti-lamin antibodies (kindly provided by Dr. Yves Raymond, Montreal). Permeabilized cells were incubated for 30 min at 30 °C with 1 μg of anti-lamin antibody in 50 % reticulocyte lysate diluted in transport buffer. After the incubation, coverslips were rinsed in transport buffer and fixed with 3 % paraformaldehyde. As a positive control, fixed cells were completely permeabilized with 0.5 % of Triton X-100 (+ Triton X-100) and then exposed to the anti-lamin antibodies.


- Triton X-100
+ Triton X-100
40 µg/ml digitonin
200 µg/ml digitonin

Next we tested if the permeabilized cells selectively imported nuclear proteins by using a naturally fluorescent protein, allophycocyanin, conjugated to the SV40 large T-antigen NLS peptide (4-8 peptides per molecule) [372-374] as a karyophilic substrate. Permeabilized cells were incubated with the import substrate in 50 % lysate in import buffer with or without ATP and an ATP-regenerating system. The fluorescence micrographs show that the fluorescent import substrate only accumulates in nuclei in the presence of ATP and was washed out of the cytoplasm in the absence of ATP (Fig. 14).

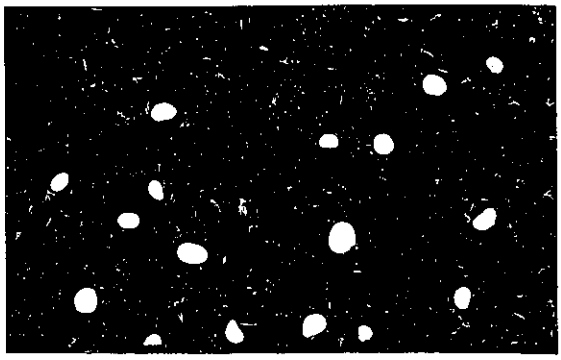
2. GR Fragment *In Vitro* Nuclear Import

With the import system working as expected, we tried unsuccessfully to reconstitute the nuclear import of *in vitro* translated GR (using non-radiolabeled methionine) added to digitonin-permeabilized cells. Import was detected by fixation of the permeabilized cells after import, permeabilization with Triton X-100 and incubating with an anti-GR antibody (BuGR). The antibody was detected by indirect immunofluorescence. We could not detect any import of GR above the background fluorescence signal of the antibody (data not shown). This was thought to be due to the low concentration of GR in the import assay, which was calculated to be less than 1 nM. To obtain more GR substrate for nuclear import we relied on a bacterial expression system to generate a 19-kDa fragment of GR consisting of the DNA-binding domain along with the hinge-region NLS, NL1. We routinely obtained a crude *E. coli* extract that contained approx. 1 mg/ml GR fragment. A concern with using such a small protein fragment is that it could diffuse through the nuclear pore and bind DNA, hindering the interpretation of our import results. To increase the size of the import substrate it was complexed with antibody (150 kDa) before adding it to the import assay. In Figure 15, we observed by indirect immunofluorescence ATP-dependent nuclear import of the GR fragment-antibody complex.

Figure 14. ATP-dependent Import of an NLS-conjugate

COS cells grown on coverslips were permeabilized with digitonin (40 μg/ml for 5 min at 4 °C) and incubated for 30 min at 30 °C with 100 nM allophycocyanin-NLS peptide conjugate (kindly provided by Dr. S.A. Adam) in 50 % reticulocyte lysate diluted with transport buffer in the presence or absence of 0.5 mM ATP and an ATP-regeneration system. After the incubation, coverslips were rinsed in transport buffer and fixed with 3 % paraformaldehyde. Samples were originally observed at 400x magnification by epifluorescence on a Zeiss Axiophot microscope. Exposure time for both panels was 15 sec.

ATP



no ATP

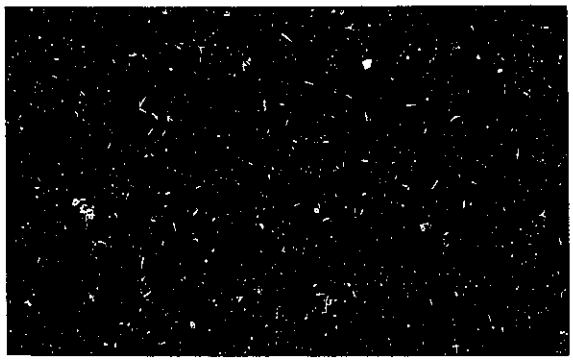
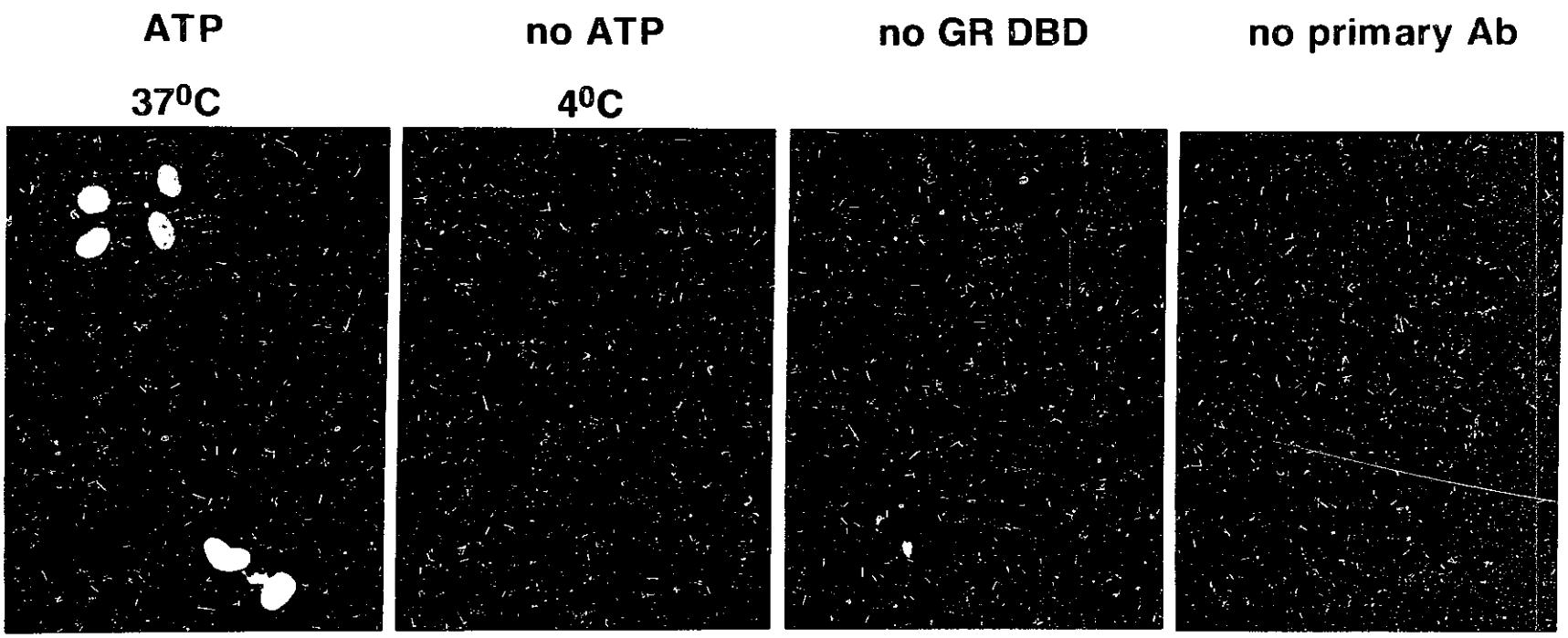


Figure 15. Nuclear Import of the GR DNA-binding Domain (GR DBD) Bound to Antibody

Bacterially expressed GR DNA-binding domain 19-kDa fragment (48 μg total protein or approx. 5 μg GR) which also contains the hinge region NLS and an epitope for the BuGR antibody was prebound to antibody (1 μg) to increase its size so not to allow passive diffusion and DNA binding of GR. Permeabilized COS cells on coverslips were incubated for 15 min at 37 °C or 4 °C with the complexed GR fragments in 50 % reticulocyte lysate diluted with transport buffer in the presence (ATP) or absence of 0.5 mM ATP and an ATP-regeneration system (no ATP). The fluorescent secondary antibody was added after fixation and Triton X-100 permeabilization of the cells. In one panel, the GR DNA-binding domain was omitted (no GR DBD), while in the last panel, the primary antibody was omitted.

Nuclear Import of GR - DBD



IV. DISCUSSION

(i) NL1-binding Proteins

1. Identification of NL1-binding Proteins

We identified two specifically crosslinked polypeptides with molecular masses of 60 and 76 kDa in both nuclear and cytosolic subfractions after binding to either the synthetic GR NLS or TR NLS. This is the first identification of mammalian NLS-binding proteins for NLSs within mammalian nuclear proteins. We have shown that the SV40 large T-antigen NLS also competes for binding to these proteins, supporting the binding proteins' role in a step of nuclear import common to nuclear proteins, whatever their origin.

Although this is the first identification of binding proteins for mammalian NLSs (GR NLS and TR NLS), several investigators have identified NLS-binding polypeptides for yeast and viral NLSs (see Table 10). A 45-kDa NLS-binding protein has been reported [389], but as we found, was judged non-specific due to the inconsistency of the ability of excess unlabeled signal to compete. The reason for this inconsistency in specificity remains unknown, although the stability of potential NLS-binding proteins and the effects of different extraction procedures may explain the variable results. Silver [396] concluded that the yeast and mammalian cells share 66- to 70-kDa and 59- to 60-kDa nucleus-associated proteins that recognize NLSs. The 60-kDa polypeptides we have identified may correspond to the one identified by other workers. It is of interest that there was much more 60-kDa crosslinked product observable in the nuclear envelope fraction, as this may indicate a role for this polypeptide in translocation across the nuclear pore. A 76-kDa NLS-binding protein has been identified in rat liver nuclear envelope by Benditt and co-workers [400],

Table 10. NLS-binding Proteins Identified with SV40-T NLS Peptides*

Species	Protein size (kDa)	Protein location[a]	Other NLSs recognized[b]	Comments	Refs
Rodent	76, 60	N+C, N+C	GR, TR	This thesis	[397, 398]
Rat	70, 60	N+C, N+C	ND		[357]
Bovine	56/54	C**	ND	Stimulates import in permeabilized cells	[349]
Rat	140, 55	N, N	EIA, NP		[389]
	55	ER	ND	Identified as PDI	-[c]
	70	ER	E1A, NP, MATα2	Copurifies with ERp72	[389][c]
	100	C	E1A, NP		[389]
Rat	140	N'	ND	Named Nopp140 (sequenced) NLS binding-activity depends on phosphorylation	[399]
Rat	76, 67, 59, 58	N, N, N, N	ND		[400]
Rat	69	N	NP	Binds anti-DDDED antibodies	[191]
				Identified as hsc70	[189]
Rat	60	NE	NP	Purified from nuclear envelopes	[401]
Human	38	N'	ND	Identified as B23	[402]
	55	C	ND	Identified as tubulin	
Human	66	C>N	protein A***		[403, 404]
Hamster	110	N'	H2B	Purified nucleolin (C23)	[405]

N.B. continued on next page.

Table 10
(cont'd)

Species	Protein size (kDa)	Protein location[a]	Other NLSs recognized[b]	Comments	Refs
Yeast	140, 95, 59	N, N, N	GAL4, NP, H2B		[406]
	70	N+C	GAL4, NP, H2B	Named NBP70 Antibodies for NBP70 prevent SV40-T ligand binding to nuclei	[406, 407] [407]
Yeast	67	N'	H2B	*NSR1* gene product (sequenced)	[408, 409]
Frog	90, 58, 53	Unknown	NP, N1/N2		-[d]
Maize	23/25	N+C	ND	Abscisic acid-responsive protein Rab17	[410]

* For our work we did not use a SV40-T peptide but a GR NLS-T peptide. **Comes from an enucleated cell, *i.e.* erythrocyte. ***Protein A appears to be an artifactual karyophile.
[a]Location: N, nucleus; C, cytoplasm; ER, endoplasmic reticulum; N', nucleolus; NE, nuclear envelope.
[b]Other NLSs recognized: glucocorticoid receptor (GR), thyroid hormone receptor (TR), adenovirus E1a (E1A), *Xenopus* nucleoplasmin (NP) and N1/N2 proteins (N1/N2), and yeast Matα2 (MATα2), Gal4 transactivator (GAL4) and histone H2B (H2B); ND, not determined.
[c]L. Yamasaki and R.E. Lanford, unpublished (reported in [411]).
[d]D.D. Newmeyer, personal communication to Yamasaki and Lanford [411].
Other abbreviations: PDI, protein disulfide isomerase; anti-DDDED, antibody to AspAspAspGluAsp peptide; NBP, NLS binding protein; NSR, nuclear signal receptor.

whereas an 80-kDa NLS-binding protein in NRK cell cytosol was identified by Adam and Gerace [349], which may correspond to the 76-kDa binding protein we identified [397, 398]. It appears that most nuclear proteins are imported by a common pathway [412] suggesting that there are common receptors or gating proteins for the various signals.

The NLS-binding polypeptides in Table 10 have been identified by NLS-affinity chromatography [401, 402, 413], crosslinking [357], ligand blotting (overlay assay) [399, 401, 405, 409], and photoaffinity labeling (u.v. crosslinking) [389, 400, 403, 414]. Some of the polypeptides are likely to be involved in nuclear import while others are believed to result from non-specific interactions, such as PDI (protein disulfide isomerase) which is both an enzyme and a chaperone [415]. PDI is capable of non-specific peptide binding like other chaperones, which are notable for their lack of substrate specificity. The purification of binding proteins on an SV40 NLS-affinity matrix led to the purification of two abundant cellular proteins, tubulin and B23/no38, which both have a net negative charge. Hence, their interaction with the positively-charged NLS may be non-specific [402].

The nuclear and cytosolic polypeptides of rat liver and mouse S49 lymphoma cells bind the NLSs present in the hinge region of GR and TR in a saturable manner. The saturating concentrations observed fell within the wide range reported by other groups for NLS-binding proteins, from 50-75 nM [357] to 20-80 μM [414]. This wide range (1000-fold) of saturating concentrations reported may be due to the low efficiency of crosslinking which differs for the different crosslinkers and conditions used; and the possibility that only a small percentage of the peptide is in the right conformation to bind to its receptor, which may also differ for various peptide constructs. It is important to note that the affinities of the synthetic NLS for binding proteins should not be used to assess the ability of the NLS to interact with the binding proteins at physiological concentrations of GR. The affinity of the

signal peptide for its binding protein(s) is expected to be greater in the context of the full-length GR because additional amino acid contacts may stabilize the structure of the NLS.

The apparent diversity of NLSs and the lack of a reliable consensus sequence led us to complete a more detailed investigation in competition experiments. These experiments demonstrated the specificity of the binding sites for NLS peptides. A mutant GR NLS was a very poor competitor, as was expected. Because of the resemblance of the NLS peptides to basic substrates or inhibitors of various protein kinases and because of evidence for a role of phosphorylation in nuclear import [260, 261], we also performed competition experiments with some known substrates for protein kinase C, protein kinase A, and calcium/calmodulin-dependent kinase. The only peptide that served as an effective competitor was CAM, a substrate for the calcium/calmodulin-dependent kinase. As calcium/calmodulin-dependent kinases of 80 and 60 kDa have been reported [416, 417], the possibility that these are identical to the 76- and 60-kDa NLS-binding proteins must be considered. These studies also revealed that the 60-kDa polypeptide demonstrates greater specificity for binding to the NLSs; thus, caution in the identification of the 76-kDa crosslinked polypeptide as an NLS-binding protein is warranted. Close investigation of the competition analysis of the 80-kDa NRK cell cytosolic polypeptide [349] reveals that the specificity of this NLS-binding protein is, as with the 76-kDa binding protein reported here, difficult to assess.

2. Subcellular Localization of NLS-binding Proteins

The observation that the same 60- and 76-kDa NLS-binding proteins are located in both cytosolic and nuclear fractions argues for the involvement of these polypeptides in shuttling proteins between the two cellular compartments, as suggested by others [349, 399]. While there is general agreement in the literature for

localization of NLS-binding proteins in the nucleus, the localization in the cytoplasm is more controversial. We and others [389] have identified NLS-binding proteins in the rat liver cytosolic fractions. Cytosolic NLS-binding proteins have also been identified in rat erythrocytes, and identical proteins purified from bovine erythrocytes have been shown to function in an *in vitro* nuclear import assay [349]. However, cytosolic binding proteins have not been reported in yeast, although immunofluorescence detected minor amounts of the 70-kDa NLS-binding protein in the cytoplasm [407]. Moreover, Pandey and Parnaik [414] did not detect cytosolic binding proteins in the rat liver, probably because different conditions were used in isolation of subfractions and crosslinking experiments. Our findings and those of others [400, 414] demonstrate that the nuclear NLS-binding proteins are at least partially localized to the nuclear envelope.

3. Targeting of Steroid/Thyroid Hormone Receptors to the Nucleus

It may be argued that cellular interactions of TR during nuclear localization differ from those of GR because its smaller size (52 kDa) allows it to enter the nucleus by passive diffusion. The recent finding that the nuclear transport of histone H1 (21 kDa) is facilitated suggests that many nuclear proteins, regardless of their size, may actively enter the nucleus [156]. Furthermore, the identification of a v-*erbA* protein with a mutation in the hinge region NLS which makes it unable to enter the nucleus strongly supports a role for the hinge NLS of TR in nuclear localization of this receptor as well [299].

GR previously unexposed to hormone is present in the cytosol in a complex with hsp90, hsp56 (FKBP59) and other proteins, whereas TR is always found in the nucleus tightly bound to chromatin [112, 113]. Whether the NLS-binding proteins correspond to any of the components of the GR-complex remains to be determined. It has been suggested that the difference in localization arises because the NLS is masked in

cytosolic GR , because hsp90 is complexed to it, and that hormone is required to make the NLS accessible before nuclear entry can occur. In the case of TR, the NLS is accessible in unliganded TR, and thus, the receptor is competent for nuclear entry in the absence of hormone. Our findings are consistent with the above suggestions, as they show that the interaction of GR and TR NLS with different NLS-binding proteins does not account for differences in localization.

The process of nuclear import appears to be a complex one, with the involvement of several proteins, acting as shuttles and receptors. In the case of the steroid/thyroid hormone receptor superfamily, there is yet another layer of complexity that will have to be addressed; several studies have identified a second NLS in the receptors. A second NLS for which there is no sequence similarity with the SV40 NLS, has been identified in GR [1, 294], TR [418], the androgen receptor [419], progesterone receptor [264, 295] and the estrogen receptor [295]. Our studies show that the hinge region NLSs from GR and TR can interact with the same cellular proteins. This suggests common interactions for nuclear receptor proteins during nuclear entry. How the other signals within the receptors which on their own are competent in nuclear targeting modulate these interactions will be of paramount interest.

