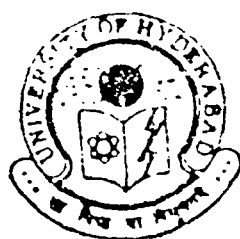


**PROTEINS THAT REGULATE THE NUCLEAR
ENTRY OF THE GOAT UTERINE
ESTROGEN RECEPTOR**

THESIS SUBMITTED TO THE UNIVERSITY OF HYDERABAD
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY



A. SAI PADMA

DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500134
INDIA

October, 1998
Enroll No. 92LSPH01

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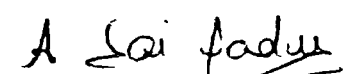
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SCHOOL OF LIFE SCIENCES
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HYDERABAD

Date: Oct. 08, 1998.

DECLARATION

I hereby declare, that the work presented in this thesis has been carried out by me under the supervision of Dr. R.V.Thampan, and that this work has not been submitted for a degree or diploma in this or any other university.


Dr. R.V. THAMPAN
Supervisor.


A. SAI PADMA
Candidate.

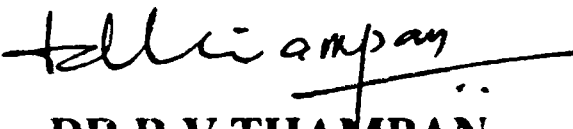
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UNIVERSITY OF HYDERABAD
HYDERABAD

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CERTIFICATE

This is to certify that Ms. A. Sai Padma, has carried out the research work embodied in this thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this university. I recommend her thesis entitled "PROTEINS THAT REGULATE THE NUCLEAR ENTRY OF THE GOAT UTERINE ESTROGEN RECEPTOR", for submission for the degree of Doctor of philosophy of this university.


PROF. K. SUBBARAO
Head, Dept. of Biochemistry


DR. R. V. THAMPAN
Supervisor


PROF. R. P. SHARMA
Dean, School of Life Sciences

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ABBREVIATIONS

AMP	Adenosine 5' monophosphate.
AR	Androgen receptor
ATP	Adenosine 5' triphosphate.
ATPase	Adenosine triphosphatase.
BCIP	5-Bromo 4-Chloro 3-indolyl phosphate.
CK	Casein kinase
DNA	Deoxyribonucleic acid.
E ₂	Estradiol.
EDTA	Ethylenediamine tetraacetic acid.
ER	Estrogen receptor.
E-RAF	Estrogen receptor activation factor.
ERE	Estrogen responsive element.
FKBP	Forskolin binding protein.
gp	Glycoprotein
GR	Glucocorticoid receptor.
GTP	Guanosine 5' triphosphate.
HBD	Hormone binding domain.
HRE	Hormone response element.
hsp	Heat shock protein.
naER	Non activated estrogen receptor.
NBT	Nitroblue tetrazolium.
NLS(s)	Nuclear localization signal(s)
NLSBP(s)	Nuclear localization signal binding protein(s).
NPC	Nuclear pore complex.
NP 40	Nonidet P 40.
PAGE	Polyacrylamide gel electrophoresis.
PEG	Polyethylene glycol.
PMSF	Phenylmethylsulphonyl fluoride.
POPOP	1,4,Bis (2,5 phenyl oxazol) benzene.
Pi	Inorganic phosphate.
PPO	Diphenyloxazole
PR	Progesterone receptor
RPM	Revolutions per minute.
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetic acid.
TRIS	Tris (hydroxymethyl) methylamine.
WGA	Wheat germ agglutinin.

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INTRODUCTION

1.0 INTRODUCTION

Estrogens regulate female reproductive functions through a nuclear receptor that belongs to a superfamily of ligand activated transcription factors that includes receptors for steroids, thyroid hormone, retinoids, prostanoids and vitamin D. In target tissues, receptors are activated following binding by hormonal ligands; the receptor hormone complex binds to specific DNA binding sites and thereafter modulate the expression of a network of specific target genes (Yamamoto, 1985; Green and Chambon, 1986; Evans and Hollenberg, 1988). Unliganded receptors for most of the steroid hormones appear to be present in cells as part of large oligomers (300 ~kDa mass, 7-10 s sedimentation constant), formed by noncovalent association of a monomeric or dimeric receptor protein with a dimer of 90 kDa heat shock protein and possibly other proteins as well. When the hormone binds to the receptor, the receptor dissociates from the 9s complex, forms a homodimer and binds to DNA at specific binding sites in the gene called hormone response elements (HREs) ultimately leading to enhanced transcription of specific genes (Beato, 1989). The estrogen responsive element (ERE) is a palindromic pair of hexameric half sites AGGTCA_nnnTGATCT (Green and Chambon, 1987).

1.1 STRUCTURE OF THE ESTROGEN RECEPTOR (ER):

The ER gene has been cloned, sequenced and the predicted amino acid sequence has been determined. The ER has 595 amino acids and a molecular mass of 66 kDa. The ER can be divided into six functional regions, from A to F.

The 'A' region is well conserved between chicken and human estrogen receptors and is required for transcriptional activation. The 'C' region is also very well conserved and contains the DNA binding domain. Deletion of this region results in the loss of DNA binding capacity of the receptor (Kumar et al., 1986). The DNA binding domain is composed of two highly conserved zinc fingers that set the receptor apart from other DNA binding proteins. These zinc fingers are generated by the co-ordination of zinc ions with four cysteine residues (Schwabe et al., 1990). This region targets the receptor to the hormone response elements (Green et al., 1988). The 'E' region is also very well conserved and contains the ligand binding domain. This domain possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiological responses. In its simplest terms, the ligand binding domain can be thought of as a molecular switch that, upon ligand binding, shifts the receptor to a transcriptionally active state. The region 'D' has 37 amino acids and functions as a hinge between the DNA binding and the hormone binding domains.

The above mentioned receptor is the classical estrogen receptor. From our laboratory it has been shown that in addition to the classical ER, there exists another ER form, called the non activated ER (naER) which has the capacity to bind estradiol with high affinity while remaining incapable of binding to the DNA (Anuradha et al., 1994). This protein also has a molecular mass of 66 kDa, like the regular ER. The structural differences between the two proteins have been demonstrated by the non identical peptide maps that the two proteins display (Zafar and Thampan, 1993; Karthikeyan and Thampan, 1996). Karthikeyan and Thampan (1996) demonstrated that the naER is primarily localized in the plasma membrane and is specifically extracted into the medium when the membranes are

exposed to estradiol. The naER has been shown to have the capacity to dimerize with the estrogen receptor activation factor (E-RAF), a cytosolic DNA binding protein with no capacity to bind estradiol (Thampan and Clark, 1981; Thampan, 1987, 1989).

Recently Mosselman et al. (1996) reported the identification and characterization of a novel human estrogen receptor (ER β), which is highly homologues with the classical ER (now known as ER α) and has an overlapping but non-identical tissue distribution. They demonstrated that this novel receptor is functional in that it interacts with (anti)-estrogens and is able to modulate estrogen-responsive receptor gene expression.

1.2 CELLULAR LOCALIZATION OF THE ER:

The intracellular localization of the unoccupied receptors has been a subject of considerable debate. In 1984, two independent reports appeared (King and Green 1984; Welshons et al., 1984), localizing the unoccupied receptors in the nucleus of the target cells. King and Green used a monoclonal antibody raised against the MCF-7 cell estrogen receptors to detect the receptors in the intracellular compartments and found that the majority of the staining was confined to the nuclear compartment.

Welshons et al., (1984), by using immuno cytochemical techniques and enucleation experiments, proved that the unoccupied estrogen receptors were predominantly nuclear in localization.

Vazquez-Nin et al., (1991) by using immunoelectron microscopic technique showed that the receptor was mainly nuclear but was also present in the cytoplasm.

This is apparently a debatable topic but a general agreement has been formed that estrogen receptors are predominantly localized in the nucleus.

1.3. INTERACTION OF ER WITH HEAT SHOCK PROTEINS AND IMMUNOPHILIN CHAPERONES:

Steroid receptors are recovered from cells in large (9S) heterocomplexes that contain both heat shock proteins (hsp) and immunophilins. Some components of the receptor heterocomplexes are proteins with established chaperone functions (eg. hsp 90 and hsp 70), and one critical function of the hsp heterocomplex is to facilitate the folding of the hormone binding domain (HBD) of the receptors into a high affinity steroid binding conformation.