4. Summary of Crosslinking Experiments

To summarize the crosslinking results, we have identified a 60-kDa binding polypeptide that binds both GR and TR NLS as well as the SV40 NLS. This is consistent with the involvement of the hinge NLS in a nuclear entry step(s) common to all nuclear proteins and accessibility of the hinge region being the determinant for nuclear entry for steroid and thyroid hormone receptors. Further, the finding that the NLS-binding site was in nuclear and cytosolic fractions suggest that it plays a role in shuttling of the nuclear receptors between the cytoplasm and the nucleus. A

76-kDa binding site with similar properties was also identified, but was judged not to bind NLSs with as great specificity. It is possible from our data and that of others to design a model for further investigation in which the NLS of GR is unmasked by binding of glucocorticoid to its receptor [100], the signal is able to bind to a general import or docking protein of 60 kDa (based on its increased association with the nuclear envelope). Subsequently, it is able to bind to a 76-kDa nuclear importer polypeptide and gain access to the intranuclear compartment (based on its ATP-enhanced binding of NLS). The 76-kDa polypeptide then shuttles back to the cytoplasm for another cycle (based on its nuclear and cytosolic localization). Future work will address the role of these binding sites in nuclear import of the nuclear receptors.

5. The 60-kDa NLS-binding Protein is Probably Not Hsp56

Our inability to crosslink the GR NLS peptides to a 60-kDa product in rabbit reticulocyte lysate, even though hsp56 is present, is not conclusive proof that these are distinct proteins (Fig. 9A). Differences in the rabbit reticulocyte lysate (EDTA, hemin, *etc.*) from the rat liver or mouse lymphoma cytosol may hinder crosslinking to the 60-kDa species. The 54- and 56-kDa species isolated from bovine erythrocytes by Gerace's group, and shown to stimulate nuclear import [349] are believed not to be hsp56 (L. Gerace, personal communication). These bovine products are believed to be representative of the class of 60-kDa NLS-binding proteins observed in the various systems tested, including ours [396]. Further experiments are needed to unequivocally identify the 60-kDa NLS-binding protein. Another candidate that requires some investigation is p60. p60 is the avian homologue of a mammalian 63-kDa protein that is part of a chaperone complex, composed of hsp90, hsp70 and p60 [420], which may also contain hsp56 [421-423]. This complex is found in rabbit reticulocyte lysate and is believed to be required for the assembly of the

untransformed steroid receptor complex. The 63-kDa protein is closely related to the yeast *sti1* gene product, a 63-kDa protein involved in the heat shock response (see [420]). The p60 and hsp56 (FKBP59) proteins are distinct [76].

The best evidence that the 76-kDa NLS-binding protein is not protein kinase C (PKC) comes from the competition experiments in which the PKC-substrates were poor competitors for crosslinking of GR NLS-L to the 76-kDa product (Fig. 10).

6. Identification of Nuclear Import Factors by Means other than Crosslinking

The crosslinking experiments performed by our group and others are a useful and valid means of identifying NLS-binding proteins. These studies have been complemented by the genetic and functional studies (discussed below). Genetic studies (see Table 11) have identified factors such as SCJ1 a homologue of *E. coli* DnaJ [424] that appear not to be directly related with nuclear import but that play a more general chaperone role in the cell. There are at least five DnaJ homologues in yeast SCJ1, YDJ1(MAS5), SIS1, SEC63, and Zuotin [425], some of which are known to be involved in endoplasmic reticulum and mitochondrial protein import. Bacterial DnaJ is known to function together with DnaK (an hsp70 homologue) and GrpE, as a molecular chaperone. SCJ1 is also a mammalian hsp40 homologue [426]. The genetic studies take an indirect approach to investigate the role of proteins in nuclear import and probably do not identify proteins directly involved in nuclear import. Any serious mutation affecting nuclear import would be lethal for the cell due to the fundamental importance of nuclear function to cell viability.

Table 11. Nuclear Import Factors Identified by Genetics

Factor	Species	Protein location[a]	Protein size (kDa)	Comment	Refs
NSP1	Yeast	NPC	100	Essential gene	[350, 427, 428]
NSP49	Yeast	NPC	49		[350, 429]
NPL1	Yeast	ER	73	Identified as SEC63	[430]
SCJ1	Yeast	ER	40	DnaJ homologue hsp40 homologue	[424] [426]
NPL3	Yeast	N	54		[350, 431]
NPL6	Yeast	N	50		[202]
NIP1	Yeast	C	100		[432]
NUP116	Yeast	NPC	116	Mutant forms seal over NPC Also called NSP116	[433] [429]

[a]NPC, nuclear pore complex; C, cytoplasm; ER, endoplasmic reticulum; N, nucleus.

(ii) In Vitro Nuclear Protein Import Models

1. *In Vitro* Binding to Isolated Rat Liver Nuclei

The immediate goal of these experiments was to demonstrate that *in vitro* translated nuclear receptor proteins could be imported into isolated rat liver nuclei in buffer. The ultimate goal was to use the system for identification of NL2 binding proteins by crosslinking of NL2, to characterize GR nuclear import *in vitro,* and to test the function of NLSBPs for NL1. I first established that the coupled transcription/translation kit programmed with GR or NL2 cDNA yielded the correct molecular weight products as detected by fluorography of an SDS-PAGE gel of the translation products, and also by Western blotting with an anti-GR antibody (Fig. 10). The conditions for transformation of the GR complex were then established. It is known that GR translated *in vitro* associates with hsp90 near the end of translation [89, 96, 434, 435], and that the lysate can reconstitute purified transformed cytosolic GR into an untransformed complex [436]. The synthesized receptors bind hormone as well as cellular GR does. Transformation of steroid receptors is measured either by the change in the receptor to a DNA-binding state (*e.g.*, binding to DNA-cellulose) or by a shift in sedimentation coefficients from an untransformed complex of 8-10 S to a transformed complex of 4 S on sucrose or glycerol gradients. Usually differences in sedimentation coefficient are detected after binding of the receptor to a radiolabeled hormone in the presence or absence of 10-20 mM $MoO_4^=$. The presence of $MoO4^=$ keeps the receptor in an untransformed state. High-salt concentrations in the absence of $MoO4^=$ are added to fully transform the receptor. The free hormone is removed by dextran-coated charcoal extraction before the labeled receptor is loaded onto a gradient for fractionation. The fractions are then counted in a scintillation counter and the sedimentation determined from the positions of known standards. However, the presence of a large excess of unincorporated [^{35}S]-methionine in the

translation product suspensions obscured determinations of the position of the radiolabeled receptor. Therefore, I ran all fractions on an SDS-PAGE gel, and scanned the intensity of the fluorogram for the GR band, as has been done for *in vitro* translated androgen receptor [365]. I tested two different transformation protocols, heat alone (25 °C, 30 min) or ligand (100-1000 nM dexamethasone, 2 h, 4 °C) combined with heat. I did not want to use high-salt to transform the receptor as this may interfere with subsequent measurements of receptor binding to nuclei. High-salt was omitted from the gradient in order to observe only the transforming effect of heat or ligand. The full-length rat GR construct we have used in our studies was missing a stretch of nineteen consecutive Q residues in the N-terminus of the receptor. These were probably removed because they hinder *in vitro* transcription/translation production of this protein. However, as this stretch of Q residues is absent in the human GR, its absence is unlikely to influence our analysis of rat GR nuclear import. Sedimentation profiles of GR and NL2 in the untransformed state showed that while GR is completely untransformed, the majority of NL2 is untransformed with a small amount (13 %) that is transformed even in the presence of molybdate (Fig. 11). Ligand and heat treatment of both translated constructs yielded more efficient transformation than heat alone. Heat transformation of GR transformed 59 % of the receptor while ligand and heat transformed 84 % of the receptor. The smaller ligand-binding domain was poorly transformed by heat alone (27 %) while ligand and heat transformed 59 %. Less of the ligand-binding domain (NL2) was transformed than GR by either method. This is most likely due to NL2's reduced affinity for hormone and the highly hydrophobic nature of the domain that may enhance hsp association and prevent transformation. It is important to note here that my definition of transformation means the disruption of the large steroid receptor-hsp complex. Another definition often used is the acquisition of DNA-binding ability. This definition could not be used as the ligand-binding domain (NL2)

does not have a DNA-binding domain. The translated products were transformed as well as, or better, than have been obtained for radiolabeled cytosolic receptor [364] or *in vitro* translated receptor [96, 435]. Although it is difficult to directly compare results as the transformation results vary for the various sources of GR, transformation protocols, and assays used for transformation, heat-transformation of cytosolic GR typically yields 20-40 % activation [364], while hormone- and heat-transformation of *in vitro* translated receptor typically yield a 30-35 % increase in binding of GR to bulk DNA [435] or a 15-20 % increase in binding to DEAE-cellulose [96]. It was previously determined by Schlatter *et al.* [89] that GR and NL2 translated *in vitro* bind to hsp90.

I adapted a filtration assay used previously for the measurement of $^{45}Ca^{++}$ binding to nuclei for measurement of GR binding to nuclei. I switched from a nitrocellulose filter, normally used in water microbiology for coliform analysis, to a hydrophilic PVDF-membrane as this membrane showed the least background binding of protein. The hydrophilic PVDF-membrane binding results agreed with the manufacturers statement that this membrane binds protein in the 1 $\mu g/cm^2$ range, two orders of magnitude lower than nitrocellulose or nylon filters. The direct spotting of lysate to the filter does not give an accurate determination of ^{35}S-labeled protein as the lysate also contains other ^{35}S counts ([^{35}S]-methionine, free or bound to tRNA). The actual amount of trichloroacetic acid precipitable counts in 3 µl of GR-containing lysate is around 400 000 d.p.m. (or 15 % of the total counts). Therefore, 2.4 % binding of ^{35}S to the nitrocellulose filter actually represents 16 % binding of labeled protein to nuclei (Table 6).

More than three times the amount of transformed receptor bound to isolated rat liver nuclei compared to untransformed receptor. This difference was not affected by exogenous BSA added to block non-specific binding. The translated receptor represented only a small percentage of the total protein in the lysate.

Therefore, the lysate components probably block some non-specific binding sites. To our surprise, there was no difference in binding in the presence or absence of 4 mM ATP. We expected a difference because nuclear import of proteins is a two-step process [159-161]; the first step is an ATP-independent binding to the nuclei while the second step is an ATP-dependent import into the nuclei. Therefore, the lack of dependence on ATP suggests that the nuclei are not competent for nuclear import. Rather, only the first step, *i.e.*, binding to nuclei is occurring to isolated nuclei. This is supported by the fact that we did not observe differences in binding of transformed receptor at 4 or 33 °C. Chilling is known to block nuclear import but still allow nuclear binding [159-161]. The small differences we sometimes observed between binding at 4 °C and 33 °C were thought to be due to differences in receptor transformation with the receptor rapidly transforming at the high temperatures in some cases. It has also been suggested [437], that a shift in the transformation equilibrium may occur upon binding to nuclei. This was supported by our inability to observe any difference in nuclear binding between heat-transformed and ligand- and heat-transformed receptors, even though sedimentation analyses had indicated that heat and ligand was more efficient in the promotion of transformation. Therefore binding of transformed GR to nuclei may promote conversion of any untransformed GR in the sample to transformed receptor, and ligand-transformation or heat-transformation results in receptors equally competent and indistinguishable in their ability to bind nuclei. The shift in equilibrium was not detected in the sucrose gradient results of receptor transformation because the gradients were performed in the absence of nuclear acceptor sites for transformed receptors. Another cause of variability between experiments may be freezing and thawing of stored receptor which may cause transformation of untransformed receptor, or inactivation of stored transformed receptor and alter the interaction with nuclei (E. LaCasse, R. Haché, G. Préfontaine, unpublished).

The peroxisomal protein, luciferase, was used as a negative control for the binding of protein to nuclei and it bound poorly to nuclei as expected. The ligand-binding domain, NL2, served as an internal control for GR binding to possibly ruptured nuclei. It bound to nuclei several fold greater than did the full-length GR. Since NL2 has no DNA-binding function this definitely ruled out any contribution of DNA binding of GR to ruptured nuclei that could expose other GR acceptor sites.

We were also surprised to find that slightly more receptors bound to mitochondrial membranes than to nuclear membranes. This suggested that the binding was non-specific. This was further supported by our (E. LaCasse, R. Haché, G. Préfontaine, unpublished) observation that transformed receptor bound non-specifically to a glutathione-agarose matrix several-fold better than untransformed receptor did. However, a recent report [438] demonstrates the import of GR into mitochondria *in vivo* and *in vitro*. GR synthesized in an *in vitro* reticulocyte system enters within minutes to added rat liver mitochondria in the form of intact GR, as demonstrated by Western blotting. Therefore, the binding of GR to both mitochondria and nuclei may be physiologically relevant.

I was unsuccessful at crosslinking NL2 bound to nuclei (data not shown), as no new crosslinked products were detected when NL2 was bound to nuclei as compared to NL2 alone. The lack of detection may be due to low yield or the lack of accessible and correctly spaced chemically reactive amino acid side chains on NL2 or the putative binding protein for the crosslinking agents used.

We conclude that isolated nuclei are not competent for ATP-dependent nuclear import of GR. However, this system can be used to study the first step of nuclear import, that of nuclear binding. Two reports of *in vitro* translated proteins (p53, E1b, thymidine kinase, nucleoplasmin and SV40 large T-antigen) being imported into isolated mouse liver nuclei [439] or cytochalasin B-enucleated yeast nuclei [440] indicate that a 20-fold nuclear accumulation is observed in the former, while greater

than 70 % of the nuclear protein is imported in the latter. These results differ from my results and those of Simons and co-workers [441]. Simons and co-workers state that isolated rat liver nuclei demonstrate only binding of GR and no transport. Furthermore, under the same conditions used to observe the temperature-dependent nuclear import of phycoerythrin-NLS conjugates, transformed GR displayed equal levels of nuclear binding at both temperatures, similar to my results. The differences in systems used by our and Simons' laboratories compared to others are many. However, one of the most critical factors is likely the quality of the nuclei for *in vitro* import which differs with the different preparation methods of the various groups. However, the early reports of *in vitro* import into isolated nuclei in which both steps were shown to be viable have not been duplicated by other groups, nor have there been follow up reports from the original laboratories.

A recent study reports the ATP-dependent nuclear transport of SV40 Vp3 does occur in isolated nuclei in the absence of added cytosol [441A]. This is demonstrated using a filtration assay similar to mine. The authors explain their success by the source of their nuclei, cultured TC7 cells in which extensive cytoskeletal fibers surround the nucleus. This differs from isolated rat liver nuclei which have few attached cytoskeletal elements. These elements are thought to play a role in nuclear import.

2. *In Vitro* Import into Permeabilized Cells

Two other systems for investigation of nuclear import have been used. The first system involves adding *Xenopus* cytosol to isolated rat liver nuclei to reseal the damaged nuclei and reconstitute import [348, 442, 443]. The second system involves digitonin permeabilization of cells. We did not attempt to adapt the *Xenopus* cytosol/rat liver nuclei system to our purposes because we felt the system was heterogeneous which would make identification and analysis of import factors more

difficult. We chose the permeabilized cell system for nuclear import which has gained widespread acceptance [192, 193, 350, 444] in recent years.

It was necessary to permeabilize the cell plasma membrane with a low concentration of digitonin (0.004 %) so as not to permeabilize the nuclear envelope at the same time. A fluorescent import substrate, allophycocyanin-NLS conjugate, demonstrated ATP-dependent nuclear import in this system. To witness the nuclear import of GR it was necessary to use a large amount of GR, more than generated by *in vitro* translation. Therefore, we produced a fragment of GR (amino acids 407-556) in *E. coli* which included the NL1 signal of GR. The 19-kDa fragment was complexed with an antibody to prevent diffusion through the nuclear pore. It would be feasible to use this reconstituted system to study GR nuclear import with GR fragments that have been produced in *E. coli*, baculovirus [109, 197, 445], or vaccinia [446].

3. Other Factors Involved in Nuclear Import Identified in Import Assays

The permeabilized-cell system and the *Xenopus* extract resealed rat liver nuclei system have been used to identify components involved in nuclear import (see Table 12). Cytosol can be depleted of factors, fractionated and reconstituted with purified factors to test their roles in nuclear import. Two NLS-binding proteins have been shown to function in *in vitro* nuclear import, p56/54 [349] and NBP70 [413], as has the molecular chaperone hsp70/hsc70 [196, 197]. A recent interesting discovery by two independent groups [444, 447] provides evidence for a GTPase, Ran/TC4, a Ras-related nuclear protein, in stimulating nuclear import of protein. Ran/TC4 does not have an NLS but it is found predominantly in the nucleus (70 %). It may 'piggyback' into the nucleus with RCC1, a regulator of chromosome condensation that carries an NLS and is a guanine nucleotide exchange factor for Ran/TC4 [444]. This finding is interesting because it provides evidence for a GTPase in nuclear import while other members of the Ras superfamily, Rab and ARF, have clearly demonstrated roles in

Table 12. Nuclear Import Factors Identified by Function or Shown to Function in Import Assay

Factor	Species	Protein location[a]	Protein size (kDa)	Comment	Refs
p62-p58-p54 complex	Rat	NPC	54, 58, 62	GlcNAc proteins (nucleoporins)	[448, 449]
NIF-1	Frog	C	Unknown	NEM sensitive	[348]
NIF-2	Frog	C	Unknown	NEM sensitive	[348]
PCG-binding factor[b]	Rabbit	C	Unknown	NEM insensitive	[450] [429]
Ran/TC4[c]	Frog Human	N>C	25	Stimulates import	[444, 447]
gp210	Rat	NE/NPC	210	Antibodies to lumenal domain of gp210 inhibit nuclear import	[451]
p180	Rat	NPC	180	GlcNAc protein interacts with cytosolic factor required for nuclear import	[450]
hsp70/hsc70	various	C+N	70	See refs. 188, 189, 192 and 193	
p56/54	Bovine	C	56, 54	NLSBP, stimulates import in permeabilized cells	[349]
NBP70	Yeast	N+C	70	NLSBP, antibodies to NBP70 prevent SV40-T ligand binding to nuclei	[407, 413]
p97	Bovine	C	97	Aids NE binding not an NLSBP	[452]

[a]NPC, nuclear pore complex; C, cytoplasm; ER, endoplasmic reticulum; N, nucleus; NE, nuclear envelope. [b]PCG-binding factor, cytoplasmic protein that binds pore complex glycoproteins modified with O-linked GlcNAc. [c]Ran/TC4 is a teratocarcinoma-derived Ras-related nuclear protein. Other abbreviations: NEM, N-ethylmaleimide; GlcNAc, N-acetylglucosamine; NLSBP, NLS-binding protein.

vesicular transport in the cytoplasm [453]. The permeabilized-cell system has recently been adapted to yeast cells [350] which will provide a powerful tool for the future because it is now possible to analyze the yeast genetic mutants by complementation assays with cytosolic extracts from wild-type or other mutant cells.

4. Summary of *In Vitro* Import Models

We have reconstituted an *in vitro* system consisting of isolated rat liver nuclei to study the first step in GR nuclear import. *In vitro* transcription/translation of GR and NL2 cDNAs yield products that can be ligand- or heat-transformed like cytosolic GR. There was approximately 10 % binding of the transformed receptor to isolated rat liver nuclei. Transformed GR bound to nuclei 3- to 6-fold better than untransformed receptor, while a non-nuclear protein, luciferase, bound poorly. Binding occurred at at 4 ^{o}C and at 30 ^{o}C and was not dependent on the addition of ATP, indicating that we had reconstituted the first step of nuclear import. NL2 bound to isolated nuclei as well. However, the binding of GR and NL2 to mitochondria raises some questions about the specificity of these interactions. Although no mitochondrial import of a GR fragment was observed in digitonin-permeabilized cells (Fig. 15). A proven *in vitro* nuclear import assay was established for GR in which the two steps of nuclear import were observed and controlled by chilling the cells, omitting ATP, or by adding wheat germ agglutinin (not performed here) which block the ATP-dependent nuclear translocation of protein. I have therefore established an *in vitro* system to study the nuclear import of GR under controlled circumstances.

(iii) Proximity of NLSs and DNA/RNA-binding Motifs

1. Initial Observation

NLSs occur anywhere in the primary structure of a protein and have been identified at the N-terminus, at the C-terminus and in between for various nuclear proteins with a functional NLS. However, I observed that within the nuclear receptor superfamily, steroid receptors, thyroid hormone receptors, and retinoic acid receptors, the NLS (NL1 in GR) either overlaps directly with DNA-binding functions or is separated from the C-terminal end of the DNA-binding domain by only a few amino acids [295]. This observation prompted me to investigate whether this close spatial relationship between the NLS and nucleic acid-binding domain was a general property of other nucleic acid-binding proteins.

2. NLSs Overlap DNA- and RNA-binding Domains in Nucleic Acid-binding Proteins

A review of the literature identified those nuclear proteins with known DNA- (Table 13) and RNA-binding domains (Table 14) and for which an NLS has been proven by an additive or negative approach. More than forty different nuclear proteins from non-homologous genes have been reported for which the positions of the DNA-binding motif and the NLS(s) are known, while only a small number of proteins with defined RNA-binding motifs and NLS(s) have been reported. I have determined the relative positions of the DNA- or RNA-binding domain to the NLS in each of these proteins. The proteins are grouped, based on the type of DNA- or RNA-binding motif, similar to the grouping in the Prosite library (*e.g.* nuclear receptor 'zinc-finger') for ease of analysis [454]. The positions of the DNA- and RNA-binding domains and of the NLS are indicated. Each of the nuclear proteins was then placed into one of four categories depending on whether the NLS and the DNA- or RNA-binding motifs are overlapping (O), immediately flanking each other (F, *i.e.* less

than ten amino acid separation), separated by less than thirty amino acids (proximal or P) or separated by more than thirty amino acids (distal or D).

The major findings from Tables 13 and 14 are as follows. First, 78 % of the proteins contain overlapping or flanking NLSs and nucleic acid-binding motifs (40 out of 51) or 76 % (32 out of 42) of proteins with DNA-binding motifs, and 89 % (8 out of 9) of proteins with RNA-binding motifs. Although homologous proteins are listed, these percentages were calculated from the non-homologous proteins so as not to give any weighted advantage to the various homologues of a protein that have a proven NLS function. Inclusion of the homologues into the calculations would increase the reported percentages. Second, the proximity of NLSs and DNA- or RNA-binding motifs was observed for several types of DNA- and RNA-binding motif in proteins of unrelated evolutionary origins. This suggests a functional basis for the association of NLS and DNA- or RNA-binding motifs. Third, the NLSs found close to DNA- and RNA-binding motifs are also representative of various classes of NLSs (see Introduction).

I have classified members of the Rel family as proteins with flanking NLS/DNA-binding domains even though according to the criteria described above they could be classed as distal because the two motifs are separated by more than 100 amino acids. The Rel homology domain, also called the NRD (NF-kB/Rel/dorsal) motif, is a roughly 300 amino acid region that is conserved from *Drosophila* to man [455]. The Rel homology domain contains the DNA-binding domain at its N-terminus next to a dimerization domain with an NLS at the C-terminal end. All three functional domains are conserved. An exception for this family was made because the dimerization domain between the NLS and DNA-binding domain may be required for high affinity DNA binding, and therefore can be considered part of the DNA-binding domain which forms with the NLS a distinct well-conserved modulatory cassette known as the Rel homology domain.

Table 13. DNA-binding Proteins with Proven Nuclear Localization Signals

DNA-BINDING MOTIF	NUCLEAR PROTEIN	(#aa)	MINIMAL DNA-BINDING DOMAIN	NUCLEAR LOCATION SIGNAL(S)	Class'	REF.
HTH homeobox	mouse Pit-1 (GHF-1)	(291)	214-273	209-252	O	[456]
	yeast Matα2	(210)	136-188	1-13		[233]
				141-159	O	[234]
ZINC FINGER nuclear receptor	human ER	(595)	179-250	256-303	F	[114, 295]
	chicken PR	(786)	410-478	490-506	F	
				477-487	O	
				second finger	F	[295]
	rabbit PR	(930)	568-633	638-642/645	O	
				614-618	O	[178,
				624-627		264]
	rat GR	(795)	438-505	497-524	O/F	[1]
				540-795		
	human GR	(777)	420-488	478-505	O/F	[294]
				521-777		
	human AR	(910)	550-615	608-625	O/F	[292,
				628-634		297]
		(919)	559-624	580-661	O/F	[293,
						296]
	rat TRα1 (c-erbA)	(410)	53-121	134-136	F	[301]
	chicken c-erbA	(408)	51-118	127-135	F	[226]
	AEV v-erbA	(639)	293-361	370-376	F	[299,
						300]
ZINC FINGER poly(ADP-ribose)pol	human PARP	(1014)	12-66	207-226	D?	[457]
			116-166			
ZINC FINGER C2H2	mouse Egr-1 (NGFI-A, Krox24, Zif268, TIS8)	(533)	331-419	315-330		[458]
				361-419	O	
				328-433	O	[459]
	yeast Swi5	(709)	538-635	635-655	F	[460]
ZING FINGER B box (RING)	xenopus nuclear factor 7	(609)	145-273	106-120	P	[208]
ZINC FINGER (GAL4)	yeast Gal4	(881)	11-38/52	1-29	O	[222]
ZINC FINGER (other)	adenovirus E1a	(289)	139-185	282/285-289		[214]
				140-185	O	[211]
	human XPAC	(273)	105-129	30-42	D	[461]
	human GATA-3	(444)	303-347	249-311	O/F	[462]
bHLH/bZIP Fos/Jun	ASV v-Jun	(296)	223-273	223-239	O	[206]
	chicken c-Jun	(310)	236-287	245-253/259	O	[206]
	human c-Jun	(340)	259-317	267-283	O	[463]
	mouse c-Fos	(380)	131-193	139-161	O	[213]
	FBJ v-Fos	(379)	131-193	139-161	O	[213]

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DNA-BINDING MOTIF	NUCLEAR PROTEIN	(#aa)	MINIMAL DNA-BINDING DOMAIN	NUCLEAR LOCATION SIGNAL(S)	Class'	REF.
bHLH/bZIP Myc	AMCV v-Myc	(416)	330-410	294-309	P	[464]
	human c-Myc	(439)	354-439	320-328		[228]
				364-374	O	[465]
	human N-Myc	(456)	381-456	337-344	P	[226]
	mouse Max (Myn)	(160)	24-103	149-157	D	[466]
	human Max	(151)	15-99	140-148	D	[467]
bHLH/bZIP (other)	EBV ZEBRA (Zta, EB1)	(245)	167-202	178-183	O	
				187-194	O	[463]
	rat CREB	(327)	269-327	287-295	O	[468]
	tobacco TGA-1A	(359)	77-114	70-139	O	[469]
	maize Opaque-2	(437)	136-284	101-135		
				223-254	O	[470]
Helix-Loop-Helix	maize R protein	(610)	190-330?	100-109	D	[235]
				419-428		
				598-610		
	human c-myb	(640)	34-86	521-528	D	[226]
Ets (HTH)	chicken c-ets-1	(485)	375-459	421-427	O	[471]
		(441)	331-415	369-388	O	[472]
				388-441	O/F	
HSF	human HSF2 (heat shock factor)	(536)	5-113	108-122	F	[219]
				195-210		
HMG BOX	mouse UBF	(795)	6 boxes	449-480	O	[258]
			401-480 (#4)			
			480-560 (#5)			
	xenopus UBF-2	(701)	5 boxes	566-592?	F	[473]
			?-563 (#5)			
NRD or RH domain	v-rel	(503)	(rel 16-304)	274-317	F ?	[474, 475]
	human p65	(551)	33-226 (rel 21-291)	300-304	F ?	[476]

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DNA-BINDING MOTIF	NUCLEAR PROTEIN	(#aa)	MINIMAL DNA-BINDING DOMAIN	NUCLEAR LOCATION SIGNAL(S)	Class'	REF.
OTHER	yeast Apn1 DNA repair enzyme	(367)	1-287?	334-367	D?	[477]
	SV40 large T-antigen	(708)	137-246	126-132	F	[154, 155]
	SV40 Vp3	(234)	221-234?	198-206	F?	[478-480]
	Agrobacterium VirE2	(556)	? unknown	212-252	O/F?	[481]
	Agrobacterium VirD2	(447)	29-?	1-37 417-434	O/F?	[482, 483]
	polyomavirus VP1	(384)	1-5	1-11	O	[484, 485]
	BPV E1 replication protein	(605)	1-299	85-108	O?	[486]
	hamster RCC1	(421)	8-?	8-24/29	O	[487]
	HSV ICP8	(1196)	564-849	1169-1196	D	[488, 489]
	murine p53	(390)	307/343-390 280-390 80-290	312-318 365-370 375-380	O/F	[490-492]
	human p53	(393)	319-393	316-325 312-323	O/F	[226, 493]
	Drosophila HP1	(206)	95-206	152-206	O	[494]
	Adenovirus DNA-binding protein	(529)	?-529	23-105	P/D	[495, 496]

Table 12. Abbreviations: O, overlapping; F, flanking; P, proximal; D, distal; HTH, helix-turn-helix; bHLH/bZIP, basic region helix-loop-helix/basic region leucine zipper; HSF, heat shock factor; HMG, high mobility group; NRD, NF-kB-Rel-Dorsal; RH, Rel homology; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; AR, androgen receptor; TR, thyroid hormone receptor; ?, refers to uncertainties with exact borders of domains, or other uncertainties (see text). 'When a protein contains multiple NLSs only the closest NLS to the DNA-binding domain is classified.

Table 14. RNA-binding Proteins with Proven Nuclear Localization Signals

RNA-BINDING MOTIF	NUCLEAR PROTEIN	(#aa)	MINIMAL RNA-BINDING DOMAIN	NUCLEAR LOCATION SIGNAL(S)	Class'	REF.
RNP/RRM	chicken nucleolin	(694)	278-682	256-273	F	[256]
	xenopus nucleolin90	(651)	236-639	271-290 209-225	F/O	[209]
	yeast NSR1	(414)	169-187 (RRM1)	139-142 159-162	F	[257]
ZINC FINGER	xenopus XFG 5-1	(420)	7 fingers (209-404)	in finger 3 (265-292)	O	[497]
OTHER	HDV p27 delta antigen	(214)	88-163 (79-107) (2-27)	35-44 69-89	F/O	[498-500]
	influenza PB1 (NS1)	(237)	19-38	34-38 216-221	O	[501, 502]
	yeast L25	(142)	62-126	11-17 18-28	P/D	[503]
	HTLV-1 Rex (pX)	(189)	1-19/77	1-19	O	[250]
	HIV-1 Tat	(86)	37-72?	48-52/61	O	[226, 504]
	HIV-1 Rev	(116)	35-50	38-45	O	[244, 504]

Abbreviations: O, overlapping; F, flanking; P, proximal; D, distal; RNP, ribonucleoprotein; RRM, RNA recognition motif. 'When a protein contains multiple NLSs only the NLS closest to the RNA-binding domain is classified.

The poly(ADP-ribose)polymerase (PARP) bipartite NLS is well conserved for human, murine, bovine, chicken and *Xenopus* PARP [453]. Because this NLS is functionally separate from the DNA-binding activity and forty amino acids away from the second 'zinc-finger', we have classified it in the D (distal) group. However, it is part of a 46-kDa proteolytic fragment that forms the DNA-binding domain which suggests that the two functions share a common protease-resistant globular domain. Therefore, it could be argued that the NLS and DNA-binding motifs should be classified as flanking [505].

Another example that requires some clarification is that of the *Agrobacterium spp.* VirE2 protein. VirE2 and VirD2 are required for the nuclear import of the *Agrobacterium* T-DNA complex into plant nuclei [151]. VirE2 is a single-stranded DNA-binding protein. Although the VirE2 and VirD2 NLSs have been mapped, their DNA-binding domain has not been precisely mapped. The VirE2 DNA-binding domain lies somewhere in the C-terminal two thirds of the protein [151]. A possible overlap with the NLS has been inferred because a point mutation in the NLS also disrupts DNA binding [151]. Likewise, the N-terminal NLS sequence of VirD2 is very close to a tyrosyl residue proposed to be involved in the covalent linkage of VirD2 to the T-DNA [506].

The NLS of SV40 Vp3 protein (amino acids 198-206, [478]) is classified as flanking or overlapping the DNA-binding domain (amino acids 221-234) because the C-terminal amino acids which constitute the major DNA-binding domain requires the C-terminal thirty-five amino acids (which also contain the NLS) for full DNA binding activity [479]. The yeast Apn1 DNA repair enzyme has been classified as distal since the DNA binding function must be within the homology domain with *E. coli* endonuclease IV which excludes the region containing the NLS. However, this does not preclude the possible contribution of the NLS region to DNA binding of Apn1. The adenovirus DNA-binding protein NLS is classified as distal because the

DNA-binding domain lies in a C-terminal chymotryptic fragment of 44-46 kDa while the NLS lies in the first one hundred and five N-terminal amino acids [496]. Take note that the overlapping domains of the heterochromatin-associated protein HP1 which mediate nuclear localization and heterochromatin binding may be mediated by protein-protein interactions rather than HP1 binding directly to DNA or to an NLSBP. HP1 has no identifiable DNA-binding motifs or NLS motifs [494].

I have considered the so-called nucleolar localization signal of Rex to be a nuclear localization signal, as both nuclear and nucleolar proteins have to be imported into the nucleus. What has been called a 'nucleolar localization signal' is probably an RNA-binding function, which is overlapping a known nuclear localization signal. Thus the RNA binding is responsible for the sub-nuclear localization and not the NLS [255-257]. As I discussed in the Introduction, it has been demonstrated clearly that the nucleolar localization of nucleolin [172, 256], NSR1 [257] and UBF [258] is due to nucleolar component-binding sequences.

It is interesting to note that the only nuclear proteins known to affect RNA export (see below) share the property that their NLSs and DNA- or RNA-binding motifs are overlapping. The overlap may be required for the proper functioning of these proteins, RCC1, the influenza virus NS1 protein, the HIV Rev protein and its functional homologue HTLV Rex [507]. RCC1 is also a guanine nucleotide release protein for a GTPase, Ran/TC4, which is involved in protein nuclear import (see [444]).

Another point of interest is that some of the proteins listed in the tables are conserved in prokaryotes. However, as might be expected the prokaryotic homologues lack an NLS. Yeast ribosomal binding protein L25 which binds rRNA in the nucleus [503] and the yeast DNA repair enzyme Apn1 [477] possess extensions with NLSs while their prokaryote homologues do not possess NLSs.

I have restricted my analysis to DNA- and RNA-binding motifs, although, NLSs may overlap with other important functional motifs of nuclear proteins. An example is the herpes simplex virus protein ICP8 for which the C-terminal twenty-eight amino acid NLS appears to overlap with a viral replication function [508].

3. Hypothesis

We have determined that NLSs and DNA- or RNA-binding domains of the majority of nuclear proteins are either overlapping or flanking. The proximity of the motifs suggests that their association confers some evolutionary advantage. We suggest that DNA- and RNA-binding motifs have evolved with an overlapping or flanking NLS for one or more of four reasons: requirement of the NLS for DNA- and RNA-binding protein localization, requirement of DNA binding in retaining proteins in the nucleus, the additional function of the NLS in DNA binding, or the need to mask both functions simultaneously.

A. Requirement of NLS for DNA- or Nuclear RNA-binding Protein Localization

A prerequisite for any DNA- or nuclear RNA-binding protein is localization to the cell nucleus. The correct localization of a DNA- or RNA-binding protein requires an NLS within the protein itself or association of the DNA- or RNA-binding protein with a protein that does contain an NLS for co-transport. Hence, DNA- and nuclear RNA-binding proteins are dependent directly on NLSs for their localization, and indirectly for their function.

B. DNA Binding, Retention and Nuclear Localization

The localization of proteins in the nucleus is dependent on the processes of nuclear import, retention in the nucleus, and export from the nucleus to the cytoplasm. The import of large nuclear proteins (larger than 70 kDa) requires an

NLS. However, it appears that if the protein does not bind to intranuclear components it is exported out of the nucleus by a default pathway [171, 172], even though the protein contains an NLS. The protein is still competent for nuclear entry but it will be recycled out of the nucleus. Thus, continued nuclear residency requires a nuclear-binding function such as DNA- or nuclear RNA-binding.

C. Additional Function of the NLS in DNA/RNA Binding

In some of the DNA-binding proteins with overlapping NLSs it is possible to introduce point mutations that disrupt one function but not the other [459, 470, 509]. An R to K mutation in the basic domain of the maize regulatory protein opaque-2 will no longer bind DNA but this mutant is nonetheless localized in the nucleus. DNA binding *per se* of NGFI-A (Egr-1, zif268, Krox-24), which has an overlapping NLS, is not required for nuclear localization. Of course a mutant which does not bind DNA is still directed to the nucleus. Another mutation which abolished nuclear localization had no effect on DNA binding. These results clearly demonstrate the overlap of two distinct and distinguishable motifs. However, possible co-operation in the action of these motifs is not ruled out. NLSs because of their basic nature may also increase DNA- and RNA-binding affinities. Hence, the NLS may have a dual function in the DNA- and RNA-binding proteins. This appears to be the case for the POU homeodomain transcription factor, Pit-1, in which a deletion of part of the DNA-binding domain destroys an NLS function [456]. The basic stretch of amino acids within this deletion that is believed to be the NLS of Pit-1 is known to make base specific and phosphate backbone DNA contacts for Pit-1 and other homeodomain proteins [510-512].

The study of NLS function in other homeodomain proteins has not received much attention because it is generally assumed that the small size of these proteins would allow their diffusion into the nucleus. However, it may be argued that all

nuclear proteins no matter how small may possibly require an NLS for efficient nuclear import. Functional NLSs have recently been identified in numerous small viral proteins (see Tables 13 and 14) and small mammalian proteins, such as histone H1 [156]. Therefore, it is possible that the majority of homeodomain proteins may require an NLSs for nuclear import. An argument against this possibility is that the putative basic NLS in homeodomains which is definitely part of the DNA-binding domain, is deleted or mutated in some homeodomain inhibitors, *e.g.*, I-POU [513], Id [514, 515], extramacrochaetae [516-518]. Nonetheless, these inhibitors dimerize with specific homeodomain proteins, presumably get into the nucleus, and inhibit DNA binding of the heterodimers. One could postulate that if the basic stretch is truly an NLS and the sole NLS for homeodomain proteins then these inhibitors must 'piggyback' into the nucleus with their dimerization partner.