1.3.1 hsp-90:

The heat shock protein (hsp) 90 family is a group of highly conserved stress proteins found ubiquitous in eukaryotes. hsp 90 is the most abundant constitutive hsp in eukaryotic cells, accounting for 1-2% of cytosolic protein and is associated with all steroid receptors (Baulieu, 1987; Pratt, 1990; Pratt et. al., 1992). hsp 90 exists in a stoichiometric ratio of 2:1 (hsp:SR) in the 9s non-transformed receptor complexes. There are reports of hsp 90 localization in cytoskeleton including actin, in membrane ruffles (Koyasu et al., 1986), microtubules (Redmond et al., 1989) and intermediate filaments (Czar et al., 1996). It has been proposed that cytoskeleton-associated hsp 90 may reflect potential protein targeting and

trafficking function of hsp 90 and its associated proteins. The hsp 90 binding of the ER prevents it from binding to DNA and at the same time maintains the receptor in a conformation required for hormone binding (Bresnick et al., 1989). The direct evidence for this came from the studies of Picard et al. (1990) who have shown that mutants having low levels of hsp 90 but normal levels of steroid receptors are not responsive to the steroid as the receptors do not bind the hormone efficiently.

1.3.2 Immunophilins:

FKBP 59 is an immunophilin as it binds Forskolin (FK 506) and rapamycin and is found associated with all the non-transformed steroid receptor complexes and is associated with hsp 90 (Tai et al., 1986; Tai et al., 1992). In the untransformed receptor complexes, two molecules of the hsp 90 and one molecule of p59 are associated with one molecule of the steroid receptor (Tai et al., 1993; Segnitze et al., 1995). The FKBP 59 binds to hsp 90 via tetratricopeptide repeat domain. It has an ATP and calmodulin binding site and it also has peptidyl prolyl isomerase activity, suggesting that it may play a role as molecular chaperone

1.4 NUCLEAR TRANSPORT OF PROTEINS:

Nuclear proteins are actively transported across the nuclear envelope. This transport is a highly selective process that can be divided into two steps. The first step is the binding to the cytoplasmic surface of the nuclear pore complex. It does not require ATP or GTP. The second step is the energy dependent translocation through the nuclear pore complex.

1.4.1 The nuclear envelope:

The nuclear envelope sequesters the genome and its activities within a unique biochemical environment, the nucleus. The nuclear envelope consists of two lipid bilayers, the outer and inner nuclear membranes, separated by a perinuclear cisternal space. The perinuclear space is continuous with the lumen of the endoplasmic reticulum (ER). The outer nuclear membrane and ER membrane are also continuous and functionally similar in that both contain ribosomes on their cytoplasmic surfaces. The nucleoplasmic surface of the inner membrane is associated with the nuclear lamina, a fibrous network that supports the nuclear envelope.

Pores traverse the nuclear envelope at sites where the inner and outer membranes are fused, thereby providing a link between the cytoplasm and the interior of the nucleus. The nuclear pores are water-filled channels within a large proteinaceous nuclear pore complexes (NPC). The nuclear pore is a large and complex structure of 124 million Daltons (Reichelt et al., 1990).

1.4.2 The nuclear pore complex:

The nuclear pore complex is a supramolecular assembly that straddles the inner and outer nuclear membranes of all eukaryotes. It is $\sim 0.13\mu\text{m}$ in diameter, $\sim 0.07\mu\text{m}$ thick and has a relative molecular mass of about 125 megadaltons (Akey, 1989., Reichelt et al., 1990). The NPC has two main functions: it allows passive diffusion of ions and small molecules through nuclear pores with a physical diameter of $\sim 9\text{ nm}$ and it mediates transport of protein and ribonucleoprotein particles through a gated channel with a functional diameter of upto 26 nm (Feldherr et al., 1984; Gerace, 1992).

1.4.2-1. The Structure of the Pore Complex:

The ultra structure of the pore-complex has been known in outline since the 1950s but recent advances in electron microscopy have revealed much more details (Akey., 1989; 90; Akey and Rademacher, 1993).

The NPC consists of four separate structural elements:

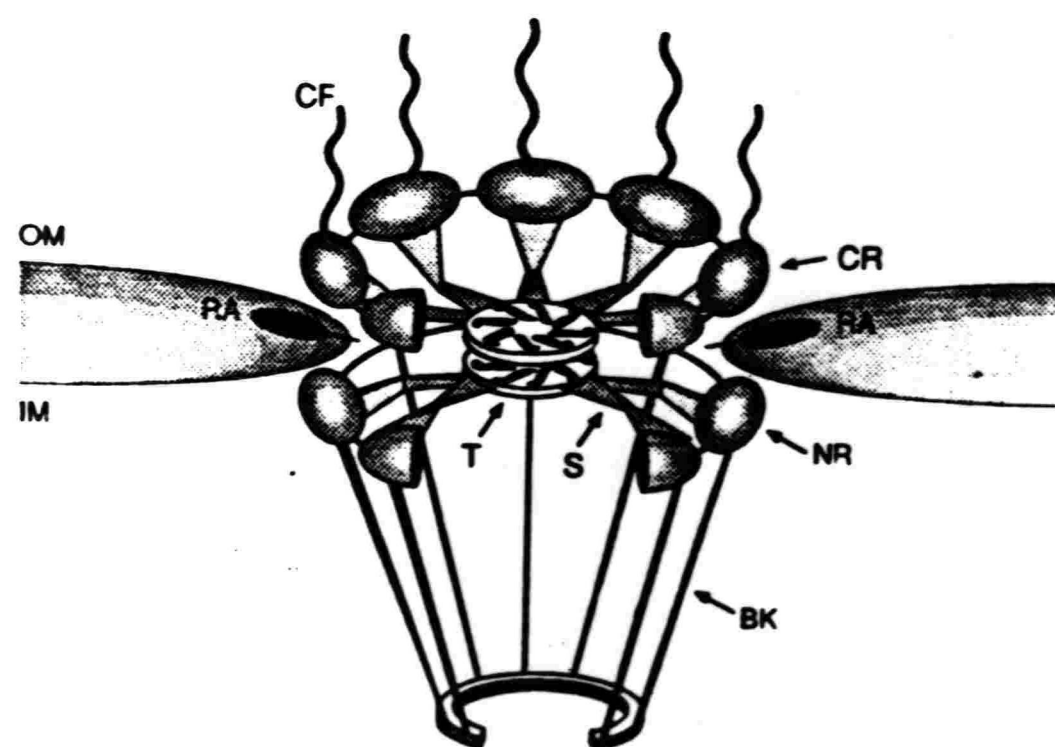
- a) **the Scaffold**, which includes the majority of the pore
- b) **the central hub or the transporter of the pore**, which appears to carry out the transport of proteins and RNA
- c) short thick **filaments** attached to the cytoplasmic side of the pore and
- d) **a nucleoplasmic basket** (Fig. 1.1)

a) the **Scaffold** appears as a stack of three closely apposed rings, the cytoplasmic ring (CR), the nucleoplasmic ring (NR), and a central ring of thick spokes (S). Each ring has an eight fold symmetry. The spokes are connected at their inner edge and support a central hub of $\sim 360\text{-}380 \text{ \AA}^0$. Interspersed between the spokes are large $90 \text{ \AA}^0$ aqueous channels, which presumably allow passive diffusion of small proteins and metabolites between nucleus and cytoplasm (Milligan, 1986). The Scaffold of the pore is thought to maintain the fusion of the two nuclear membranes that creates the $900 \text{ \AA}^0$ opening in the nuclear envelope, provide the $90 \text{ \AA}^0$ diffusion channel and support the smaller central transporter that regulates actual import and export.

b) **The central hub or transporter** ($360\text{-}380 \text{ \AA}^0$) is a proteinaceous ring. Akey (1990; 92) predicted that the central transporter would consist of two irises of eight arms each. The two irises are predicted to be stacked atop one another and to

Figure 1.1 Structural model of the nuclear pore complex.

A structural model of the nuclear pore complex as presented by Forbes, 1992 (with a portion in the front of the pore cut to show the details). The globular components of the cytoplasmic ring (CR) of the pore are connected to the spokes (S) as are the globular components of the nucleoplasmic ring (NR). The spokes are separated by 9 nm channels and support a central transporter (T). The transporter consists of two rings of 8 arms each. Radial arms (RA) extend into the luminal space between the outer (OM) and inner (IM) nuclear membranes. Cytoplasmic filaments (CF) are shown extending from the cytoplasmic ring. A basket-like structure of filaments (BK) extends from the nucleoplasmic ring of the pore.



open sequentially, each like the diaphragm of a camera, to let a nuclear protein or RNA through (Akey and Goldfarb, 1989). Elucidation of the structure of the central transporter of the nuclear pore has been derived from analyzing nuclear pores by rapid freezing technique (Akey and Goldfarb., 1989., Akey 1990; 91; 92). This rapid freezing technique has allowed retention of a structure (the central plug) that was often lost in previous studies. The transporter can be resolved within individual pores into one of the four recognizable and distinct forms. Each form has an eightfold symmetry and it is assumed that they represent sequential intermediates in nuclear import in the following order :1) a nuclear protein (K) first binds at the periphery of the transporter ring;

2) it then moves to the central channel where it docks and induces the channel to open;

3) in response to the import signal, the first iris opens, lets a protein pass into the pore;

4) the second iris would then open, further passage would ensue;

5) the first iris closes, followed by the second, and translocation is complete .