D. Co-ordinate Regulation: Masking of Both NLS and DNA-binding Functions and Accessibility

NLSs have been shown to be present in regions of the protein that are accessible to antibodies, *e.g.*, in GR [519] and E1a protein [520]. Furthermore, when an NLS was inserted into various regions of the cytosolic protein, pyruvate kinase, its function was dependent on the flanking sequences [221]. Flanking sequences determine ultimately whether or not the sequence is exposed, distorted or folded into the protein. It is believed that to be functional, NLSs must be exposed so that they can interact with the molecular chaperones involved in nuclear import. The DNA-binding domain also must be exposed so that it can interact with DNA. It would be reasonable for both motifs to be placed close to each other in a protein sequence that is exposed and not buried inside the protein as part of the structural scaffold of a domain. Furthermore, the DNA- and RNA-binding motifs of a protein are often domains because they are of sufficient length (30-100 amino acids) to form a stable

structure. NLSs are short stretches of amino acids (4-30 amino acids), too short to form an independent structure and therefore, may be part of a larger sequence that forms a structural or globular domain. For example, the NLS of NGFI-A is proposed to be dependent on the overall structure of the DNA-binding domain [459], and the bipartite NLS of the androgen receptor is dependent on the structure of the second 'zinc finger' of the DNA-binding domain [293].

Regulation of the function of a protein is sometimes achieved through masking of an NLS or of DNA binding. Hence, another reason for the close association of NLS and DNA-binding motifs may be to the facilitate mutual or simultaneous masking of both functions that occurs in some inactive proteins. The NLS and DNA-binding functions are masked either by the tertiary or quaternary structure of the protein, or by another factor. The reason for the masking of both functions is not at first obvious. One would think that it would be necessary to inactivate only one function in order to inactivate a transcription factor. However, there may be reasons for masking both functions at once. The DNA-binding domain if exposed in the cytoplasm may bind RNA or may be inappropriately altered by post-translational modifications that will affect negatively its function at a later time.

Transcription factors like the members of the Rel family [455, 521] and some members of the nuclear receptor superfamily become 'anchored' in an inactive complex in the cytoplasm [269] in which the NLS and DNA-binding domain is masked. Recently, this type of anchoring has been described for human heat shock factor 2 (HSF2). HSF2 is believed to reside in the cytoplasm of non-stressed cells because it is folded over onto itself through hydrophobic coiled-coil interactions or through interaction with some negative regulator [219] which appears to result in the masking of the NLS and DNA-binding domains. Heat-shock disrupts this interaction and allows HSF2 to trimerize exposing its NLS and DNA-binding domains [217-219]. Heat-shock inducible translocation of the transcription factor to the nucleus occurs,

followed by binding to heat-shock response elements of heat shock genes. HSF1 also displays stress-induced DNA-binding activity, oligomerization and nuclear localization [217]. HSF1 and HSF2 overexpressed in transfected 3T3 cells both display constitutive DNA-binding activity, oligomerization, and transcriptional activity and therefore by deduction, nuclear localization.

4. Modular, Divergent and Convergent Evolution Mechanisms

A protein domain is usually defined as a linear segment of a protein that can form an independently folded and recognizable structure, and the term is used here to define any part of a protein that can be defined as structurally distinct from the rest. Many proteins are composed of modular domains derived from discrete blocks of amino acids that may have well-defined and separable functions [522]. For instance, nuclear receptors are part of a large superfamily of transcription factors which all share a similar modular design [20]. Sequence comparisons of the chick and human estrogen receptors resulted in the identification of six distinct regions of sequence similarity, termed A to F [29]. All regions except the small N-terminal A region and the small C-terminal F region are conserved for all members of the nuclear receptor superfamily [20]. The modular design obtained from sequence data agrees with previous biochemical proteolytic studies of steroid receptors [19, 25]. The separable functions of each modular domain of these receptors is clearly demonstrated by experiments in which the whole DNA-binding domain of one receptor has been replaced with that of another receptor to change the DNA-binding specificity to the original receptor [523], or in which the ligand-binding domain has been fused to many other transcription factors and shown to confer hormonal regulation of transcription for the resulting chimera [305, 306]. The DNA-binding domain of nuclear receptors is composed of two 'zinc-finger' motifs. A motif is defined as any recognizable sequence that is conserved.

The association between NLSs and DNA- or RNA-binding motifs in one domain for DNA- and RNA-binding proteins may have occurred throughout evolution by a modular mechanism. Some modular domains move about within and between proteins during evolution giving rise to new proteins. The modular theory of evolution in the above context can be better understood in an example in which a module coding for a 'zinc-finger' has fused with the coding region of another gene. If this other protein is a cytoplasmic protein, the DNA-binding 'zinc-finger' would most likely not confer any new function to this protein and would not confer any selective advantage. Hence, this chimeric protein would not be selectively conserved. If a module coding for a 'zinc-finger' and an NLS were fused to another gene's coding region then it is more likely that this protein would gain an additional function as the new protein now is also able to translocate to the nucleus. The co-transfer of both NLS and DNA- and RNA-binding regions increases the protein's chances of conferring some new selective advantage and increases its chances of being conserved. DNA- or RNA-binding proteins with an overlapping or flanking NLS provide a more efficient module that confers both new and co-required properties to the chimeric partner. This module is more likely to be vertically transmitted and to be represented in greater proportion in the examples of DNA- and RNA-binding proteins in eukaryotic cells. Alternatively, a family of proteins is more likely to rapidly evolve, and in large numbers, if it can 'reproduce' using the modular type of evolution.

The overlap of function would impose a considerable constraint on the evolution of such bifunctional domains. Such a constraint for NLS and DNA-binding domains has been proposed by van der Krol and Chua to account for the high degree of similarity of the bifunctional basic domain of bZIP proteins [469]. The numerous examples of NLSs sequences found next to or integrated within 'zinc-finger' domains (Table 13) is consistent with the proposal of Gashler *et al.* that NLSs and C2H2-zinc-

fingers (both typified by basic residues) have coevolved [458]. Further support for such a mechanism comes from phylogenetic analysis of RNA-binding proteins for which RNA-binding domains have evolved together with other auxiliary domains, such as serine-arginine-rich (SR-rich) domains [524].

Another possibility is that NLS and DNA-binding motifs were once identical. It is possible that nuclear import has evolved by recruiting some general properties of DNA- and RNA-binding proteins and that this original motif has diverged to give two proximal specialized (and independent ?) functions that further increase both DNA-binding and nuclear import efficiency.

A third possible mechanism to account for the overlap of NLS and DNA-binding domains in unrelated proteins, is convergence. Many of the transcription factors possess another NLS outside the DNA-binding region. It has been shown that one NLS is sufficient for the proper localization of a nuclear protein although multiple NLSs have been shown in some systems to result in more efficient nuclear import [157]. The NLS near or overlapping the DNA-binding domain may have evolved by a convergent mechanism. The NLS near the DNA-binding domain would provide an evolutionary advantage as in this position it simultaneously improves nuclear localization (by providing a second import signal within the DNA-binding domain) and DNA binding (by increasing the binding affinity). However, it is important to note that sequence convergence has never been proven [525], and what is described above could equally have arisen from divergent evolution.

5. Conclusion

NLSs are required for the import of a large number of, if not all, DNA- and RNA-binding proteins into the nucleus. A survey of members from each of the major DNA- and nuclear RNA-binding families with proven NLS sequences was conducted. NLSs and DNA- or RNA-binding motifs directly overlap or are immediately adjacent

in 78 % of these nuclear proteins. The proximity of the two motifs appeared independent of the class of nucleic acid-binding protein and of the type of NLS. We postulate that the apparent linkage of the two motifs may be governed by one or more of the following physiological requirements: one, a protein must possess both NLSs and DNA-binding motifs to ensure the nuclear localization of a protein which must act in the nucleus; two, continued nuclear occupancy of a protein is a consequence of both nuclear import (NLS-mediated process) and retention (DNA binding); three, the basic nature of a flanking or overlapping NLS may enhance DNA binding, and four, the closeness of the two motifs may allow co-ordinate regulation. Either modular, divergent or convergent evolution may be invoked to explain the mechanism for this association.

Establishing the mechanism whereby NLSs and DNA/RNA-binding motifs became closely associated with each other is not testable as modular theories of evolution and other theories of evolution are not readily testable by direct experimentation [526]. However, the reason for the close proximity of the two motifs suggested is amenable to experimental investigation. It would be of interest, for example, to test whether the separation of the DNA-binding motif from the NLS would have detrimental consequences for the function of the nuclear protein. Although several groups have shown that mutations in an overlapping NLS can destroy DNA binding [151, 300, 456], it is not known to what extent the functions overlap, or how the specific amino acid residues contribute to the kinetics of nuclear import and/or DNA-binding affinity.

PART II: Microsomal Dexamethasone-binding Sites

I. INTRODUCTION

1. Mechanism of Steroid Hormone Action

The mechanism of steroid hormone action is of vital importance to our understanding of control of growth and development via altered cellular function. Although much knowledge exists about the molecular events associated with steroid hormone effects on gene expression [9, 527] there is still very little known about several steps in the mechanism of steroid hormone action [6-8].

The classical two-step model of steroid hormone action assumes passive diffusion of the hydrophobic steroid molecule across the plasma membrane [6-8]. In the first step, the steroid molecule binds to its intracellular receptor and causes the transformation of the steroid receptor from a non-DNA binding form to one that binds specific DNA regulatory elements. In the second step, binding of the steroid-receptor complex to DNA mediates the expression of a specific subset of genes. This altered gene expression is what is believed to account for most physiological and pathophysiological effects of steroid hormones.

2. Bioavailability of Steroids

In the circulation 90 % of the steroids are bound to high affinity (*e.g.*, binding globulins) and low affinity (*e.g.*, serum albumin) transporters. The binding globulins may deliver steroids to cells by binding to surface receptors [528, 529]. However, it is generally assumed, though unproven, that steroids enter cells by passive diffusion through the plasma membrane by virtue of their small size and lipophilic properties. Since the steroid receptor is intracellular, the entry and accumulation of active hormone within the cell is obviously required for receptor

binding. Therefore, any process that can influence the intracellular accumulation of steroids would directly affect receptor occupancy and the magnitude of the hormonal response [530].

3. Identification of Membrane Steroid-binding Sites

There have been claims for transport mechanisms for steroid [531-533] and thyroid hormones [534] across the plasma membrane based on thermodynamic data. In addition, many groups have presented data for steroid-binding sites on plasma membranes [535-545], microsomes [546-553], and the nuclear envelope [554-556, 288] as proof of membrane-steroid interactions. For a general review of steroid binding to membranes refer to Szego and Pietras [557]. Even though the identity or function of most of theses sites is still not known there is some evidence for steroid receptor association with membranes, *e.g.*, estrogen and androgen receptors on microsomal membranes [558-561], 'progesterone receptors' on plasma membranes of *Xenopus* oocytes [562] and on sperm plasma membranes [563]. Other possible steroid binding sites include microsomal membrane 'drug-metabolizing enzymes' which are proposed to regulate the steady state levels of ligands, including steroids, that affect growth, homeostasis, differentiation, and neuroendocrine functions [564].

In 1987, Gametchu's laboratory demonstrated the presence of a steroid receptor-like antigen externally disposed on the plasma membrane of the mouse lymphoma cell line S49 [565]. This antigen was detected by direct immunofluorescence using an anti-GR antibody [566]. Upon further characterization Gametchu's group reported the identification of several high molecular weight forms of GR associated with the plasma membrane [567, 568]. The presence of this membrane-associated GR (mGR) was correlated with increased sensitivity to glucocorticoid-induced lymphocytolysis [565]. In addition, they identified GR-like antigens associated with human leukemic cell plasma membranes

using a different antibody to GR [569]. Grote *et al.* [570] have provided evidence for a plasma membrane-bound GR in rat liver cells, while Liposits and Bohn [571] reported the association of GR immunoreactivity with cell membranes and transport vesicles in hippocampal and hypothalamic neurons of the rat.

4. Non-genomic Effects of Steroids

Certain effects of steroid hormones occur too rapidly (within seconds) to be accounted for by genomic effects which require more time for mRNA induction and translation to protein. The rapid effects are referred to as non-genomic effects. Genomic effects may be seen as rapidly as ten minutes but are typically in the hours to day range [572]. While the principle effects of steroids appear to be via a genomic route, the multiple effects that appear to occur via a non-genomic route are probably of physiological relevance [573]. These non-genomic effects appear to be mediated by membrane-binding sites. Genomic effects of steroids are blocked by inhibitors of RNA and protein synthesis while non-genomic effects are not affected by these inhibitors [573]. The plasma membrane has been postulated to play a role in non-genomic effects of steroids because of its key role in transducing environmental cues to the cell interior [557, 573-578]. For example, progesterone metabolites with anaesthetic properties potentiate the γ-aminobutyric acid (GABA)-induced activation of $GABA_A$ receptor of central nervous system neurons, a plasma membrane multisubunit receptor constituting a Cl^- channel [579-581].

Other examples of non-genomic effects include the breakdown of the germinal vesicle of *Xenopus laevis* oocyte induced by extracellular progesterone [582-584]. This cell appears to lack intracellular steroid hormone receptors. Furthermore, progesterone injected directly into the oocyte is ineffective in inducing oocyte maturation [585, 586], which provides evidence that the effect of progesterone is mediated by some other mechanism that does not involve the progesterone receptor.

The action of progesterone on calcium ion influx and its role in the sperm acrosome reaction has received much interest lately [563]. Sperm must undergo the acrosome reaction (a specialized form of exocytosis in sperm head membranes) before they can penetrate the zona pellucida and fertilize the egg. Before sperm can undergo the acrosome reaction they must proceed through a process called capacitation. Progesterone and 17α-hydroxyprogesterone are able, within seconds, to elevate intracellular Ca^{++} and elicit the acrosome reaction in capacitated sperm. This is thought to occur via a cell surface steroid receptor on human sperm that is distinct from the nuclear progesterone receptor. Unlike the nuclear receptor, this cell surface receptor is not stimulated by potent synthetic progestigins [*e.g.*, promegestone (R5020), norethynodrel, megestrol acetate, cyproterone acetate] and is only weakly antagonized by the anti-progestins RU486 (mifepristone) and ZK98.299 [563]. The plasma membrane receptors were visualized in the head of the sperm and not the midpiece or tail by use of progesterone conjugated to FITC-labeled BSA [563]. Recent evidence suggests the involvement of a unique human sperm steroid receptor/Cl^- channel complex in the progesterone-initiated acrosome reaction [587].

5. Rationale

None of the studies described above provides compelling evidence for membrane involvement in steroid hormone action. Before my arrival in the laboratory, Yvonne Lefebvre had been characterizing, and isolating membrane steroid-binding components in order to investigate their role in steroid hormone action. The goal was to directly identify the binding molecules before using them in reconstitution assays. Previously, androgen receptor [556, 588] and GR had been identified on the nuclear envelope [289], low-affinity binding sites for glucocorticoids had been identified on rat liver plasma membranes [541] and nuclear envelopes [288], and multiple thyroid hormone-binding sites had been identified on

rat liver nuclear envelopes [589]. To shed light on the intracellular dynamics of the glucocorticoid hormone and its receptor, we wished to investigate glucocorticoid interactions with male rat liver microsomes.

6. Goals

The three goals of this aspect of my research were: first, to characterize [^{3}H]-dexamethasone-binding sites in isolated male rat liver microsomes from intact and adrenalectomized animals; second, to further characterize the localization of glucocorticoid-binding sites in microsomal subfractions; and third, to identify the microsomal subfraction dexamethasone-binding sites with the covalent affinity reagent [^{3}H]-dexamethasone 21-mesylate [16].

II. METHODS AND MATERIALS

1. Animals

Adult male Sprague-Dawley rats weighing 225-250 g were obtained from Charles River Canada (Montreal, QC) and maintained on a diet of Purina Lab Chow (Ralston-Purina, St. Louis, MO) and tap water *ad libitum*. The rats were killed by decapitation and exsanguination. The livers were quickly removed and placed in two volumes of ice-cold homogenization buffer (0.25 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl pH 7.6). Bilateral adrenalectomy under ether anaesthesia was performed at Charles River Laboratories (Montreal, QC). Adrenalectomized animals subsequently received 0.9 % NaCl in their drinking water. They were used for experiments 7-9 days after adrenalectomy.

2. Chemicals

[6,7(N)-^{3}H]-dexamethasone (49.9 Ci/mmol) and [6,7(N)-^{3}H]-dexamethasone 21-mesylate (49.9 Ci/mmol) were purchased from NEN-Dupont (Boston, MA). Unlabeled steroids were from Steraloids Inc. (Wilton, NH) except for cholesterol and triamcinolone acetonide, which were from Sigma Chemical Company (St. Louis, MO). Sucrose (Ultra-pure) was from Schwartz-Mann (ICN, Mississauga, ON). TAPS (N-tris-[hydroxymethyl] methyl-3-amino propane-sulfonic acid), Trizma base (Tris), 5'-adenosine monophosphate (5'-AMP), glucose-6-phosphatase, and protease inhibitors were from Sigma. All chemicals for polyacrylamide gel electrophoresis and Western blotting were from Bio-Rad Laboratories (Richmond, CA). BSA (bovine serum albumin, fraction V) was purchased from Sigma. All other chemicals were of reagent grade or better.

3. Microsome Preparation

Microsomes were prepared after differential centrifugation by the method of Omrani *et al.* [590] with the following two modifications: substitution of DTT for β-mercaptoethanol in the homogenization buffer, and the addition of a final wash, *i.e.*, resuspension of microsomes in homogenization buffer or 25 mM TAPS-NH_4OH pH 8.6 and centrifugation at 51 520 x g for 30 min before final resuspension in the appropriate buffer. Briefly, 8-10 g of liver were minced in three volumes of homogenization buffer (0.25 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH 7.6). The mixture was transferred to a 40-ml all-glass Dounce homogeniser (Kontes Scientific Glassware, San Leandro, CA) and homogenized with twenty strokes of the loose-fitting pestle. All steps were carried out at 0-4 °C, as quickly as possible. The homogenate was centrifuged at 1000 x g (2700 rev./min) in a Beckman JA 17 rotor for 10 min. The resultant supernatant was centrifuged at 8816 x g (8000 rev./min) for 10 min. The supernatant was transferred to a 38.5 ml quick-seal tube and centrifuged at 165 000 x g (43 000 rev./min) for 50 min in a Beckman VTi 50 rotor. The microsomal pellet thus obtained was resuspended in buffer using a syringe with an 18-gauge needle. Resuspension was achieved after bringing the volume to approx. 30 ml with buffer (homogenization buffer or 25 mM TAPS-NH_4OH pH 8.6 buffer, depending on the assay) and homogenizing in a Dounce homogeniser with twenty strokes of the tight-fitting pestle. The suspension was centrifuged at 51 520 x g (20 000 rev./min) in a Beckman JA 20.1 rotor for 30 min. The supernatant was discarded and the microsomal pellet was resuspended in the appropriate buffer to a final volume of approx. 20 ml.

4. Microsomal Subfractionation Procedure

The procedure was a modification of a method to prepare bovine anterior pituitary microsomes [591]. Male rat liver microsomes from 8-10 g starting material

in 20 ml homogenization buffer (8 % sucrose, density = 1.03 g/ml) were layered on discontinuous sucrose gradients in four 38.5 ml ultracentrifuge tubes. The gradient consisted of 5 ml homogenate layered on top of 10 ml of each of 32, 40 and 50 % (w/w) sucrose, giving steps of defined densities 1.14, 1.18 and 1.23 g/ml, respectively (confirmed on a refractometer (Fisher Scientific) to give indexes of refraction of 1.3861, 1.4005, 1.4200 at 20 °C, respectively). Gradients were brought to volume with homogenization buffer and centrifuged for 14-16 h at 4 °C at 80 000 x g_{ave} (25 000 rev./min) in a Beckman SW28 rotor. Membrane bands were collected separately and diluted to 8-10 % sucrose (approx. refractive index of 1.3448) with homogenization buffer lacking sucrose, and were centrifuged at 80 000 x g_{ave} (25 000 rev./min) for 1 h. Pellets were resuspended in approx. 1 ml of either homogenization buffer without DTT at pH 7.6 or in 25 mM TAPS-buffered solution at pH 8.6 for binding assays. Fractions were sometimes frozen in liquid nitrogen for up to five days, with no loss of binding (data not shown) and were thawed on ice prior to binding assays.

5. Protein Determination

The protein content was determined by the method of Lowry *et al.* [376], using BSA as a standard. Linearity with respect to protein concentration was obtained between 0-100 μg BSA (fraction V) in 500 μl of ddH_2O. Color development was for 30-35 min. Absorbance of tubes was read at 660 nm. Marker enzyme assays (see below) and Lowry assays were performed on freshly prepared fractions.

6. Marker Enzyme Analysis

Each assay was performed at three different protein concentrations to check that product formation was linear with protein content. In each case, blanks without substrate and without enzyme were included.

(A) 5'-nucleotidase

The marker enzyme for plasma membrane, 5'-nucleotidase (EC 3.1.3.5), was assayed by the method of Goldfine *et al.* [592]. The P*i* liberated was quantified by the method of Ames [593] using disposable phosphate-free glassware. A standard curve was obtained with 5-200 µl of 1 mM KH_2PO_4 brought up to 300 µl with buffer (50 mM Tris, 10 mM $MgCl_2$, pH 8.5). Specific release of P*i* from AMP was calculated by subtracting the absorbance at 820 nm of sample in buffer (50 mM Tris, 10 mM $MgCl_2$, pH 8.5) from the absorbance of sample in buffer with 5 mM AMP.

(B) Glucose-6-phosphatase

The marker enzyme for rat liver and kidney endoplasmic reticulum, glucose-6-phosphatase (EC 3.1.3.9) [594] was assayed by the method of Swanson [595] and the P*i* released determined [593]. The specifically released P*i* was determined by the method of Ames by subtracting the absorbance at 820 nm of the tube without glucose-6-phosphate from that with glucose-6-phosphate and finding the corresponding P*i* amount from a standard curve performed in parallel.

7. Preparation of Steroid Solutions

Stock [^{3}H]-dexamethasone was stored in absolute ethanol at a concentration of 0.1 mCi/ml. One day prior to assay, an appropriate aliquot of [^{3}H]-dexamethasone was removed to another tube, evaporated under nitrogen or dried *in vacuo* using a Speed-Vac (Savant, Farmingdale, NY) and resuspended in the required amount of buffer. The steroid solution was sonicated in a Brandsonic 12 water bath (SmithKline, Shelton, CN) for 10 min, and then 5-10 µl aliquots were taken and counted in plastic miniatures vials after incubation overnight in 5 ml of Ready-Protein scintillation fluid (Beckman) at room temperature. The steroid solution was stored at 4 °C.

Unlabeled dexamethasone was solubilised and stored in absolute ethanol at -20 °C, at a concentration of 3.925 mg/400 µl. On the day of the assay, appropriate aliquots were taken from the stock solution, once it had warmed to room temperature, and the required buffer volume was added. The ethanol concentration in the solution was adjusted to give the appropriate final ethanol concentration in the assay tube (typically no more than 1 %). All other unlabeled steroids were solubilised and stored in absolute ethanol at -20 °C, at a concentration of 2 mg/ml, except for 17β-estradiol at 1 mg/ml.

Stock [^{3}H]-dexamethasone 21-mesylate was stored at 1 mCi/ml in absolute ethanol at 4 °C. On the day of the assay an aliquot was dried down *in vacuo* and resuspended in buffer with a small amount of ethanol (5 %, v/v) to aid in solubilization. An aliquot was taken and counted to determine the concentration from the specific activity before addition to assay tubes.

8. Binding Assays

Preliminary experiments (see Results section) determined the binding conditions used below, which are based on previous experiments by our laboratory [541, 288]. Membrane suspensions containing 100-200 µg protein were incubated in triplicate at 0-4 °C with [^{3}H]-dexamethasone in a total volume of 250 µl of homogenization buffer pH 7.6 containing 1 % ethanol without DTT, or 25 mM TAPS pH 8.6 containing 1 % ethanol, with or without a 1000-fold excess of unlabeled dexamethasone or other unlabeled steroid. Binding was stopped by adding 1 ml of ice-cold buffer, containing 1 % ethanol but no DTT, and separation of the bound from the free steroid by draining the incubation tubes onto presoaked 24-mm GF/C glass fiber filters (Whatman, Maidstone, England) placed on a twelve-place vacuum manifold (Millipore, Bedford, MA). Filters were rinsed four times with 2 ml of the buffer and placed in the bottom of 20-ml glass scintillation vials. Counts were

solubilised with 500 µl of NCS tissue solubiliser (Amersham, Oakville, ON) by incubating at room temperature overnight. To each vial was added 10 ml of Ready-Protein scintillation fluid (Beckman, Palo Alto, CA) and the vials were left in the dark overnight to reduce chemiluminescence before radioactivity was determined by scintillation counting on a Beckman LS3801. The efficiency of counting was 43-47 % as determined by the quench correction curve using the H-number method to determine the quench parameter. Specific binding was determined by subtraction of non-specific binding from total binding. Non-specific binding was always less than 10 % of the total binding.

9. Gel Electrophoresis

SDS-PAGE was performed using the discontinuous buffer system of Laemmli [358]. Vertical slab gels (18 x 16 x 0.15 cm) consisted of a 3.9 % stacking gel and a 7.5 % running gel. Samples in SDS-sample buffer (63 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS (w/v), 0.05 % β-mercaptoethanol (v/v) and 0.0013 % bromophenol blue (w/v)) were denatured by heating for 5 min at 96 °C. Samples, denatured in SDS-sample buffer, were sometimes stored at -20 °C. Samples were run at 20 mA/gel to stack for 20 to 60 min, and then at 30 mA/gel to separate.

Gels were stained for 1 h in a solution of 0.2 % Coomassie Brilliant Blue R-250, 7 % acetic acid and 50 % methanol and destained with 2-3 changes of a solution of 7 % acetic acid and 50 % methanol followed by several changes of a solution of 7 % acetic acid and 5 % methanol with gentle shaking.

10. Preparation of GR-containing Cytosol

Cytosol was prepared as described by Eisen *et al.* [596]. Rats (200-250 g), adrenalectomized 3-4 days prior, were decapitated and the livers quickly removed and placed in ice-cold buffer consisting of 25 mM HEPES, pH 7.6, containing 1.5 mM EDTA,

10 % glycerol, 0.1 M NaCl, and 20 mM sodium molybdate. All subsequent operations were performed at 0-4 °C. The livers were rinsed, finely minced with scissors and then homogenized in a Teflon/glass Potter-Elvehjem homogeniser using ten vertical strokes. The homogenate was centrifuged immediately at 100 000 x g for 65 min. After centrifugation, the lipid layer at the top of the tube was carefully removed and the remaining clear supernatant was used as cytosol. Aliquots of the cytosol were also diluted 1:1 with SDS-sample buffer and heat-denatured for 3 min at 96 °C for SDS-PAGE.

11. Western Analysis (Immunoblotting)

Proteins samples for immunoblotting were electrophoresed on a 7.5 % gel along with cytosol prepared from four-day post-adrenalectomy animals [596], as a positive control for staining of GR with the monoclonal antibody M7 [597]. Prestained molecular weight standards (Rainbow Markers, Amersham) were run on gels for blotting to visualize the efficiency of transfer and to provide only an estimate of molecular weight, since the dye affects the mobility of the markers. Proteins were transferred electrophoretically onto nitrocellulose filters based on the procedure by Towbin *et al.* [375]. Prior to electroblotting the gels were soaked in transblot buffer (192 mM glycine, 25 mM Tris, pH 8.3, containing 20 % methanol and 0.1 % SDS), for 20-30 min. The nitrocellulose sheets were also soaked in transblot buffer for 20-30 min. Whatman 3MM filter paper and Scotch-Brite foam pads were soaked in buffer and a sandwich made with a foam pad, 3-4 filter papers, the gel, the nitrocellulose sheet, 3-4 filter papers and another foam pad. Care was taken to avoid trapping air bubbles between the layers. The sandwich was placed into the Bio-Rad transblot cell with the gel closest to the cathode (-) and the nitrocellulose closest the anode (+). Transblotting was done overnight in a cold room at 30-35 V and then for 30 min at 70-100 V. The nitrocellulose sheet was removed and briefly air-dried for 15

min. The sheet was immersed briefly in 100-200 ml of tris-saline (20 mM Tris pH 7.5, 500 mM NaCl). The nitrocellulose was soaked in 100-200 ml of 1.5 % powdered skim milk in tris-saline for 30-60 min to block residual binding capacity of the sheet [598]. The sheet was then washed with tris-saline twice for 10 min. The sheet was soaked for another 5 min with 1 % BSA in tris-saline. The primary antibody, M7, was added to the solution away from the sheet to give a proper final dilution factor of 1:400, and incubated overnight at 4 °C. The antibody solution was removed and the blot washed in 100-200 ml of 0.05 % Tween-20 in tris-saline twice for 30-60 min. Then washed twice only in tris-saline for 10 min. The blot was incubated overnight at 4 °C with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution). The blot was washed with two changes of tween-tris-saline for 30-60 min and two more washes of 10 min in tris-saline. The antibody was detected with the horseradish peroxidase color development reagent containing 4-chloro-1-naphthol in the presence of 0.015 % H_2O_2. This gives a purple staining that was stopped by flooding and rinsing the blot with tris-saline. The blot was stored in a light-proof container to prevent fading.

12. Affinity Labeling

Affinity labeling of microsomes were based on previous labeling experiments with plasma membranes [541] and nuclear envelopes [289]. DTT was omitted because it interferes with the affinity labeling. Microsomes and subfractions (200 µg/tube) were incubated with 220 nM [^{3}H]-dexamethasone 21-mesylate for 6 h at 4 °C in 250 µl of 25 mM TAPS pH 8.6 . The incubation was terminated by the addition of 750 µl ice-cold buffer and centrifugation for 3 min at 356 000 x g_{ave} (100 000 rev./min) in a Beckman TL-A ultracentrifuge. The pellet was washed two more times and finally resuspended in SDS-sample buffer and heat denatured. Samples were run on SDS-PAGE using 7.5 % running gels, and stained to allow scanning on an LKB Ultroscan XL

Enhanced Laser Densitometer (Pharmacia). The gels were cut into 2-mm slices, dissolved in 150 µl 50 % H_2O_2: 0.8 % NH_4OH (19:1, v/v) by heating to 70 °C for several hours [599]. The H_2O_2 was neutralized with 1000 U of catalase and 25 mg of ascorbic acid in a total of 500 µl before adding 10 ml of Ready-Protein and scintillation counting. Efficiency of counting was 36-38 %.

III. RESULTS

1. Marker Enzyme Analysis of Microsomes

Microsomes are a population of membrane vesicles, consisting primarily of plasma membrane and endoplasmic reticulum, that are obtained by mechanical homogenization of the cell and differential centrifugation. Membrane purity was verified by assaying for marker enzymes [600, 601]. Microsomal preparations yielded a specific activity of 5'-nucleotidase (plasma membrane marker) and glucose-6-phosphatase (endoplasmic reticulum marker) that is slightly enriched (approx. 2-fold, see Fig. 6A and 6B) in the microsomal fraction as compared to the homogenate. Approximately 6-10 % of the total 5'-nucleotidase activity of the homogenate was recovered in the microsomes while approx. 10-18 % of the total glucose-6-phosphatase activity was recovered in the microsomes (Fig. 1). Total recovery of glucose-6-phosphatase was 83.9 % of the starting homogenate, and 5'-nucleotidase recovery was 66.5 % (Fig. 1).

2. Preliminary Binding Experiments

First, we determined equilibrium conditions for the binding of [^{3}H]-dexamethasone to male rat liver microsomes. Dexamethasone was used in these studies because it binds to GR with high affinity and specificity and because it does not bind to transcortin (corticosteroid-binding globulin or CBG) or to serum albumin. Dexamethasone is also not readily metabolized. The chemical structure of dexamethasone is shown in Appendix A. Our previous studies of dexamethasone interactions with the rat liver plasma membrane had shown that considerable degradation of dexamethasone-binding sites occurred at 22 °C and at 37 °C [541]. Therefore, time course studies of the microsomal fraction were carried out at 0-4 °C to reduce thermolability (Fig. 2). Incubations were in TAPS buffer pH 8.6 as we had

established in previous affinity labeling studies with dexamethasone 21-mesylate [541, 289] that this buffer gave optimal results. Other preliminary binding studies showed that there was no difference in numbers of binding sites measured when the incubations were carried out in homogenization buffer (Tris-buffered sucrose) at pH 7.6 or in TAPS buffer at pH 8.6 (Table 1). However, there was a difference in the affinity of dexamethasone for the microsomal binding sites in the two buffer systems. A lower affinity of the binding site for dexamethasone in TAPS buffer (pH 8.6) was observed as compared to the Tris-buffered sucrose (pH 7.6) containing Mg^{++}. Similar to our results, Omrani *et al.* [590] found that the omission of magnesium ion reduced microsomal dexamethasone-binding affinity by about 50 %, but still did not affect the total number of dexamethasone-binding sites. Tris-buffered sucrose resulted in a greater stability of the binding site which was thought to be due to the inclusion of Mg^{++}. However, we have not ruled out the effect of pH or sucrose (a known stabilizing agent) on the binding-site stability. Maximum binding in TAPS buffer (pH 8.6) had been attained at 8 h and remained stable to at least 16 h. Thereafter binding declined (Fig. 2), while binding was stable for up to 45 h in Tris-buffered sucrose containing Mg^{++} (data not shown). Therefore, equilibrium conditions for further studies of the binding of [^{3}H]-dexamethasone to rat liver microsomes in TAPS buffer were 8-10 h incubations at 0-4 ^oC. Despite better affinity in Tris-buffered sucrose, we still preferred to use TAPS buffer to ensure that the affinity labeled sites were the same as the binding sites.

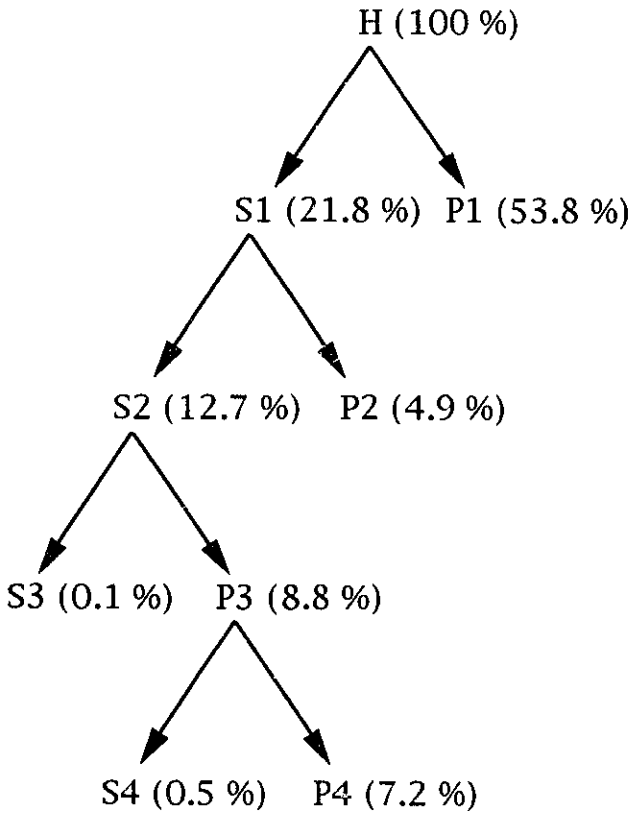
3. Characterization of Microsomal Dexamethasone-binding Sites

Specific binding of [^{3}H]-dexamethasone to rat liver microsomes demonstrated saturability (Fig. 3A) and hence was subjected to Scatchard analysis (Fig. 3B). The analysis revealed a single class of binding sites with a K_d of 68 nM and a B_{max} of 23 pmol/mg protein. Analysis of binding to microsomes obtained from animals which

Figure 1. Flow-chart of the Distribution of Marker Enzyme Total Activity in the Preparation of Microsomes

Data are expressed as a percentage of the homogenate (H) total activity, for microsomes (P4) and all the intermediate purification steps, *e.g.*, pellet (P1) and supernatant (S1) after the first centrifugation spin. After each centrifugation, an aliquot of material is put aside for enzyme assays and the total volume of the fraction is measured so as to calculate the total enzyme activity for that fraction. Activities are measured for the plasma membrane marker, 5'-nucleotidase, and the endoplasmic reticulum marker, glucose-6-phosphatase. Total recovery is the sum of activities for all the branch endings.

5'-NUCLEOTIDASE



GLUCOSE-6-PHOSPHATASE

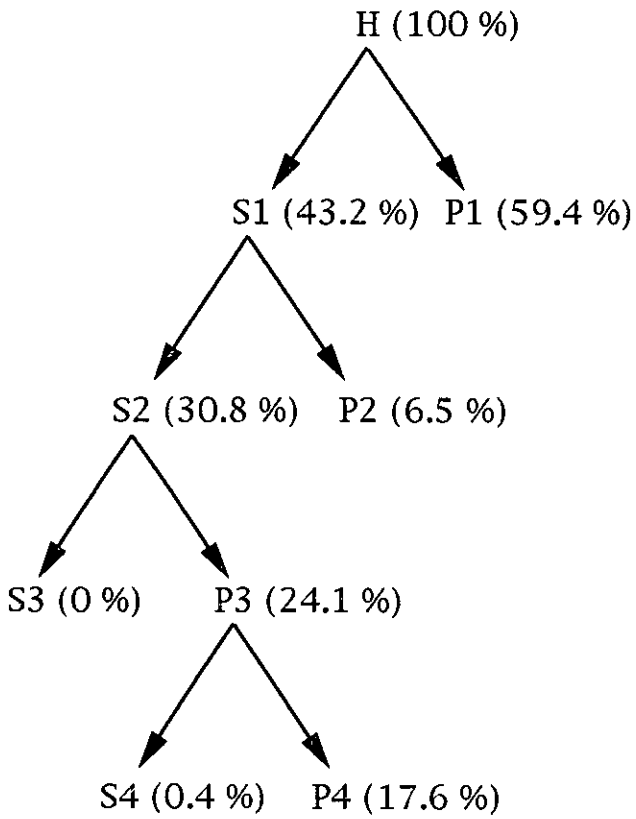


Figure 2. Time-dependent Association of [^{3}H]-Dexamethasone with Male Rat Liver Microsomes

Membranes (200 μg protein/tube) were incubated in triplicate with 50 nM [^{3}H]-dexamethasone in the presence or absence of 50 μM unlabeled dexamethasone at 0-4 °C. Specific binding (▲) for each time point was calculated from the difference of [^{3}H]-dexamethasone binding in the presence (O, non-specific) and absence (●, total binding) of excess unlabeled dexamethasone.