Such a mechanism would explain why the pore has such high fidelity of transport and does not allow the inward leakage of inappropriate proteins. The arms of the irises are further suggested to be mechano-ATPases to explain their proposed ability to pivot during the opening of the pore.

Recent electron microscopic evidence indicates that the pore also is in contact with important accessory structures. Individual pores appear to be connected to one another by the nuclear lamina and by an additional set of pore-connecting fibers (Stewart and Whytock, 1988; Allen and Douglas, 1989). On the

cytoplasmic face of the pore thick fibers ($\sim 33\text{\AA}$ diameter) that extend into the cytoplasm have been observed by a low voltage scanning electron microscopy (Ris, 1991). Several strong indications are available that such filaments exist (Scheer et al., 1988) and are a staging area for nuclear proteins to bind prior to transport (Richardson et al., 1988). On the nucleoplasmic side of the pore, a large basket-like structure has also emerged from the scanning electron microscopic studies of Ris (1990; 1991). This basket disassembles in the absence of Ca^{+2} and reforms when Ca^{+2} is added (Jarnik et al., 1991; Jarnik and Aebi, 1991). This basket structure appears to consist of eight filaments extending from the nucleoplasmic ring of the pore.

1.4.2-2. Nuclear pore complex proteins:

The nuclear pore complex could be considered as an organelle composed of a unique set of proteins necessary for transporting macromolecules across the nuclear envelope. Association of the nuclear pore complex proteins with both the nuclear membrane and the underlying lamina has made it difficult to isolate the pore complex in pure form. The first pore complex protein to be identified was the abundant high molecular weight concavalin- A reactive glycoprotein gp 120, which is located on the pore margin, largely inside the perinuclear cisternae. Subsequently a series of nucleoporins were discovered by virtue of their N-acetyl glucosamine containing O- linked carbohydrate moieties that make them reactive to wheat germ agglutinin (WGA). Evidence for the pore complex location of a newly identified polypeptide might include a) immunogold labeling, b) co-fractionation with pore-complexes enriched fraction, c) punctuate immuno fluorescence staining of the nuclear periphery.

Identification of approximately 20 genes encoding nucleoporins in yeast and a handful of others in higher eukaryotes has led to the classification of these proteins into several different groups based on sequence motifs contained within their primary aminoacid sequences. Two classes of phenylalanine/glycine (FG) repeats, GLFG and FXFG subtypes, have been identified. A number of nucleoporins contain one or the other or a combination of these repeats. Thus far, the in vivo function of the FG repeat regions has not been elucidated. It has, however, been shown that some soluble nuclear transport factors interact with these FG repeat domains in vitro (Iovine et al., 1995; Rexach et al., 1995). Thus, the FG repeat domains may play a role in targeting the soluble transport factors to the nuclear pore.

Several nucleoporins do not contain FG repeats but contain other sequence motifs. These include the coiled coil domains found in Nup1p, Nup57p and p62 (Davis 1995, Wimmer et al., 1992), leucine zipper found in Nup107 (Radu et al., 1994) and zinc finger domain found in Nup153 (Sukegawa et al., 1993).

Since the nucleoporins are both the structural and functional components of the nuclear pore complex, it seems likely that many of them will serve general function and that some may play more specific roles in the transport of different classes of macromolecules. A number of genes encoding nucleoporins have been identified in a screen for *S.cerevisiae* mutants defective in the export of poly (A) RNA from the nucleus (Amberg et al., 1992). The nucleoporins Nup100p, Nup116p and Nup145p contain putative RNA binding domains. Some nucleoporins are also directly implicated in protein import. The nucleoporin that falls most readily into this class is Nsp1p. In vitro studies suggest that the protein

import defect observed in Nsp1 mutants arises from a decrease in the docking of substrate to the nuclear envelope as well as from an inability to translocate substrate across the pore (Schlenstedt et al., 1993).

There are some more evidences to indicate that some nucleoporins play a specific role in protein translocation.

1) co-incident labeling of nuclear pore complexes with WGA- gold and nucleoplasmin-gold particles suggests that nucleoporins interact directly with proteins prior to their passage through the pore (Akey and Goldfarb, 1989)

2) Antibodies that recognize a subset of nucleoporins block nucleoplasmin import and RNA export (Featherstone et al., 1988).

3)WGA blocks uptake of proteins into the nucleus (Yoneda et al 1987; Dabauvalle et al., 1988). The effect of WGA is not due to the occlusion of the channel, because a dextran through the pore is not blocked (Finlay et al.,1987).

Instead, pore complexes assembled in vitro without WGA- binding proteins are morphologically intact but are unable to import large proteins properly (Finlay and Forbes, 1990). These reconstituted pores fail to bind NLS bearing proteins. Addition of WGA- binding pore complex proteins restores binding and import.

Understanding precisely what role each nucleoporin plays in macromolecular transport will probably require a model of the entire pore complex. There are some limited approaches to assigning in vivo function to a particular class of nucleoporins in eukaryotes. One method consists of depletion of xenopus egg extracts of nuclear pore components using WGA or antibodies prior to nuclear envelope assembly (Finlay et al 1991; Newmeyer et al., 1986; Powers et al., 1995). Such an approach has been used to demonstrate that the vertebrate

nucleoporin complex p62-p58-p54 is required for docking of proteins at the nuclear pore (Finlay et al., 1991). These experiments are, however, restricted by the availability of specific tools to deplete each nucleoporin.

Some of the nucleoporins are situated exclusively on the cytoplasmic side of the nuclear pore complex such as Nup 358 and Nup 214, most likely as constituents of 50-nm-long fibers emanating from the NPC into the cytoplasm. Other nucleoporins such as Nup 153 and Nup 98 are located exclusively on the nucleoplasmic side, as components of the nuclear basket structure. Still another repeat-containing nucleoporin p62, appears to be located in the center of the NPC.

1.4.3 Nuclear localization signals/sequences:

Proteins destined to be targeted into the nucleus following translation in the cytoplasm contain specific signals in their primary sequences. The most extensively studied signals are termed Nuclear Localization Signals (NLSs) and, unlike other signal sequences, can be located anywhere in the primary sequence of the protein. These nuclear localization signals were first suggested by De Robertis et al. (1978) that the nuclear proteins must contain in their primary structure a signal that enables them to accumulate selectively in the nucleus. Two criteria define NLSs: deletion or mutation causes cytoplasmic accumulation of a normally nuclear protein and when fused to a non nuclear protein, the NLS directs the protein to the nucleus. In many cases both the criteria can be taken into account to identify the NLSs. NLSs have now been identified in a large number of nuclear proteins. There is no single consensus among the many NLSs that have been identified to date. However there are some general descriptive rules that NLSs

- 1) are typically short sequences, usually not more than 8-10 aminoacids,

- 2) contain a high proportion of positively charged aminoacids (lysine and arginine) often associated with proline,
- 3) can reside in any exposed region of a nuclear protein
- 4) are not removed following localization and
- 5) can occur more than once in a given protein.

Some proteins do not possess their own NLS and enter the nucleus via co-transport with another protein (Dingwall 1982; Zhao and Padmanabhan 1988).

Although no strong consensus has emerged from analysis of NLSs, many contain the sequence Lys-Arg/Lys-X-Arg/Lys (Chelsky, 1989). The most extensively studied NLS of this class is that of the SV 40 large T antigen, which comprises the sequence pro.lys.lys.lys.arg.lys.val. A single point mutation of lys-128 to Thr or Asn dramatically reduces the efficiency of this sequence to direct nuclear localization (Lanford and Butel, 1984; Kalderon et al., 1984), while other mutations in surrounding residues have a lesser effect (Kalderon et al., 1984).

The presence of the N- terminal flanking region of the SV40 large T antigen NLS in association with its NLS dramatically enhances nuclear transport. This region is distinguished by a phosphorylation site (Rihs and Peters, 1989). This phosphorylation site was identified to be a casein kinase (CK) II site and a survey of other nuclear proteins shows that all nuclear proteins have CK II sites in the vicinity of the NLS at a distance of 10-30 aminoacids. Phosphorylation might modulate the affinity of NLS for its receptor (Rihs et al., 1991).

Robbins et al.(1991) further showed that the nucleoplasmin NLS is composed of two interdependent regions of basic aminoacids: mutations in either alone have no effect on nuclear localization activity. The nucleoplasmin NLS is non functional only when both domains are mutated. A nucleoplasmin like motif is seen in several proteins such as p53, N1/N2, No38 and the steroid receptors.

1.4.3-1 Steroid hormone receptor NLSs:

The NLSs of the members of the steroid/thyroid hormone receptor super family are located in the hinge region (region D) between the DNA binding and the hormone binding domains.

Nuclear targeting of β -galactosidase fusion protein has provided evidence for the existence in the hER and rat GR of a constitutive NLS resembling the SV40 T-antigen NLS prototype (Picard et al.,1990; Picard and yamamoto, 1987). In addition, the existence of hormone-dependent NLS has been reported for the rat GR (Picard and Yamamoto, 1987), but no such signal was found in hER (Picard et al., 1990).

Ylikomi et al. (1992) chose to analyze steroid hormone receptor NLSs in their natural aminoacid sequence context, because

- 1) the efficiency of NLSs is sensitive to variations in the protein context; thus a given NLS may become inactive when placed in a different environment even within the same protein (Roberts et al., 1987)
- 2) the β -galactosidase marker that is commonly used for heterologous nuclear targeting studies is a tetrameric protein, while steroid receptors are assumed to be

Figure 2.14. Schematic representation of the structure of the protein. The protein is composed of three main regions: the DNA-binding domain (DBD), the hinge region, and the homeodomain (HD). The DBD is located at the N-terminus and is responsible for binding to the DNA major groove. The hinge region is a flexible linker between the DBD and the HD. The HD is located at the C-terminus and is responsible for the protein's function as a transcription factor. The protein is shown in a ribbon diagram, with the DBD in blue, the hinge region in yellow, and the HD in red.

(256-260) RKDRR	(265-273) KHKRQR	(299-303) KRSKK
pNLS 3	pNLS 2	pNLS 1
---(DBD)---	---Hinge region---	---(HBD)---

dimers (Kumar and Chambon, 1988) and the consequences of multimerization on NLS efficiency is unknown.

3) The possible coöperation between several nuclear targeting sequences and the effect of other domains of the protein on their activity can be assessed only in their natural environment.

Thus Ylikomi et al., (1992) reported the identification and cooperation of three constitutive and one estrogen-inducible hER nuclear targeting sequence which they termed as proto-NLSs (p-NLSs), as all of these sequences have characteristics of 'classical' NLSs, while none of them individually was sufficient for nuclear targeting of the wild-type receptor, and have to co-operate to become efficient in nuclear targeting. Thus, they considered them as proto-NLSs.

The three p-NLSs are constitutive in the sense that they promote transport of ER even in the absence of the hormone. An additional p-NLS, which is hormone inducible p-NLS is seen in the hER hormone binding domain, which on its own is not sufficient for ensuring efficient nuclear accumulation, but can cooperate with the constitutive p-NLSs. This inducible p-NLS was active only in the presence of estrogen. This may apparently contributes for the nuclear accumulation of the wild type receptor in the presence of the hormone (Ylikomi et al., 1992).

In the progesterone receptor, three p-NLSs , two of which are located within and directly adjacent to the second zinc finger, cooperate with each other and a weak hormone-inducible p-NLS in the PR HBD. No masking of p-NLSs by

the HBD was observed for ER and PR, while the ligand free glucocorticoid receptor HBD inhibited the activity of both homologous and heterologous NLSs

1.4.4 Soluble transport factors in nuclear protein import: a general survey

1.4.4-1) NLS receptors:

Proteins are targeted to the nuclear pore via an interaction in the cytoplasm between the NLS within the protein and a soluble NLS receptor. The NLS receptor can be identified and purified by virtue of its capacity to bind a normal but not a reversed NLS on a protein or an affinity column and confirmed as an NLS binding protein (NLSBP) by its capacity to support nuclear import of transportants, in the presence of other requisite factors, in either permeabilized cells or resealed isolated nuclei.

Several proteins were identified that specifically recognize the synthetic wild type NLS peptide of SV-40 T antigen. Chemical cross-linking revealed two proteins of 60 kDa and 70 kDa that are mainly cytoplasmic but are also associated with the nuclear envelope and found within the nucleus (Adam et al., 1989). Yamasaki et al., (1989) identified two cytoplasmic proteins of 100 and 70 kDa and two nuclear proteins of 140 kDa and 55kDa by photoaffinity crosslinking. By a similar method, Li and Thomas (1989) demonstrated the interaction of NLS with a 66 kDa nuclear protein and Benditt et al., (1989) described four NLS binding proteins in detergent extracts of nuclear envelopes. In yeast, Silver et al., (1989) identified two proteins of 70 and 59 kDa that on western blots bind synthetic NLS peptides coupled to human serum albumin (HSA). Imamoto-Sonobe et al (1990) identified in the rat liver a 69 kDa protein which binds to SV40 large T antigen NLS. Adam and Gerace (1991) demonstrated the identification of NLSBPs by a

functional assay for the first time. They identified a 55 kDa NLSBP, which was sensitive to N-ethyl maleimide (NEM), a sulphhydryl alkylating agent. Stochaj et al. (1991) purified a 70 kDa NLSBP from the yeast *Sacharomyces cerevisiae*.

However, recent advances in research in yeast and higher eukaryotes have led to the isolation of a heteromeric dimeric complex required for targeting the NLS-containing proteins to nuclear pores. Gorlich et al. (1994) identified a 60 kDa protein termed importin- α by fractionation of *Xenopus* cytosol in conjunction with an in vitro import assay. Several other studies have also identified this subunit of the NLS receptor (Adam et al., 1991; Imamoto et al., 1995; Moroianu et al., 1995a). The second subunit of the NLS receptor is a protein of approximately 95 kDa molecular mass termed importin- β . Like importin- α this protein was identified by a number of complementary approaches (Chi et al., 1995; Iovine et al., 1995; Koepp et al., 1996; Radu et al., 1995). While both the subunits of the NLS receptor can interact with NLS sequences, the binding of importin- α is significantly tight.

1.4.4-2 GTPase Ran(ras related nuclear protein):

It is homologous to small G-proteins like Ras. It was identified as a cytosolic factor required for the import of NLS-containing substrates into the nucleus (Melchior et al., 1993; Moore and Blobel, 1993). GTP hydrolysis by Ran seems to be an important step in the nuclear transport in living cells.

1.4.4-3 NTF2:

Another protein that has been implicated in nuclear transport by both in vitro (Moore and Blobel, 1994; Paschal and Gerace, 1995) and in vivo (Corbett et al.,

1996) experiments is the small Ran binding protein that has been referred to as pp15 (Bohn et al., 1980), p10 (Moore and Blobel, 1994) and nuclear transport factor 2 (NTF2; Paschal and Gerace, 1995). This protein interacts both with Ran (Moore and Blobel, 1994) and with nuclear pore protein p62 (Paschal and Gerace, 1995), and is localized at the nuclear rim. The role of this protein is not understood completely till now.

There is no general agreement on the requirement of cytosolic factors for nuclear transport. A number of in vitro assays have been published and most of them do not require any cytosolic factors in addition to the NLSBPs for nuclear accumulation (Markland et al., 1987; Imamoto-Sonobe et al., 1988; Silver et al., 1989; Kalinich and Douglas, 1989; Parnaik and Kennedy, 1990; Nirmala and Thampan, 1995).

1.4.5 Mechanism of nuclear transport:

Although proteins are produced in the cytoplasm, those that participate in nuclear functions must be translocated into the nucleus, a process known as nuclear protein import. Some of these proteins are subsequently transported back into the cytoplasm via a specific process termed nuclear protein export. The physical separation of nuclear and cytoplasmic functions bestows upon the eukaryotic cell a mechanism for regulation of cellular processes that is not available to prokaryotes. Compartmentalization is a regulatory mechanism where an activator may be sequestered from its activation target. There are many transcription factors that are sequestered in the cytoplasm and are transported to the nucleus only in response to a cellular signal. The macromolecular traffic crossing the nuclear envelope must be meticulously regulated both to maintain the

normal state of the cell and to respond to intracellular signals that mediate cell growth and other essential process.

Molecules with a diameter of <9 nm diffuse freely through the pore equilibrating between the nucleoplasm and the cytoplasm (Paine et al., 1975; Lang et al., 1986). Molecules with $>$ diameter 9 nm enter the nucleus by active transport (Dingwall et al., 1982; Feldherr et al., 1983;1984).