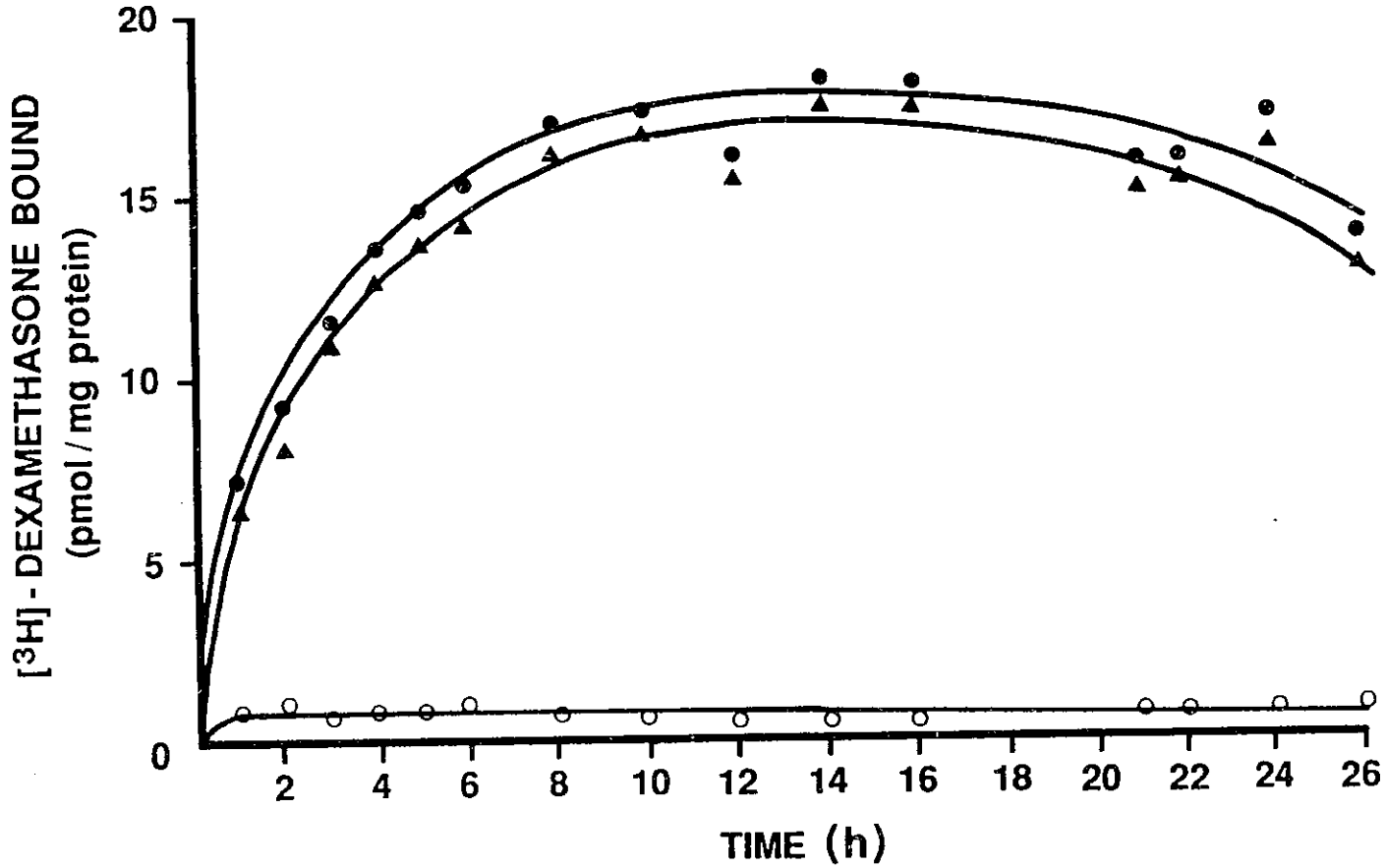

[3H]-DEXAMETHASONE BOUND
(pmol/mg protein)
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME (h)

Table 1. Scatchard Analysis of [^{3}H]-Dexamethasone Binding to Male Rat Liver Microsomes and Subfractions in Two Different Buffer Systems

Fraction	TAPS			Tris-buffered sucrose		
	K_d (nM)	B_{max} (pmol/mg)	r	K_d (nM)	B_{max} (pmol/mg)	r
M	101	27.8	-0.701	47.4	26.5	-0.645
P1	61.0	18.6	-0.855	25.0	20.7	-0.955
P2	121	56.4	-0.681	73.0	54.0	-0.750
P3	89.2	28.4	-0.865	41.9	18.1	-0.913

Rat liver microsomes (M) and subfractions (P1, P2, P3) (200 μg protein/tube) were incubated (in triplicate) at 0-4 °C with [^{3}H]-dexamethasone (1.0-210 nM) in the presence or absence of a 1000-fold excess of unlabeled dexamethasone for 12-17 h in 250 μl TAPS-NH_4OH buffer pH 8.6 containing 1 % ethanol or 17-20 h in Tris-buffered sucrose pH 7.6 (0.25 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris-HCl, pH 7.6). Specific binding was determined by subtracting non-specific binding (tubes with a 1000-fold excess of unlabeled dexamethasone) from total binding (tubes without excess unlabeled dexamethasone). Scatchard plots were subjected to linear regression analysis and the dissociation constant (K_d), binding capacity (B_{max}) and correlation coefficient (r) calculated. *N.B.*, we did not determine the binding parameters for fraction P4 because of a lack of material.

had been adrenalectomized showed that there is no change in the affinity of these sites upon glucocorticoid depletion, but there is an approximately 75 % decrease in the binding capacity (Fig. 3B and Table 2).

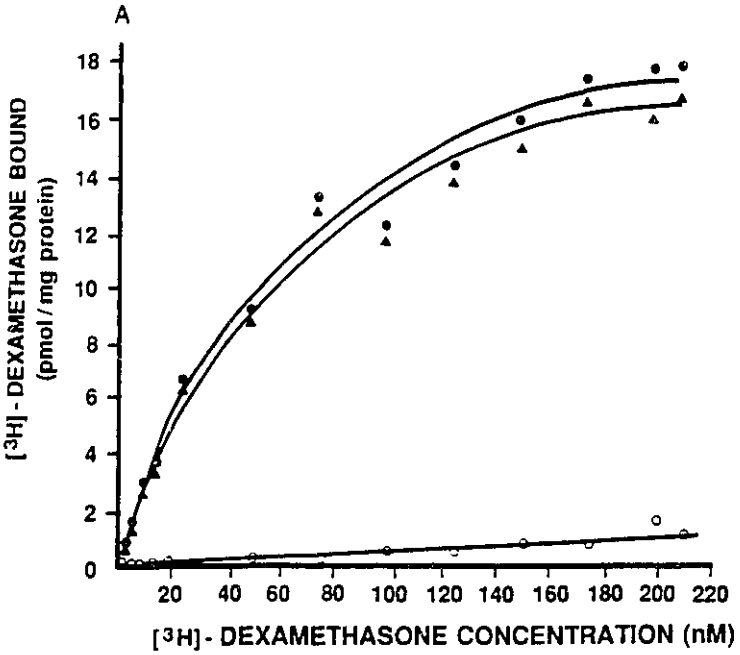
The steroid specificity of the microsomal [^{3}H]-dexamethasone-binding sites was assessed by competition analysis with unlabeled steroids (Table 3). The natural glucocorticoids, corticosterone, deoxycorticosterone and cortisol, competed for the [^{3}H]-dexamethasone-binding sites with the same potency as unlabeled dexamethasone. The glucocorticoid analogue, triamcinolone acetonide, which competes well for the binding of dexamethasone to GR [602], was a relatively poor competitor for these sites. The progestin, progesterone, competed as effectively as dexamethasone while the estrogen, 17β-estradiol, was a poorer competitor and the androgen, testosterone, was an even poorer competitor. Cholesterol, the steroid precursor, did not compete for the binding of [^{3}H]-dexamethasone to the rat liver microsomes.

4. Identification of Microsomal Dexamethasone-binding Sites by Affinity Labeling

We next affinity labeled microsomes with the anti-glucocorticoid [^{3}H]-dexamethasone 21-mesylate [16] in order to enable identification of the binding sites after SDS-PAGE. [^{3}H]-dexamethasone 21-mesylate is usually used to identify GR on SDS-PAGE gels because the mesylate function forms a covalent thioether bound with sulfhydryl groups in the ligand-binding domain of the receptor [16, 596]. As mentioned above, the efficiency of affinity labeling was greater at pH 8.6 than at pH 7.4 for both plasma membrane and nuclear envelope sites [541, 289]. Therefore, microsomes were also labelled at pH 8.6. Figure 4A shows the radioactivity profile of an SDS-polyacrylamide gel of microsomes after affinity labeling with [^{3}H]-dexamethasone 21-mesylate. The profile clearly shows two major specifically labeled peaks, one at approximately 45 kDa which sometimes appeared as a doublet and one at

Figure 3. Analysis of [^{3}H]-Dexamethasone Binding to Rat Liver Microsomes

Rat liver microsomes (200 μg protein) were incubated with [^{3}H]-dexamethasone (1-210 nM) in the presence or absence of a 1000-fold excess of unlabeled dexamethasone for 10 h at 0-4 °C. A. Saturation curve of binding to liver microsomes from intact rats. Specific binding (▲) was determined by subtraction of non-specific binding (O) from total binding (●). B. Scatchard analysis [603] of specific [^{3}H]-dexamethasone binding to liver microsomes obtained from intact rats (●) and from adrenalectomized rats (O) subjected to linear regression analysis. Note the parallel slopes (same affinity) but different intercepts (binding capacity).


A
[3H] - DEXAMETHASONE BOUND
(pmol / mg protein)
0
2
4
6
8
10
12
14
16
18
20
40
60
80
100
120
140
160
180
200
220
[3H] - DEXAMETHASONE CONCENTRATION (nM)

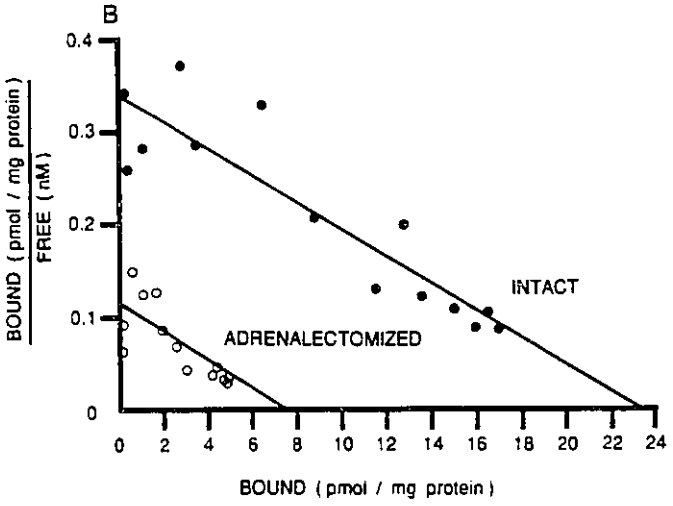

B
BOUND (pmol / mg protein)
FREE (nM)
0
0.1
0.2
0.3
0.4
INTACT
ADRENALECTOMIZED
0
2
4
6
8
10
12
14
16
18
20
22
24
BOUND (pmol / mg protein)

Table 2. Parameters of [^{3}H]-Dexamethasone Binding to Male Rat Liver Microsomes and Subfractions from Intact and Adrenalectomized Animals

	Fraction	K_d (nM)	B_{max} (pmol/mg)	r
Intact animal	M	101	27.8	-0.701
	P1	61.0	18.6	-0.855
	P2	121	56.4	-0.681
	P3	89.2	28.4	-0.865
Adrenalectomized animal	M	57.6	6.7	-0.780
	P1	52.1	4.2	-0.844
	P2	46.9	15.2	-0.979
	P3	82.6	13.1	-0.791

Rat liver microsomes (M) and subfractions (P1, P2, P3) (200 μg protein/tube) from intact or adrenalectomized male rats were incubated (in triplicate) at 0-4 °C with [^{3}H]-dexamethasone (1.0-210 nM) in the presence or absence of a 1000-fold excess of unlabeled dexamethasone for 8-10 h in 250 μl TAPS pH 8.6 containing 1 % ethanol. Specific binding was determined by subtracting non-specific binding, *i.e.*, tubes with a 1000-fold excess of unlabeled dexamethasone from total binding, *i.e.*, tubes without excess unlabeled dexamethasone. Scatchard plots were subjected to linear regression analysis and the dissociation constant (K_d), binding capacity (B_{max}) and correlation coefficient (r) calculated.

Table 3. Specificity of [^{3}H]-Dexamethasone Binding to Male Rat Liver Microsomes and Subfractions

% Inhibition of total binding												
	M			P1			P2			P3		
Unlabeled hormone competitor (μM)	8	16	40	8	16	40	8	16	40	8	16	40
Dexamethasone	97.9	98.0	98.3	96.5	96.4	96.3	96.4	97.8	98.0	94.6	93.8	96.2
Triamcinolone acetonide	0	0	14.1	0	1.8	17.4	7.6	17.2	34.5	0	0	12.1
Cortisol	98.1	98.6	98.6	96.6	98.1	98.3	97.9	97.7	97.7	97.5	97.7	97.8
Corticosterone	97.2	98.2	98.7	96.6	97.0	96.4	97.6	98.1	98.2	97.2	97.5	97.5
Deoxycorticosterone	95.8	97.4	98.3	95.6	96.8	97.8	96.0	97.4	98.0	95.1	94.7	94.6
Progesterone	98.4	98.6	98.7	98.3	98.4	98.3	98.0	98.3	98.2	97.2	97.1	96.9
Estradiol-17β	65.3	83.2	90.5	82.4	90.6	91.6	81.8	90.1	91.6	70.8	82.0	ND
Testosterone	46.7	63.4	82.0	58.4	69.9	84.4	54.8	68.9	83.7	48.5	63.6	79.1
Cholesterol	0	0	0	0	0	7.0	3.1	6.7	11.2	0	6.0	7.9

Rat liver microsomes (M) and subfractions (P1, P2, P3) (100 μg protein/tube) were incubated with 40 nM [^{3}H]-dexamethasone for 10 h at 0-4 °C in the presence of 8, 16 and 40 μM (200-, 400- and 1000-fold excess) of the unlabeled steroid. The data presented here are expressed as percent inhibition of total binding by an excess of unlabeled competitor. (ND = not determined)

approximately 66 kDa. In one experiment, protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 10 U/ml aprotinin) were added to buffers used in the preparation of the microsomes. The polypeptide labeling pattern was not altered by the protease inhibitors, suggesting that the affinity-labeled polypeptides are not degradation products of higher molecular weight polypeptides (data not shown). Next we examined the profiles of affinity-labeled microsomes obtained from adrenalectomized animals (Fig. 4B). This figure shows that both specifically labeled entities were reduced upon adrenalectomy, indicating a dependency on glucocorticoids. We had previously shown the presence of the 66-kDa entity in serum [541] and thus suspect that it is in the microsomal preparation at least in part as a result of serum contamination. Furthermore, its response to adrenalectomy was variable; in some experiments the specifically labeled band at 66 kDa was not reduced by adrenalectomy. The 45-kDa entity, on the other hand, was not present in serum and its response to adrenalectomy was very reproducible. Thus this polypeptide doublet resembled the site identified in the [^{3}H]-dexamethasone binding studies. The 66-kDa site may be a site which is affinity labeled by dexamethasone 21-mesylate, but which is not a dexamethasone-binding site.

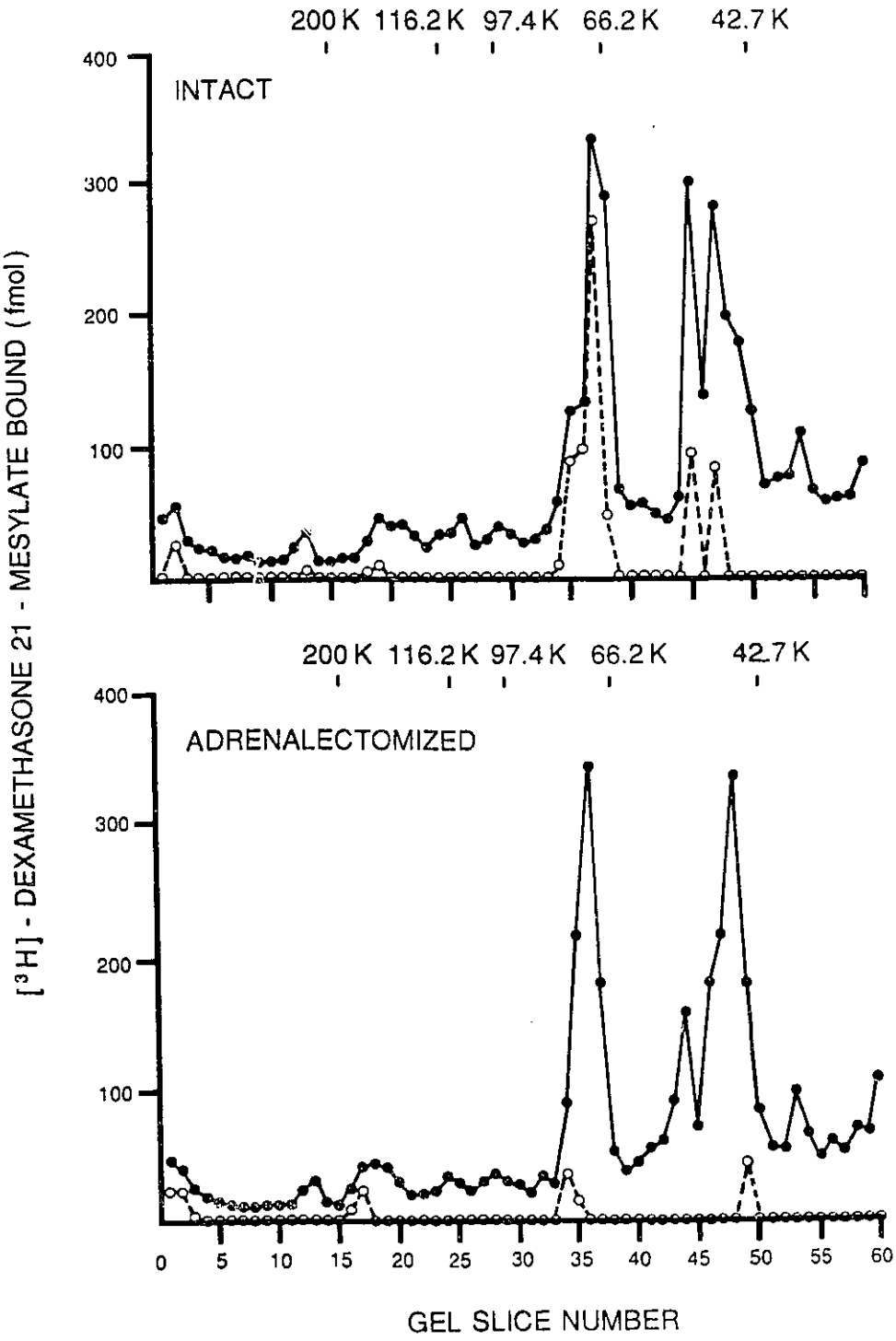
5. Sublocalization of Microsomal Dexamethasone-binding Sites

In order to further localize the dexamethasone-binding sites, microsomes were first subfractionated on a discontinuous sucrose density gradient. Then [^{3}H]-dexamethasone binding to the subfractions was characterized followed by identification of polypeptides affinity labeled with [^{3}H]-dexamethasone 21-mesylate.

The subfractionation procedure yielded four subfractions: white particulate material obtained at the 1.03/1.14 g/ml interface (P1), dark yellow material at the 1.14/1.18 g/ml interface (P2), light yellow material at the 1.18/1.23 g/ml interface (P3) and the pellet (P4). The amber color is due to abundant microsomal enzymes

Figure 4. Affinity Labeling of Male Rat Liver Microsomes with [^{3}H]-Dexamethasone 21-mesylate

Membranes (200 μg protein) were incubated with 220 nM [^{3}H]-dexamethasone 21-mesylate for 6 h at 0-4 °C in 25 mM TAPS-NH_4OH pH 8.6 to determine total binding. Non-specific binding was determined by the addition of [^{3}H]-dexamethasone 21-mesylate to membranes which previously had been incubated with 220 μM unlabeled dexamethasone for 6 h and subtracted from total binding to give specific binding. The affinity-labeled proteins were heat-denatured in SDS-sample buffer and run on 7.5 % SDS-PAGE. The gel was cut into 2-mm slices and dissolved in 150 μl of 50 % hydrogen peroxide-0.8 % ammonia (19:1, v/v) at 60-70 °C. After the addition of 1000 U of catalase and 25 mg of ascorbic acid in a total volume of 500 μl, the sample was counted in 10 ml of Ready-Protein. Total binding (●) and specific binding (○) are shown for microsomes from intact or adrenalectomized rats. Molecular mass standards are myosin (200 000 Da), β-galactosidase (116 250 Da), phosphorylase B (97 400 Da), bovine serum albumin (66 200 Da) and ovalbumin (42 699 Da).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200 K 116.2 K 97.4 K 66.2 K 42.7 K
ADRENALECTOMIZED
400
300
200
100
0 5 10 15 20 25 30 35 40 45 50 55 60
GEL SLICE NUMBER
[3H] - DEXAMETHASONE 21 - MESYLATE BOUND (fmol)

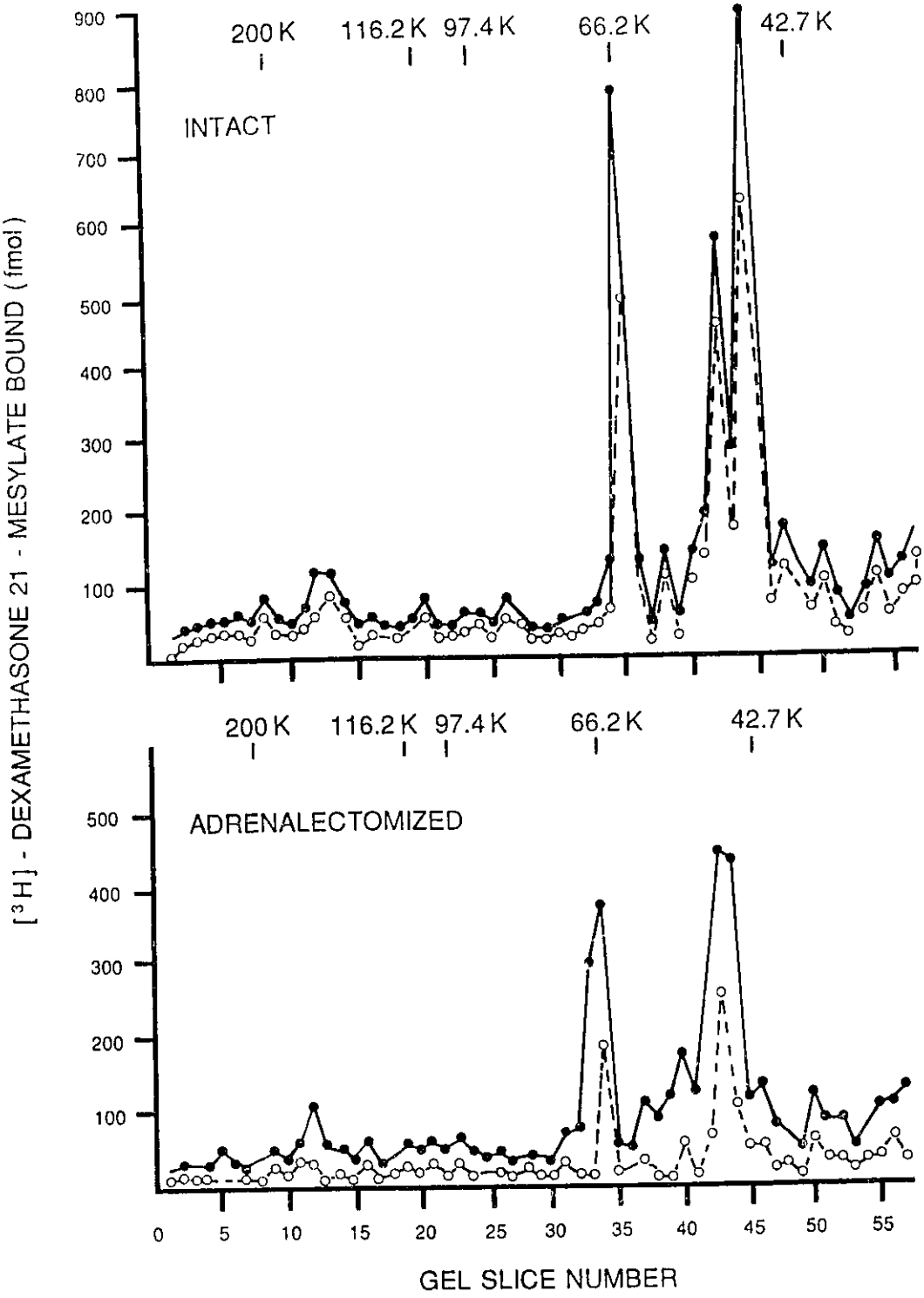
which contain cytochrome P-450. Binding studies with the subfractions showed that the affinities for [^{3}H]-dexamethasone (Table 2), the response to adrenalectomy (Table 2) and the steroid specificities as determined by competition analysis (Table 3) of the microsomal subfractions and the microsomes were similar. Subfractions were then affinity labeled with [^{3}H]-dexamethasone 21-mesylate. The radioactivity profile obtained from an SDS-PAGE of each microsomal subfraction resembled the labeling of the microsomal fraction in that two specifically labeled peaks were seen, one at 66 kDa and a doublet at 45 kDa. As an example, the radioactivity profile of microsomal subfraction P2 after affinity labeling is shown in Fig. 5A . The affinity labeling pattern obtained after adrenalectomy of the animals is shown in Fig. 5B and is seen to resemble closely that obtained from microsomes of adrenalectomized animals.

Figure 6A shows that the plasma membrane was recovered predominantly in the lighter fractions, as the 5'-nucleotidase specific activity was highest in subfraction P1 and decreased as the density of the subfractions increased. The endoplasmic reticulum, as monitored with glucose-6-phosphatase, was found in all subfractions; however, the glucose-6-phosphatase specific activity was highest in subfraction P2 (Fig. 6B). As the profiles of enrichment of the two marker enzymes were distinct, we concluded that this subfractionation would allow us to determine whether dexamethasone-binding sites were restricted to either the plasma membrane or to the endoplasmic reticulum.

Figure 6C shows that P2, which contained the greatest enrichment of glucose-6-phosphatase activity possessed the greatest number of [^{3}H]-dexamethasone-binding sites per mg membrane protein as deduced from Scatchard analysis (Table 2). As in the [^{3}H]-dexamethasone-binding studies, there is a correlation of the glucose-6-phosphatase specific activity with the enrichment of the binding of the 45-kDa site per mg protein in the various microsomal subfractions, as determined with the affinity labeling reagent, dexamethasone 21-mesylate (Fig. 6D).

Figure 5. Affinity Labeling of Male Rat Liver Microsomal Subfraction P2 with [^{3}H]-dexamethasone 21-mesylate

Membranes (200 μg protein) were incubated with 220 nM [^{3}H]-dexamethasone 21-mesylate for 6 h at 0-4 °C in 25 mM TAPS-NH_4OH pH 8.6 to determine total binding. Non-specific binding was determined by the addition of [^{3}H]-dexamethasone to membranes which previously had been incubated with 220 μM unlabeled dexamethasone for 6 h and subtracted from total binding to give specific binding. The affinity-labeled proteins were heat-denatured in SDS-sample buffer and run on 7.5 % SDS-PAGE. The gel was cut into 2-mm slices and dissolved in 150 μl of 50 % hydrogen peroxide-0.8 % ammonia (19:1, v/v) at 60-70 °C. After the addition of 1000 U of catalase and 25 mg of ascorbic acid in a total volume of 500 μl, the sample was counted in 10 ml of Ready-Protein. Total binding (●) and specific binding (○) are shown for membranes from intact or adrenalectomized rats.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66.2 K
42.7 K
ADRENALECTOMIZED
500
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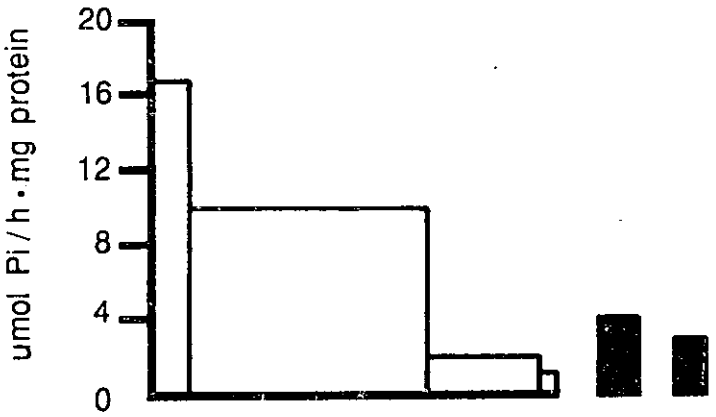
GEL SLICE NUMBER
[3H] - DEXAMETHASONE 21 - MESYLATE BOUND (fmol)

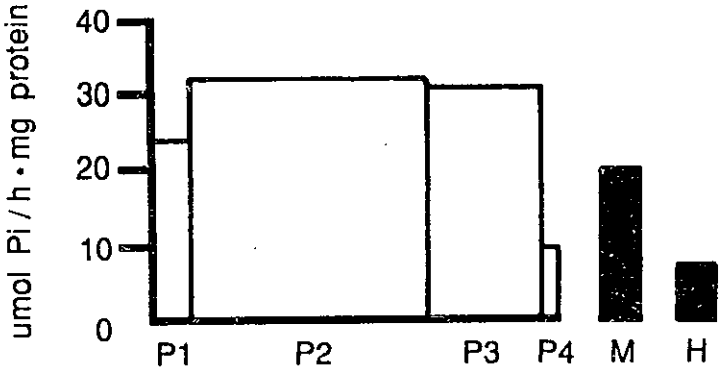
Figure 6. Discontinuous Sucrose Density Gradient Fractionation of Rat Liver Microsomes

Particulate material was recovered at density interfaces 1.03/1.14 (P1), 1.14/1.18 (P2), 1.18/1.23 (P3) and pellet (P4). Values are also reported for the homogenate (H) and the microsomes (M). The horizontal axis indicates the relative protein content of the individual fractions as a proportion of total microsomal protein. A. 5'-nucleotidase specific activity is shown as a marker of plasma membrane content. B. Glucose-6-phosphatase activity is shown as a marker of endoplasmic reticulum content. C. Binding capacity of microsomal dexamethasone- binding sites is shown (calculated from Scatchard plots). D. Binding capacity of 45-kDa affinity-labeled entity as determined by measuring the area under the curve from plots of specific binding counts for the 45-kDa band. Values are expressed as the means of triplicate determinations which differed from the mean by $\pm$ 10 %.

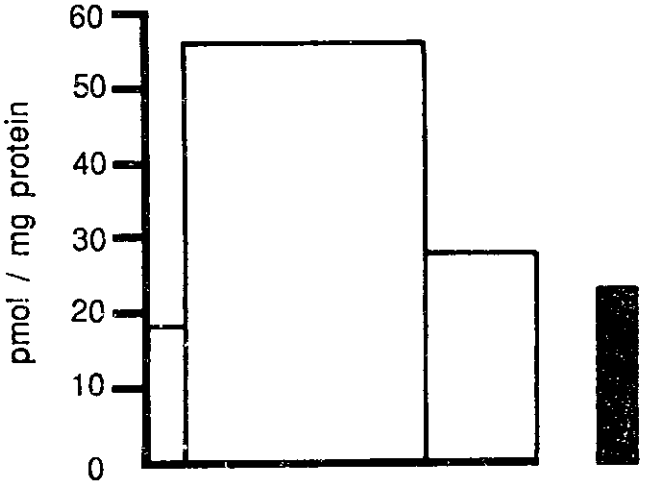
A. 5' - NUCLEOTIDASE



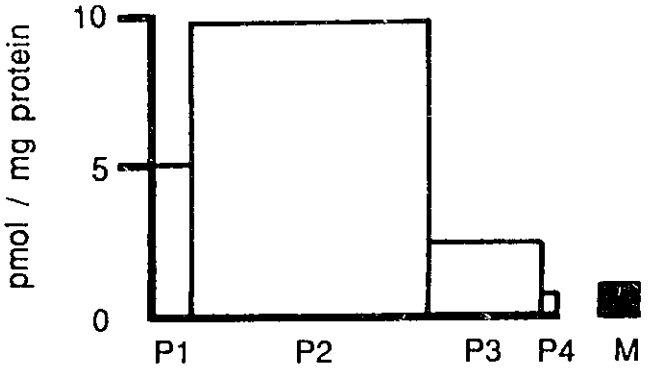
B. GLUCOSE - 6 - PHOSPHATASE



C. BINDING CAPACITY (Bmax)



D. AFFINITY LABELED
45 kDa ENTITY



6. Immunoblotting of the Affinity-labeled Polypeptides

As a possible candidate for a glucocorticoid-binding site is GR, a Western blot analysis of 300-420 μg microsomal proteins with the anti-GR antibody, M7 [597] was obtained. While rat liver cytosol (25-150 μg) enriched in untransformed GR [596] gave a positive result as expected, the immunoblotting of the rat liver microsomes was negative (results not shown). Molecular weights for actin have been reported which range from 42 kDa to 48 kDa. We therefore also investigated whether the 45-kDa entity might be an actin isoform. However, while the anti-actin antibody (kindly provided by Dr. Michael McBurney, University of Ottawa) identified a cross-reacting species, the antigen ran at 42 kDa [604]. There was no cross-reactivity in the region of the dexamethasone 21-mesylate binding, *i.e.*, 45 kDa, and a doublet was not observed (data not shown).

IV. DISCUSSION

1. Role of Membrane Steroid-binding Sites

The major focus of study in steroid hormone action for many years has been on identifying steroid receptors and the genes they control [9, 527], and to a lesser extent, on defining the steps of the classical two-step model of steroid hormone action [6, 7]. This area of research has been greatly advanced by the cloning of hormone receptors for all known classes of steroid hormones and has led to the discovery of some new hormones [8, 20, 40]. The results of this focus has led to a narrow view of what steroid hormone action consists, and ignores alternative models of steroid hormone action. Membrane-steroid interactions may play some transport or regulatory role in mediating genomic effects or may explain non-genomic effects of steroid hormones [557, 573-578, 605]. Membrane interactions during steroid hormone action have been long postulated by many groups to play various roles, from steroid transport [531-533], to effecting second messenger pathways [542, 557, 606], to altering membrane electrical activity in the central nervous system [538, 574, 577, 607], and finally to explaining anaesthetic properties of some steroids [608].

2. Microsomal Dexamethasone-binding Sites

We have identified by Scatchard analysis of [^{3}H]-dexamethasone binding to male rat liver microsomes and microsomal subfractions one class of dexamethasone-binding sites. Omrani *et al.* [590] and Ambellan *et al.* [553] have also reported a class of dexamethasone-binding sites on rat liver microsomes having a similar affinity and binding capacity to the site reported here. We have previously reported the presence of dexamethasone-binding sites in plasma membranes [541] and nuclear envelopes [288] from rat liver. Both of those membrane systems, however, possessed two classes of dexamethasone-binding sites, one class displaying an affinity similar to the site

reported here and one class displaying a higher affinity. It is possible that a higher affinity lower capacity site is present on the microsomes but is masked by the higher capacity site in this membrane fraction and is not detected with our methods.

The dexamethasone-binding sites exhibited a broad range of steroid specificity (binding equally well to all natural twenty-one-carbon steroids tested) in contrast to that reported for cytosolic GR [609]. Plasma membrane [540, 541] and nuclear envelope [288] dexamethasone-binding sites have been characterized which show similar steroid specificity to the site described here. Further, the broad specificity of membrane-associated steroid-binding proteins is not unique to glucocorticoid systems as such sites have been reported for estrogen on anterior pituitary microsomes [546] and tri-iodothyronine on rat liver nuclear envelopes [589]. Adrenalectomy did not affect the affinity of these sites but resulted in a marked decrease in the number of binding sites.

Affinity labeling of rat liver microsomes with the electrophilic reagent [^{3}H]-dexamethasone 21-mesylate identified two specifically labeled polypeptides, one at 66 kDa and a doublet at 45 kDa. The 45-kDa site responds to adrenalectomy in the same way as the [^{3}H]-dexamethasone-binding sites and, therefore, probably is identifying the dexamethasone-binding sites. A possibility is that the 45-kDa doublet results from degradation of GR, as known proteolytic fragments of GR at 66-, and 45/42-kDa exist. However, three reasons suggest that this is not the case: first, immunoblotting with an anti-GR antibody did not detect the presence of GR; second, triamcinolone acetonide competes effectively with dexamethasone for the cytosolic GR [602] whereas triamcinolone acetonide was a poor competitor in these studies; and third, GR is a high affinity glucocorticoid binder (K_d of 1-10 nM) [19], unlike our dexamethasone-binding site (K_d of 47-121 nM). These findings suggest that these sites are not GR. However, the 45-kDa entity may be a proteolytic fragment of GR, which has lost the immunoreactive site by degradation. This possibility must be

considered as the region of the immunogenic domain of the rat GR (amino acids 119-273) containing the M7 epitope [23] is distinct from the site labeled with dexamethasone 21-mesylate (C^{656}) [610]. We have also considered the possibility that the 45-kDa polypeptide is actin, because of its similar molecular weight, Mg^{2+} dependence, membrane association, abundance and putative role in transport. We were unable, however, to detect a 45-kDa microsomal polypeptide which cross-reacted with anti-actin antibody. Corticosteroid-binding globulin (transcortin) is another candidate, although this is less likely because dexamethasone does not bind serum globulins and affinity labeling of rat serum with [^{3}H]-dexamethasone 21-mesylate did not label a 45-kDa entity [541]. A fourth possible identity for the 45-kDa polypeptide is that it is a drug- and steroid-metabolizing enzyme (such as cytochrome P-450-containing enzymes) which are quite abundant in liver microsomes. Dexamethasone is not itself readily metabolized. However, it may bind to a microsomal catabolic or conjugating enzyme. The 45-kDa low affinity dexamethasone-binding site resembles very much the microsomal low affinity glucocorticoid-binding site (LAGS) identified by Chirino *et al.* [547]. This membrane site is also reported not to bind triamcinolone acetonide [547], as are other membrane-associated low affinity dexamethasone-binding sites on the plasma membrane [541, 542]. The LAGS protein has not been purified nor cloned. However, its hormonal regulation has been extensively studied [611, 612].