Historically, nuclear protein import has been described as a two step process: an energy-independent binding at the nuclear pore followed by an energy-dependent translocation into the nucleus (Newmeyer and Forbes, 1988). Advances in our understanding of the import mechanism has led to the realization that this process can actually be divided into more distinct and specialized steps

- 1) recognition of the transport substrate in the cytoplasm,
- 2) targeting to the nuclear pore complex,
- 3) translocation through the nuclear pore,
- 4) release of the transport substrate at the nucleocytoplasmic face of the pore complex.

1.4.5-1) Recognition:

Proteins are targeted to the nuclear pore via an interaction in the cytoplasm between the NLS within the protein and a soluble NLS receptor. Recent advances have led to the isolation of a heterodimeric complex required for targeting NLS-containing proteins to nuclear pores. The first subunit is the importin- α of approximately 60 kDa. The second subunit is a protein of approximately 95 kDa termed importin- β . While both subunits of the NLS receptor can interact with NLS

sequences, the binding of importin- α is significantly tight. This finding in combination with the observation that importin- β interacts with repeats contained in several nucleoporins has led to the proposal of a model in which importin- α is primarily responsible for binding to NLS-containing proteins. Importin- β then targets the complex to the nuclear pore (Gorlich et al., 1995). Moroianu et al., (1992) showed that importin- α (Karyopherin- α) alone cannot bind to the nuclear envelope of digitonin-permeabilized cells while importin- β (Karyopherin- β) can bind without importin- α being present.

By immunofluorescence microscopy of methanol fixed cell, Moroianu et al.(1995) findings gave a very important information about importin α and β . Importin β was localized to the cytoplasm and the nuclear envelope and was absent from the nuclear interior. Recombinant importin- β can bind directly to nuclear envelope of digitonin permeabilized cells. In contrast, recombinant importin- α did not bind unless importin- β was present. Likewise in an import reaction with all recombinant transport factors (importin- α , importin- β , Ran and p10) import depended on importin- β . Localization of the exogenously added transport factors after a 30 minutes import reaction showed importin- β at the nuclear envelope and importin- α , Ran and p10 in the nuclear interior. In an overlay assay with SDS/PAGE resolved and nitrocellulose transferred proteins of the nuclear envelope, ^{35}S -labelled importin- β bound to atleast four peptide repeats containing nucleoporins-Nup358, Nup214, Nup153 and Nup98. This result indicates a division of labor, the α subunit of the importin heterodimer serves in NLS recognition, and the β subunit mediates docking to the peptide repeat containing nucleoporins. Most interesting is the finding that the importin- α enters the nucleus and is retained there, whereas importin- β doesnot enter the nucleus.

Immunofluorescence localization with anti-Karyopherin- β (importin- β) antibodies showed primarily cytoplasmic staining and a strong nuclear rim staining but no significant intranuclear staining.

Nirmala and Thampan (1995a) identified and purified a protein of molecular mass 55 kDa which helps in the transport of estrogen receptor from the cytoplasm to the nucleus in the goat uterus. This 55 kDa protein (p55) alone can mediate both the functions 1) recognition of NLS on estrogen receptor 2) docking of the complex to nuclear membrane. From this study it is evident that there is no general agreement for the requirement of multiple factors for the binding step, since it can be fulfilled by a single factor.

1.4.5-2) Targeting to the nuclear pore:

Very little is known how the import complex is targeted to the nuclear pore. It is thought that the cytoplasmic filaments identified in higher eukaryotes may serve to concentrate the transport substrate at the face of the nuclear pore. Recent high-resolution electron microscopic studies provide support for this hypothesis (Pante and Aebi, 1996).

Tubulin and actin coprecipitated with the estrogen receptor when goat uterine cytosol was immunoprecipitated with antibodies against estrogen receptor antibody (Zafar and Thampan, 1993). Similar result was also observed when the immunoprecipitation was carried out using antibodies against tubulin and actin. Tubulin and actin were also demonstrated to be associated with other steroid receptors. (Sanchez et al., 1988; Miyata et al., 1991). Nirmala and Thampan (1995a) showed that p55 bound to both actin and tubulin with very high affinity.

This interaction of both the ER and the p55 with the cytoskeletal elements of the cell suggests the possible involvement of cytoskeletal proteins in the transport of ER into the nucleus.

The nucleoporins on the filaments that extend into cytoplasm acts as docking sites for the NLS-receptor complex at the periphery of the nuclear envelope (Wilken et al., 1995). Recently a direct biochemical interaction between the isolated transport factors and peptide repeat-containing nucleoporins has been demonstrated. Nup 358, located at or near the tip of the cytoplasmic fibers has been shown to contain four Ran-binding sites (wu et al., 1995). The repeat containing nucleoporins also contain binding sites for p10. It has been proposed that the repeat-containing nucleoporins serve as a stationary phase and the transport factors as the mobile phase in the transport across the NPC (Radu et al., 1995).

1.4.5-3) Protein translocation into the nucleus:

Once transport substrates are targeted to the nuclear pore, they must undergo translocation through the pore complex. Mechanism of translocation through the transporter of the NPC was proposed by Akey (1990) in the double iris model based on electron microscopic studies of the NPC. The gating events results in the dilation of the first iris to allow the protein inside. The protein passes through the transporter and the first iris closes and the second iris open at the nucleocytoplasmic side to let the protein move into the nucleoplasm. WGA may prevent the dilation of the first iris by cross linking the subunits of the iris.

The factors which play role in the translocation step were identified to be 1) GTPase Ran: GTP hydrolysis by Ran seems to be important step in the nuclear transport (Moore and Blobel, 1994). In cell-free import assays by using either isolated nuclei or digitonin permeabilized cells ATP or ATP regenerating system can substitute the GTP and GTP hydrolysing factors (Ran) (Newmeyer et al., 1986; Richardson et al., 1988; Nirmala and Thampan, 1995b).

2) Rna1p/Ran GAP1: like many other cellular G- proteins, the rate of GTP hydrolysis by isolated Ran is negligible (Bischoff et al., 1994). Thus, in vivo, a catalyst is required to enhance the rate of this reaction. Proteins that enhance the rate of GTP hydrolysis mediated by G- proteins are called GTPase activation proteins (GAPs). Studies using yeast strain *S.cerevisiae* shows that the Ran GAP, Rna1p is absolutely required for protein import into the nucleus.

3) prp 20p/RCC1: another critical regulator of Ran function is the nucleus-localized nucleotide exchange factor for Ran (Bischoff, 1991). Exchange factors catalyze the release of GDP from the GTP binding protein and consequently the regeneration of the GTP- bound form of the protein.

4) NTF2: Since it interacts most tightly with the GDP-bound form of Ran, it seems likely that it may act as a timing mechanism for the recycling of the Ran protein to the GTP- bound form by the exchange factor.

5) hsp 70: As with many other cellular transport processes, some studies have implicated the role of chaperone hsp 70 in nuclear protein import .(Imamoto et al., 1992; Shi and Thomas, 1992). The functional role played by hsp 70, however, remains unknown.

Nirmala and Thampan (1995a) showed that the p55-dependent translocation of the goat uterine ER into the nucleus is mediated by a 12-14 kDa protein(s) present in the nuclear membrane near the nuclear pore complex.

The general model drawn from all these studies is that NLS-containing proteins interact with the importin- α -importin- β heterodimer in the cytoplasm via direct binding to importin- α . Importin- β then targets the complex to the nuclear pore periphery, where it interacts with repeat containing nucleoporins. Current thinking dictates that Ran must be in the GDP-bound state to promote import of this complex to the nucleus. The importin-NLS-bearing protein complex is translocated into the nucleus through the nuclear pore. Dissociation of the transport complex occurs when the importin complex interacts with Ran in the GTP-bound state, which is generated in the nucleus by the exchange factor prp 20p.

The above mentioned model was drawn on the basis of general studies of in vivo and in vitro transport assays with xenopus egg extracts or permeabilized mammalian cells and the genetic systems using *S.Cerevisiae*.

Nirmala and Thampan (1995 a;b) have shown that the estrogen receptor transport into the nucleus can be separated into two distinct steps: the p55-mediated transport and binding of ER to the nuclear membrane, followed by an ATP- dependent 12-14 kDa protein(s) mediated translocation of ER into the nucleus. The p55 has inherent ATPase activity and it is proposed that the energy released during this ATP hydrolysis is utilized in the nuclear transport of the ER.

From the observations mentioned above, it may be inferred that no single general mechanism can account for the transport of all the proteins. There exists selective differences in the transport of different proteins, probably indicating the specificity and selectivity that should be associated with the transport of individual proteins.

1.5 REGULATION OF NUCLEAR IMPORT:

The control of nuclear uptake provides a powerful means to regulate the activity of transcription factors and other proteins with nuclear activity. The various strategies for inhibition of nuclear uptake seem to center around the access of nuclear localization signals of proteins to the receptors involved in protein delivery to the nucleus. Possession of the nuclear localization sequence while probably necessary, is not sufficient to ensure nuclear entry. For example, the nuclear targeting sequence may be masked by subunit interactions or binding with other proteins. Ligand binding could expose (or mask) the patch, or protein phosphorylation would modify the effectiveness of the nuclear targeting sequence. Even if the patch of nuclear targeting sequence is exposed and active, other signals on the protein could negate its effect by anchoring the protein in the cytoplasm, and such anchoring could be regulated in different ways. Examples of transcription factors that undergo inducible nuclear uptake are the glucocorticoid receptor (Picard and Yamamoto, 1987), the α interferon-regulated factor ISGF3 (Levy et al., 1989), the yeast protein SW 15 (Moll et al., 1991), the drosophila morphogen *dorsal*, and the nuclear factor *κB* (NF-*κB*) (Baeuerle and Baltimore, 1988a).

NF-*κB* is a ubiquitous mammalian transcription factor whose activity is regulated at the level of its intracellular location. It is a tetrameric protein which

consists of two 50 kDa and two 65 kDa subunits. In stimulated B lymphocytes, NF- κ B is nuclear, binds to the DNA and regulates the transcription of κ immunoglobulin light chain genes. However, in pre-B cells, where κ light chains are not expressed, NF- κ B is cytoplasmic. The cytoplasmic form of NF- κ B is associated with another protein, I- κ B, and this complex is unable to bind to the DNA. Phosphorylation disrupts the I κ B-NF- κ B complex and NF- κ B enters the nucleus (Ghosh and Baltimore, 1990).

NF- κ B, *dorsal* and the *rel* oncogenes have a high degree of sequence similarity extending over 300 aminoacids in the N-terminal half of the proteins (Gilmore, 1990). All three proteins have an SV-40 T antigen-like NLS toward the end of the region of homology. In addition, all three proteins have a conserved serine approximately 20 aminoacids before the nuclear localization sequence. Phosphorylation at this serine mediates nuclear localization either by disrupting the interaction with cytoplasmic factor, or conversely, by effecting the NLS activity by altering the local charge density or the conformation of the protein.

The glucocorticoid receptor is a zinc finger-type DNA-binding protein of steroid receptor superfamily that regulates transcription of genes in response to a given steroid hormone. In the absence of the hormone, the receptor remains within the cytoplasm. In the presence of the hormone, the receptor rapidly translocates into the nucleus where it binds to DNA and performs its regulatory role. Translocation into the nucleus depends on interaction of the receptor with hormone. The NLSs within the receptor are nonfunctional in the absence of the hormone because they are obscured by the heat shock protein, hsp 90, bound to the glucocorticoid receptor in the cytoplasm (Sanchez et al., 1985). Hormone binding

to the receptor dissociates it from hsp 90 and NLSs become exposed, which then results in receptor translocation into the nucleus.

Rihs et al., (1991) demonstrated that the nuclear transport of recombinant proteins in which short fragments of the SV 40 T- antigen are fused to the aminotermminus of *Escherichia coli* β - galactosidase is dependent on both the nuclear localization sequence (NLS, T-antigen residues 126-132) and a phosphorylation site containing sequence (T-antigen residues 111-125). While the NLS determines the specificity, the rate of the transport is controlled by the phosphorylation site containing sequence.

A survey of other nuclear proteins shows that all nuclear proteins have CK II sites in the vicinity of the NLS at a distance of 10-30 aminoacids. Phosphorylation might modulate the affinity of NLS for its receptor (Rihs et al., 1991). In many nuclear proteins cdc 2 kinase sites are seen adjoining the CK II sites. The phosphorylation of the kinase site near the residue switches off the transport and thus is proposed to have a major regulatory role in nuclear retention and nuclear export. Jans et al., (1991) proposed that a CcN motif consisting of a CK II site, cdc 2 kinase site and a NLS may be a general element of nuclear transport regulation.

1.6 hsp 70:

In eukaryotes, members of the conserved hsp 70 family of proteins are present in the cytoplasm and within organelles where they act as chaperones of protein folding and traslocation. Members of the hsp 70 family bind in an ATP dependent manner to unfolded regions in proteins or to hydrophobic peptides and

ATP hydrolysis facilitates release of the protein. hsp 70 is known to be associated with GR (Diehl et al., 1993); PR (Kost et al., 1989) and AR (Veldschote et al., 1992) heterocomplexes but there are no reports on its association with the ER. The site of hsp 70 interaction with the receptor is the HBD. Although hsp 70 is required for assembly of receptors into a complex with hsp 90 (Smith et al., 1992; Hutchison et al., 1994) it is not known whether hsp 70 plays a role in receptor action or cycling after the heterocomplex is formed. In contrast to the bound components, hsp 90 and the immunophilin FKBP 52, hsp 70 has not been recovered in cross-linked receptor heterocomplex (Segnitz et al., 1995; Rexin et al., 1991; Alexis et al., 1992). Based upon the failure and the fact that some hsp 70 is bound to immunomatrix independent of receptor (Rexin et al., 1991) it has been argued that hsp 70 nonspecifically sticks to the receptors as a persistent component of receptor-hsp 90 complex. It is entirely possible that the hsp-70 bound receptors represent a fraction of the receptors that are in the process of hetero complex assembly or help up in the assembly process.

1.7 hsp-25:

The biological roles of hsp 25 are unknown, however, it has been suggested that this protein may play a role in thermotolerance and regulation of gene expression. Moreover, like the high molecular weight hsps, hsp 25 may be involved as a molecular chaperone.

SCOPE OF THE THESIS

The nuclear transport of the estrogen receptor is known to be effected in a two-step process. The first step is mediated by a 55 kDa cytosolic protein, which recognizes ER in the cytosol and transports it to the nuclear membrane. This step in transport is ATP-independent. The second step is mediated by a 12-14 kDa protein(s) present in the nuclear membrane, which recognizes the p55-ER complex and translocates the ER into the nucleus in a ATP-dependent manner. The transport of estrogen receptor from the cytoplasm to the pore complex might be aided by microtubular and/or microfilament network as the p55 binds to both tubulin and actin. The *in vitro* transport of ER by p55 is independent of the presence of the hormone, estradiol (E₂) (Nirmala and Thampan, 1995). However, the current studies reveal that there is an apparent role for estradiol in the process, which manifests prior to the recognition of the ER-NLS by p55.

In the absence of the hormone, estrogen receptor is predominantly nuclear in its localization (King and Green, 1984., Welshons et al., 1984). But exposure of cells to estradiol results in a significant and reproducible increase in nuclear accumulation of hER (Ylikomi et al., 1992). An estrogen-inducible p-NLS was found in the hormone binding domain (HBD) of the hER in addition to the three constitutive NLSs. The inducible and the constitutive ER p-NLSs cooperate in the presence of estrogen and influence the nuclear translocation of ER when the cells are exposed to estradiol (Ylikomi et al., 1992). Nuclear co-translocation experiments indicated that *in vivo* the stability of ER dimers is hormonally controlled

The experimental work presented in this thesis serves to understand the regulatory mechanism involved in the nuclear transport of estrogen receptor under the influence of estradiol. Consequently a couple of cytosolic proteins of molecular mass 28 and 73 kDa were identified and isolated apart from the p55. With the studies on these proteins what has been achieved is the knowledge that the estradiol-influenced receptor entry into the nucleus is highly regulated. The p28 antagonizes the ER-p55 interaction, apparently through the masking of the NLS on the ER. This inhibition is reversed by yet another, 73 kDa protein, the p73. Estradiol causes the release of p73 from ER and facilitates p28 dissociation from the NLS site on the ER. p55 occupies the NLS and mediates the transport. This, apparently is the main role played by the hormone in the nuclear transport of the ER.

The second step of the nuclear transport of ER is found to be mediated by the 12-14 kDa protein(s) of nuclear membrane/pore complex origin. It was of interest to isolate and purify the protein(s) and to know the mechanism associated with the translocation step at the nuclear pore complex. The results, presented in this thesis, provide a new insight into the mechanism of nuclear transport of the estrogen receptor. It is expected that these studies would serve to enhance the current knowledge on the biology of estrogen receptor.

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 MATERIALS.