Unpublished microsequencing results from our laboratory on a 45-kDa plasma membrane dexamethasone-binding site which is likely the same as the 45-kDa microsomal site suggest a few possibilities as to the nature of this 45-kDa site. Initially, we failed to get any microsequence data on the 45-kDa site. Therefore, it was subjected to *S. aureus* V8 protease digestion [613], and the digested products separated on SDS-PAGE. The digested products were electroblotted onto PVDF-membranes and sent to the Alberta Peptide Institute (Edmonton) for

microsequencing [614]. Two of the samples yielded some sequence information while the others failed to yield any information either due to amino-terminal blocking or, more likely, due to insufficient material. The sequences obtained are best guesses and likely to be in error because the assignment of the residues was made on yields of below 1 pmol, the lower operating limit of the instrument. A further complication was added by finding multiple residues in many cycles. This suggests that the gel bands were not discrete peptides. However, a twelve and a fourteen amino acid peptide sequence were obtained, and are as follows:

$$^{1}(S/I)XT(L/P)V(Y/I)DDXX(Q/N)M^{12}$$

$$^{1}SAP(L/P/N)(I/V/N)(Q/E)S(I/E/A)(T/N)(Q/E)XEMA^{14}$$

where X signifies a failure to make a specific assignment. Multiple residues are shown in parentheses. The two sequences were compared against the NBRF-PIR database (release 27) using the program PROSCAN from DNASTAR. No perfect matches were found. However, a large number of similar sequences existed because of the degenerate sequences used to perform the search. It was reasoned that both peptides derive from the same protein. Therefore, any protein showing similarity with one or the other peptide should be found on the lists of similar proteins to both peptides. This restricted the possible similarities to a few families of proteins that include cytochrome P-450-containing enzymes, penicillin-binding proteins, H^{+}-transporting ATP synthase, anthranilate synthase, and nitrogenase molybdenum-iron proteins. All of theses families have members in the 40- to 50-kDa range. This lends further support to the notion that the 45-kDa site may be a microsomal steroid-metabolizing cytochrome P-450-containing enzyme. Another candidate worth investigating is P-glycoprotein (product of the multidrug-resistance gene) which shows some similarity with only one of our peptides. P-glycoprotein is a plasma membrane protein of 170 kDa. However, it is known to bind steroids in some cases [530, 615].

The 66-kDa site, unlike the 45-kDa site, was not altered consistently by adrenalectomy. Rat serum albumin has a M_r of 66 kDa and Simons has shown that, although [^{3}H]-dexamethasone does not bind to serum albumin, [^{3}H]-dexamethasone 21-mesylate labels serum albumin [616]. We have shown that affinity labeling of ammonium sulfate-precipitated serum resulted in a heavily labeled polypeptide of 66 kDa [541]. Therefore, the 66-kDa entity is probably rat serum albumin. Its presence in the microsomal preparation could be a result of serum contamination and of its synthesis in the liver. The fact that it is being contributed by two different pools would make variations in the data more likely. We have previously shown that the 45-kDa sites are located in the plasma membrane [541], but the microsomal subfractionation studies presented here show that these sites are not localized solely to the plasma membrane. They are also present in the endoplasmic reticulum, and are at least ten times more abundant (B_{max} of 23 pmol/mg for microsomes, which still contains plasma membranes, versus a B_{max} of 2.6±3 pmol/mg for purified plasma membranes [541]). If this 45-kDa site is an endoplasmic reticulum cytochrome P-450-containing enzyme then a 1-2 % contamination of purified plasma membranes with endoplasmic reticulum components could possibly account for the 45-kDa site being present in plasma membrane fractions. Cytochrome P-450-containing enzymes are also found in the nuclear envelope, as detected by Western analysis of purified nuclear envelopes from rat liver with an anti-cytochrome P-450 antibody (from Dr. Yves Raymond, Montreal) (data not shown). The outer nuclear envelope is contiguous with the rough endoplasmic reticulum. This would account for the 45-kDa site being also present on the nuclear envelope. Some cytochrome P-450-containing enzymes are regulated by dexamethasone [617], and would be expected to show a response to adrenalectomy similar to our 45-kDa site.

We did not identify which microsomal subfractions contained the rough and smooth endoplasmic reticulum, although we would expect more rough membranes in

the heavier fractions. Ambellan *et al.* [553] suggested a rough endoplasmic reticulum localization for their binding site. However they did not measure the plasma membrane contamination. Recently, Quelle *et al.* [540] suggest a correlation of increased dexamethasone binding with the enrichment of the plasma membrane marker. The many differences in their experimental design make comparisons with our results difficult, but their procedure in which fractions enriched in glycoproteins were prepared would favor plasma membrane purification. A potential problem with their system is that the lectin column used would purify 'right side out' plasma membrane vesicles, and 'inside out' endoplasmic reticulum and Golgi vesicles. Hence, the glucose-6-phosphatase marker enzyme could not bind the membrane impermeant glucose-6-phosphate [618]. This would lead to an underestimation of the amount of endoplasmic reticulum components.

3. Conclusion

We have identified a glucocorticoid-dependent rat liver microsomal polypeptide of 45 kDa which binds glucocorticoids. We have previously identified this binding site on the purified plasma membrane preparations of rat liver [541]. In this study, we show that it is also present in the endoplasmic reticulum. The polypeptide may have a regulatory or transport role in steroid hormone action. Ultimate proof of its role in transport will come from the purification of this polypeptide and reconstitution into liposomes for transport studies. Alternatively, further purification, microsequencing and/or cloning will inform us of other possible functions for this binding site based on sequence similarity with other proteins of known function.

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GENERAL CONCLUSION TO PARTS I AND II

While defining the mechanism of nuclear import of the cytoplasmic GR, I identified a 45-kDa membrane binding site for glucocorticoids, and two cytosolic and nuclear 60- and 76-kDa binding proteins for the GR NLS. These binding proteins likely play key roles in the nuclear import of GR by regulating the intracellular concentration of glucocorticoid hormone, and chaperoning the receptor into the nucleus.

We may be able to isolate enough 45-kDa protein from microsomes or its subfraction P2 for microsequencing. The procedure involves taking the flow-through from a triamcinolone acetonide affinity column (to which the protein does not bind) and passing it over a dexamethasone affinity column (to which it does bind) and collecting the eluate fraction. This will separate the 45-kDa binding protein from other glucocorticoid binding proteins present in the microsomes.

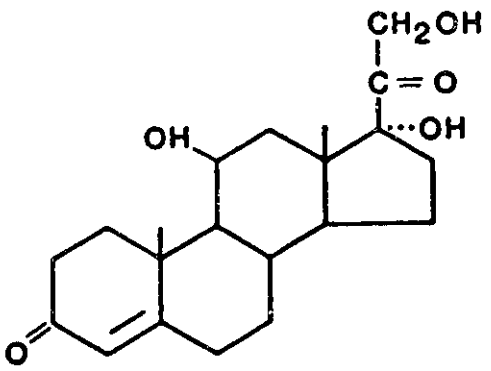
It appears from my results that the 60- and 76-kDa NLS-binding proteins are common components in the nuclear protein import pathway as various NLS peptides compete for GR NLS binding to these sites, and other groups have identified similar molecular weight NLS-binding proteins. The function of NLS-binding proteins will be studied once the proteins have been purified and added to an *in vitro* nuclear import system. A purification scheme is underway in the laboratory and involves conjugating biotin to the cysteinyl residue of the peptide, followed by crosslinking of the peptide to the binding proteins and purification of the complex on a streptavidin column. Alternatively, antibodies generated against these binding proteins can be used to deplete cytosol, or to neutralize the nuclear sites for study of their function in import.

The most striking feature of GR nuclear import is that it is hormone dependent. The ligand-induced nuclear translocation regulates the activity of GR

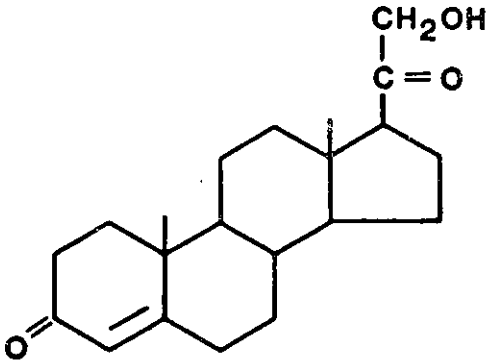
since GR must be in the nucleus to act. Future studies will address the role of the hormone-dependent NLS in GR nuclear import, using both the permeabilized cell system, and cell transfection studies. These studies will help provide answers to the following questions central to glucocorticoid hormone action. Why is GR cytoplasmic while most other nuclear receptors are nuclear? What is the relevance of GR or accessory factor phosphorylation/dephosphorylation to receptor recycling and the continued action of hormone in the cell? These challenges may be met with the knowledge and tools at hand.

APPENDIX A. Chemical Structure of Glucocorticoid Agonists and Antagonists

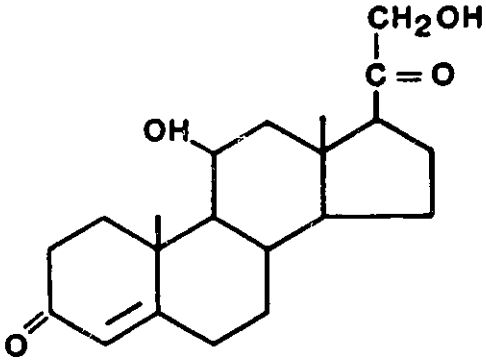
This figure provides the chemical structure of the principal steroids used in this thesis (dexamethasone and dexamethasone 21-mesylate). The figure was taken from Clark, Schrader and O'Malley, 1992 [10].



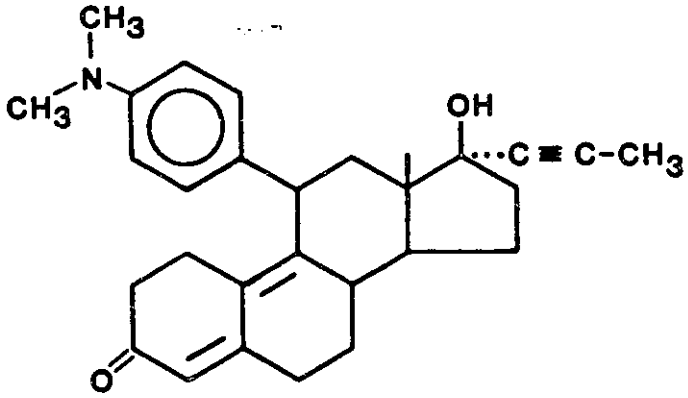
Cortisol



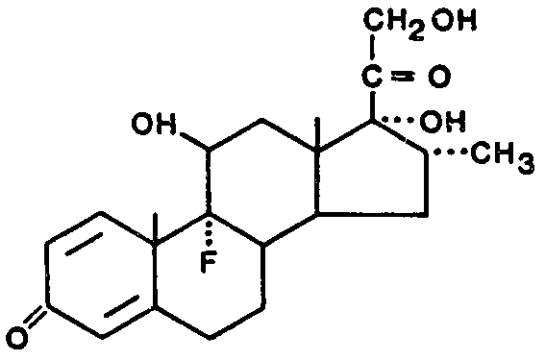
Progesterone



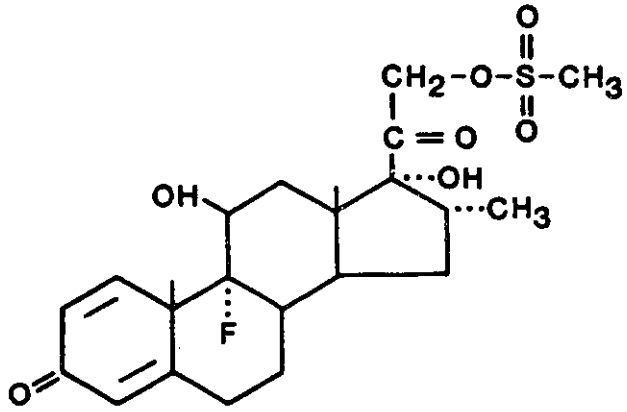
Corticosterone



RU-38486



Dexamethasone



Dexamethasone Mesylate

APPENDIX B. Abbreviations

Throughout this thesis the standard one-letter code is used for amino acid abbreviations. Two-letter abbreviations are used for provinces and states.

ADX	adrenalectomized
Ah receptor	arylhydrocarbon receptor
ammediol	2-amino-2-methyl-1,3-propanediol
AMP	adenosine monophosphate
AR	androgen receptor
Arnt	arylhydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
ATSP	ATP-stimulated GR translocation promoter
ATCC	American Type Culture Collection
bFGF	basic fibroblast growth factor
bHLH	basic region helix-loop-helix
B_{max}	maximum binding capacity
BS^3	bis(sulfosuccinimidyl)suberate
BSA	bovine serum albumin
bZIP	basic region leucine zipper
C4	four cysteinyl residues
C2H2	two cysteinyl residues and two histidyl residues
CaaX	motif of cysteine with two aliphatic amino acids followed by any amino acid
CBG	corticosteroid-binding globulin (transcortin)
cDNA	complimentary deoxyribonucleic acid
D	distal (in Tables 13 and 14 only)
DBD	DNA-binding domain

ddH_2O	doubly-distilled and deionized water
dexamethasone	1,4-pregnadien-9α-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione
dexamethasone 21-mesylate	1,4-pregnadien-9α-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione-21-methylsulfonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ER	estrogen receptor (or endoplasmic reticulum, only in Tables 10 and 11)
ERE	estrogen-response element
F	flanking (in Tables 13 and 14 only)
FCS/FBS	fetal calf serum/fetal bovine serum
FITC	fluorescein isothiocyanate
FK506	a macrolide drug/immunosuppressant
FKBP	FK506-binding protein
GABA	γ-aminobutyric acid
gave	average g force
GlcNAc	N-acetylglucosamine
GR	glucocorticoid receptor
GRE	glucocorticoid-response element
GTP	guanosine triphosphate

GTPase	guanosine triphosphatase
H-7	1-(5-isoquinolinesulfonyl)-2-methyl piperazine
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HMG	high mobility group
hnRNP	heteronuclear ribonucleoprotein particle
HPLC	high performance liquid chromatography
hsp	heat-shock protein
HTH	helix-turn-helix
HTLV	human T-cell leukemia virus
HSF	heat-shock factor
IODO-GEN	1,3,4,6-tetrachloro-3α,6α-diphenylglycouril
IPTG	isopropyl β-D-thiogalactopyranoside
K_d	dissociation constant
LAGS	low-affinity glucocorticoid-binding site
mGR	membrane-associated GR
MMTV-CAT	mouse mammary tumor virus promoter driving the expression of the chloramphenicol acetyl transferase gene
MR	mineralocorticoid receptor
MTI-III	macromolecular-translocation inhibitor III
NBRF-PIR	National Biomedical Research Foundation Protein Identification Resource
NE	nuclear envelope
NEM	N-ethylmaleimide
NL1	nuclear localization signal 1 of GR

NL2 nuclear localization signal 2 of GR

NLS nuclear localization signal

NLSBP NLS-binding protein

NLS-L long form NLS

NLS-T NLS with a threonyl substitution

NPC nuclear pore complex

NRD NF-κB/*Rel*/*dorsal* motif

O overlapping (in Tables 13 and 14 only)

OIG octyl-β-D-thioglucopyranoside

P proximal (in Tables 13 and 14 only)

P1, P2, P3, P4 pellet of microsomal subfraction 1, 2, 3 or 4

PARP poly(ADP-ribose) polymerase

PBS phosphate-buffered saline

PDI protein disulfide isomerase

PHA phytohemagglutinin

P_i orthophosphate (inorganic)

PKA protein kinase A

PKC protein kinase C

polII U snRNA uracil-rich small nuclear RNA transcribed by RNA polymerase II

POU Pit-Oct-Unc (common) motif

PPO 2,5-diphenyloxazole

PR progesterone receptor

PVDF polyvinylidine difluoride

RAR retinoic acid receptor

RH *Rel* homology

RNA ribonucleic acid

RNAase	ribonuclease
RNP	ribonucleoprotein particle
RRM	RNA recognition motif
rRNA	ribosomal RNA
RU486/RU38486	17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(prop-1-ynyl)-estra-4,9-dien-3-one
SA	specific activity
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SV40 large T-antigen	simian virus 40 large tumor-antigen
TAPS	N-tris-[hydroxymethyl]methyl-3-aminopropane-sulfonic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
T-DNA	transferred DNA (in plants)
TEMED	N,N,N',N'-tetramethylethylene-diamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid
TR	thyroid hormone receptor
triamcinolone acetonide	1,4-pregnadien-9α-fluoro-11β,16α,17α,21-tetrol-3,20-dione-16,17-acetonide
Tris	tris[hydroxymethyl]aminomethane or 2-amino-2-hydroxy-methylpropane-1,3-diol
tRNA	transfer RNA
U snRNP	uracil-rich small nuclear ribonucleoprotein
u.v.	ultraviolet
VDR	vitamin D receptor

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PUBLICATIONS AND ABSTRACTS:

(1) Full Papers:

E.C. LaCasse, G.M. Howell and Y.A. Lefebvre (1990) Microsomal dexamethasone binding sites identified by affinity labelling. J. Steroid Biochem. 35, 47-54.

E.C. LaCasse and Y.A. Lefebvre (1991) Nuclear and nuclear envelope binding proteins of the glucocorticoid receptor nuclear localization peptide identified by cross-linking. J. Steroid Biochem. Molec. Biol. 40, 279-285.

E.C. LaCasse, H.A. Lochnan, P. Walker and Y.A. Lefebvre (1993) Identification of binding proteins for nuclear localization signals of the glucocorticoid and thyroid hormone receptors. Endocrinology 132, 1017-1025. (Erratum appears in Vol. 133, p. 2760)

E.C. LaCasse and Y.A. Lefebvre, Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins. Submitted (1994).

(2) Abstracts

LaCasse E.C., Howell G.M. and Lefebvre Y.A., Affinity labelling of microsomal dexamethasone binding sites. (Abstract of poster presented at CFBS Meetings, Calgary, June 14-17, 1989.)

LaCasse E.C., Howell G.M. and Lefebvre Y.A., Microsomal dexamethasone binding sites identified by affinity labelling. (Abstract of poster presented at Endocrine Society Meeting, Seattle, June 21-24, 1989.)

LaCasse E.C., Golsteyn E.J. and Lefebvre Y.A., Identification of a nuclear protein that interacts with a nuclear localization sequence of the glucocorticoid receptor. (Abstract of poster presented at Eastern Great Lakes Regional Endocrine Meeting, Rochester, October 17, 1989.)

Lefebvre Y.A. and LaCasse E.C., Nuclear binding proteins for the nuclear localization signal of the glucocorticoid receptor identified by cross-linking. (Abstract of poster presented at CFBS Meetings, Halifax, June 14-16, 1990.)

LaCasse E.C., Golsteyn E.J. and Lefebvre Y.A., Identification and partial characterization of glucocorticoid receptor nuclear localization signal-binding proteins of the nucleus and nuclear envelope. (Abstract of poster presented at Endocrine Society Meeting, Atlanta, June 20-23, 1990.)

LaCasse E.C. and Lefebvre Y.A., Nuclear and nuclear envelope binding proteins of the glucocorticoid receptor nuclear localization peptide identified by cross-linking. (Abstract of poster presented at VIII International Congress on Hormonal Steroids, The Hague, September 16-21, 1990.)

LaCasse E.C., Lochnan H.A. and Lefebvre Y.A., Targetting of steroid and thyroid hormone receptors to the nucleus: signal binding proteins. (Abstract of poster presented at CFBS Meetings, Kingston, June 9-11, 1991.)

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