2,4,6,7-³H] estradiol 17 β (sp.act, 101 Ci/mmol) was obtained from Amersham. Monoclonal anti-hsp25 (clone LAP-28), monoclonal anti-hsp 70 (clone BRM-22) and anti-actin antibodies, 5-Bromo 4-chloro 3-indolyl phosphate (BCIP), Nitroblue tetrazolium (NBT), Fluorescein isothiocyanate (FITC) isomer I, estradiol 17 β , Triton X-100, Nonidet P-40, Quercetin, CM-Sephadex, DEAE-Sephadex, protein A-Sepharose, 4-chloro-1-naphthol, unprocessed wheat germ and the dialysis bags were obtained from Sigma chemical Co. Sepharose 4B, Sephadex G-25 and Sephadex G-100 were purchased from Pharmacia. DEAE cellulose was obtained from Whatman. Nitrocellulose membranes were purchased from Schleicher and Schuell Inc.,USA. Concavalin A and HRPO (Horse radish peroxidase) or alkaline phosphatase coupled goat anti-rabbit IgG and alkaline phosphatase coupled rabbit anti-mouse IgG were purchased from Genei, Bangalore. Phenyl methyl sulphonyl fluoride and silver nitrate were purchased from E-Merck, Germany. Estrogen receptor NLS peptide (amino acid sequence from 256-303) and HBD peptide of ER (amino acid sequence from 302-320) were synthesized at the Rajiv Gandhi Center for Biotechnology, Trivandrum. Routine chemicals used were of the analytical grade and were purchased from local commercial establishments.

Distilled water used in these studies: The raw water was first subjected to pressure filtration in order to remove suspended particles. This was then deionized with the help of a commercial (Purewater Systems) de-ionizer. The deionized

water was subjected to single distillation in a glass distillation unit. This water was re-distilled using a quartz-glass distillation unit.

2.2 BUFFERS.

1) TEM buffer: 50 mM Tris-HCl pH7.6
1 mM EDTA,
12 mM Monothioglycerol,
0.2 mM PMSF

2) TEMN buffer: 50 mM Tris-HCl pH 7.6,
1 mM EDTA,
12 mM Monothioglycerol,
50 mM NaCl,
0.2 mM PMSF.

3) TCKM buffer: 50 mM Tris-HCl pH 7.6,
1 mM CaCl_2 ,
20 mM KCl
2 mM MgCl_2 ,
0.2 mM PMSF.

TCKM buffer was made as a 5X buffer and stored at 4°C. It was diluted to X and the following buffers were made for the isolation of nuclei.

Buffer A: TCKM (X)+ 250 mM sucrose.

Buffer B: TCKM (X)+ 250 mM sucrose+0.05% Triton X 100 .

Buffer C: TCKM (X)+ 340 mM sucrose.

4. Nuclear transport

assay buffer: 50 mM Tris-HCl pH 7.6,
3 mM CaCl_2 ,
5 mM MgCl_2 ,
25 mM KCl,
500 mM Sucrose,
4 mM ATP.

5) TMC buffer: 2 mM Tris-HCl pH 8.0,
0.5 mM β -mercaptoethanol,
0.2 mM CaCl_2 .

- 6)Imidazole buffer:** 10 mM Imidazole pH 8.0,
0.5 mM β -mercaptoethanol,
0.1 mM CaCl_2 .
1 mM ATP.
- 7)PM buffer:** 10 mM Sodium phosphate pH 6.5,
10 mM MgCl_2 .
- 8)Towbin buffer:** 25 mM Tris-HCl pH 8.3,
192 mM Glycine,
20% methanol.
- 9) TNN bufffer:** 10 mM Tris-HCl pH 7.6,
150 mM NaCl,
0.1% NP-40.
- 10)TMg buffer:** 10 mM Tris-HCl, pH 7.6,
6 mM MgCl_2 .
- 11)ScintillationCocktail :**5 g PPO, 500 mg POPOP in Toluene:Triton X-100 (67:33 V/V).

2.3 PREPARATION OF GOAT UTERINE CYTOSOL.

Goat uteri were obtained from a local slaughter house, transported on ice to the laboratory and kept frozen at -75°C until further use. Goat uterine cytosol was prepared following the procedure of van der Hoeven (1981). The uteri were homogenized in the cold using a polytron (PT-45-80) homogenizer at setting 4 for 30 seconds (X 4)with sufficient time in between to prevent heating of the homogenate. The homogenate was filtered through a nylon mesh. The filtrate was centrifuged at 10,000 X g for 30 minutes. The fat layer was carefully aspirated off and the supernatant was filtered through glass wool. Polyethylene glycol (PEG) 6000 was added to the filtrate to a final 5% concentration. The filtrate was stirred in the cold for 30 minutes. The stirring was monitored at a specific speed in order

to avoid frothing of the filtrate. The microsomes which were precipitated with 5% PEG were pelleted at 10,000 X g for 30 minutes. The post-mitochondrial supernatant (cytosol) was filtered again through the glass wool.

2.4 ISOLATION OF GOAT UTERINE NUCLEI:

Goat uterine nuclei were isolated following the procedure developed by Thampan (1985). Goat uteri were obtained from the slaughter house, transported on ice to the laboratory and used immediately for the isolation of nuclei. The uteri were cleaned off the adhering fat tissue and was minced very finely. A 20% homogenate was made in buffer A using a Polytron homogenizer at setting 4 for 30 seconds four times with sufficient time in between to prevent heating of the homogenate. The homogenate was filtered through a fine nylon mesh and through a nylon membrane of 80-120 microns. The filtrate was then centrifuged at 800 X g for 10 minutes. The nuclear pellet was washed with the buffer B followed by two washes with the buffer A to remove Triton X-100. The nuclei were suspended in buffer A and 5 ml of this suspension was layered over 30 ml buffer B and centrifuged at 800 X g for 10 minutes. The final pellet contained purified nuclei with 80% yield as observed under a phase contrast microscope.

To obtain nuclei with the nuclear membranes intact, the above procedure was followed except that the nuclei were not treated with the buffer B which contained 0.05% Triton X 100 which solubilizes the nuclear membranes.

2.5 NUCLEAR TRANSPORT ASSAY FOR ER TRANSPORT INTO THE NUCLEUS.

The nuclei to be used in the transport assay were isolated with their membranes intact or without membranes as mentioned in the previous section. The

nuclei were suspended in 2X assay buffer. The ER was isolated following the procedure described by Zafar and Thampan (1993) (This procedure is detailed in the section 2.10)

100 μ l of the ER preparation was incubated overnight with 20 nM ^3H -estradiol (50 μ l). The unbound hormone was removed following adsorption of free hormone to dextran coated charcoal (100 μ l) (1% charcoal and 0.1% dextran in TEMN buffer). 200 μ l of ^3H -E₂-ER complexes (containing ~2 μ g) were incubated with goat uterine nuclei (250 μ l) in the presence of 50 μ l aliquot of the fractions containing the p55 for 30 minutes at 30°C in a water bath. The incubation was terminated by transferring the assay tubes to an ice bath and dilution of the incubation mixture by the addition of 2 ml ice cold assay buffer. The nuclei were sedimented at 5,000 X g for 2 minutes. The nuclei were washed once again with 2 ml assay buffer and finally the radioactive hormone associated with the nuclei was extracted with 1 ml distilled ethanol. The ethanol extracts were transferred to glass vials containing 10 ml scintillation cocktail. The radioactivity was measured using a Wallac liquid scintillation counter.

2.6 FLUORESCENCE ASSAY OF THE ER TRANSPORT INTO THE NUCLEUS:

ER was labeled with FITC as described in section 2.15. Goat uterine nuclei were isolated as described above and were suspended in the nuclear transport assay buffer. The nuclei were incubated with FITC-ER (5 μ g) in the presence or absence of p55, p28 and p75 at 30°C. Aliquots of the incubation mixture were transferred to a glass slide and covered with a glass coverslip. The transport of ER

into the nucleus was observed using a Nikon fluorescence microscope and photographed using Ilford 400 ASA film.

2.7 CYANOGEN BROMIDE ACTIVATION OF SEPHAROSE-4B:

Sepharose 4B was activated following the procedure of March et al., (1974) with some modifications. Sepharose 4B was washed with double distilled water (DDW), followed by 1M sodium bicarbonate, pH 10.9. The volume of Sepharose 4B was measured and two volumes of 1M sodium bicarbonate, pH 10.9 was added. The slurry was stirred slowly on ice to keep the temperature at 4°C. The following operations were all carried out in a fume hood. The rate of stirring was increased and 0.05 volumes of cyanogen bromide solution (2 mg CNBr/ml acetonitrile) was added. The slurry was stirred vigorously for 10 minutes at 4°C. The slurry was poured into a sintered glass funnel and washed with 10 volumes each of 0.1 M sodium bicarbonate, pH 9.5; double distilled water and 0.4 M sodium bicarbonate, pH 9.5. After the last wash, the slurry was filtered to a moist cake. The activated Sepharose 4B was transferred to a bottle containing one volume of the protein solution. The protein to be coupled to Sepharose 4B was dissolved in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M sodium chloride. Coupling was done at 4°C for 20 hours with gentle stirring on a rotary shaker. The protein solution was removed, Sepharose was washed with 1 M glycine and stirred with 1 M glycine for an additional four hours to mask the unreacted groups. The protein-Sepharose was washed with 20 volumes each of 0.1 M sodium acetate, pH 4.0 and 0.1 M sodium bicarbonate, pH 8.3; both the buffers contained 0.5 M sodium chloride. The matrix was subsequently washed with the TEMN buffer and stored at 4°C in TEMN buffer containing 0.02% sodium azide.

2.8 PREPARATION OF ss-DNA-SEPHAROSE.

DNA was isolated from goat liver following the procedure of Marmur (1961). DNA (1g) was dissolved in 25 ml of 0.5 N sodium hydroxide and the solution was left in a boiling water bath for 15 minutes. The DNA solution was cooled on ice and 25 ml of 0.5 N HCl was added, followed by 50 ml of 0.4 M sodium bicarbonate pH 9.5. This was mixed with 100 ml CNBr activated Sepharose 4B and the coupling was carried out as described above.

2.9 PREPARATION OF ACTIN-SEPHAROSE.

Actin was purified from the goat skeletal muscle following the procedure of Pardee and Spudich (1982). Acetone powder of the skeletal muscle was made as follows. 250 g muscle was washed with double distilled water and minced finely. The mince was washed extensively with distilled water and extracted for 10 minutes in 500 ml ice cold 150 mM potassium phosphate buffer pH 6.5 containing 100 mM potassium chloride. This was filtered under vacuum. The residue was extracted by stirring successively in 1) 1000 ml of 50 mM sodium bicarbonate for 10 min; 2) 500 ml of 1 mM EDTA pH 7.0 for 10 min; 3) twice with 500 ml of double distilled water for 5 min and 4) five times each with 250 ml of acetone (at room temperature) for 10 mins. Each step was followed by filtration under vacuum. The final residue was lyophilized in order to remove traces of acetone. The acetone powder was obtained and stored at -70°C until further use.

Ten grams of acetone powder was extracted with 250 ml TMC buffer containing 0.2 mM ATP for 30 mins. The mixture was centrifuged at 15,000 X g for 20 mins. The supernatant was aspirated very carefully, leaving the turbid layer in the centrifuge tube. To the supernatant potassium chloride, magnesium chloride

Lower gel:	5%	7.5%	10%	12%	15%
Acrylamide	6.7	11.7	13.3	16.7	20.0
Distilled water	22.7	19.4	16.0	12.7	9.5
Lower Tris (4X)	10.0	10.0	10.0	10.0	10.0
10% APS	0.6	0.6	0.6	0.6	0.6
TEMED(μ l)	20	10	10	10	10

upper gel	3%
Acrylamide	1.6
Distilled water	5.7
Upper Tris (4X)	2.5
10%APS	130 μ l
TEMED	10 μ l

Standard gels were run at a constant current of 30 mA and the samples in mini gels at a constant voltage of 60 V through the stacking gels and 100 V through the separating gels. When the tracking dye reached the end of the gels, the run was terminated and the gels were transferred to the gel boxes containing the fixer. Standard proteins of known molecular mass were loaded into one of the wells to enable the calculation of the molecular mass of the unknown proteins.

2.23 SILVER STAINING OF SDS GELS.

The gels were stained with silver nitrate following the procedure of Blum et al.(1987). The solutions required for this procedure were all prepared fresh just before use. All the solutions were made in double distilled water.

- 1) **Fixer:** 50% methanol, 12% glacial acetic acid and 100 μ l 37% formaldehyde/200 ml.
- 2) **50% ethyl alcohol.**
- 3) **Thiosulphate solution:** 40 mg sodium thiosulphate was dissolved in 200 ml double distilled water.
- 4) **Silver nitrate solution:** 400 mg silver nitrate was dissolved in 200 ml double distilled water. 150 μ l of 37% formaldehyde was added to this solution immediately before use.
- 5) **Developer:** 12 gm of anhydrous sodium carbonate and 100 μ l of 37% formaldehyde was dissolved in 200 ml double distilled water.
- 6) **12% glacial acetic acid.**

The gels were transferred to a staining tray containing 200 ml of the fixer and were left on a shaker for one hour. The gels were washed with 50% ethanol for 30 minutes. The ethanol was removed and the gels were treated with the thiosulphate solution for one minute. The treated gels were washed thrice with distilled water for about twenty seconds each in order to remove thiosulphate. The gels were then incubated with the silver nitrate solution for twenty minutes with continuous shaking. The silver nitrate solution was then drained off and the gels were washed thoroughly with double distilled water as mentioned above. The gels were then developed with the Na_2CO_3 solution (developer) till protein bands were seen, washed with double distilled water and left in 12% glacial acetic acid to stop staining.

2.24 WESTERN BLOTTING.

Western blotting analysis was performed according to the procedure developed by Towbin et al. (1979) and Yonezawa et al. (1988). The proteins were separated on mini SDS-PAGE gels. After electrophoresis the gels were immersed in cold Towbin buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol) for half an hour. The dimensions of the gel were taken and the nitrocellulose membrane of the same size was cut and equilibrated in Towbin buffer. Six Whatman No.3 sheets of the same size as of the gel were taken and equilibrated along with the fiber pads in Towbin buffer. A sandwich was made as follows: the grey panel, fiber pad, 3 layers of Whatman paper, gel, nitrocellulose membrane, 3 layers of Whatman paper, fiber pad and the white panel. Care was taken to remove any air bubbles trapped between the gel and the nitrocellulose membrane. The sandwich was clamped tightly and immersed in the electrophoresis tank filled with ice cold Towbin buffer, with the grey panel facing the cathode. The transfer was performed in a Trans Blot Electrophoretic cell (BIO-RAD 170-3910). The transfer was done at 70V (0.25A limit) for three hours. While The transfer was going on, the buffer in the tank was stirred to prevent local heating. After the transfer, the blots were air dried and washed with TNN buffer for 10 minutes. The blots were blocked with 3% BSA in TNN buffer for one hour, at room temperature. The blots were incubated overnight with the primary antibody in 3% BSA in TNN buffer. The blots were washed with TNN buffer and were re-incubated with anti-rabbit IgG (anti-mouse IgG in case of monoclonal primary antibody) coupled with horse radish peroxidase (HRPO) or alkaline phosphatase (AP) in 3% BSA in TNN buffer for two hours at room temperature. The unbound antibody was washed with TN buffer (TNN buffer without NP40) and stained with 4-chloro, 1-naphthol as substrate (10% methanol, 0.25% H_2O_2 and 0.3 mg/ml 4-chloro, 1-naphthol in TN

buffer) in the case of horse radish peroxidase-IgG, or, with 5-bromo 4-chloro 3-indolyl phosphate (BCIP) nitroblue tetrazolium (NBT) (0.85% NBT in dimethyl formamide and 5% BCIP in 50 mM Tris-HCl, pH 9.0 containing 150 mM NaCl and 100 mM MgCl₂ in the case of alkaline phosphatase-IgG.

2.25 GEL FILTRATION ANALYSIS.

A Sephadex G-100 column (75 X 1.5 cm) was used to perform gel filtration analysis in order to determine the Stokes radius (R_s) of the purified protein following the procedure of Siegel and Monty (1966). The Sephadex G-100 column was calibrated with the gel filtration markers (blue dextran and glycine) and proteins of known Stokes's radii (bovine serum albumin (BSA), ovalbumin (OV) and cytochrome C (CYT C)). The column was equilibrated with TEM buffer containing 0.3 M NaCl. The marker proteins were dissolved (4 mg/ml) in the equilibration buffer and about 300 μ l of this solution was applied carefully onto the column. Fractions collected were subjected to measurement of absorbance at 280 nm. The k_d (distribution coefficient) of the protein was calculated and a standard graph of k_d versus R_s of the marker proteins was made.

$$k_d = \frac{V_e - V_o}{V_t - V_o}$$

where,

V_o = Volume of buffer used to elute blue dextran,

V_t = Volume of buffer used to elute glycine,

V_e = Volume of buffer used to elute a given protein V_{eBSA} , V_{eOV} , V_{eCYT-C} etc.

The k_d of the unknown protein was also monitored similarly and the Stokes radius of the protein was calculated with the help of a standard graph.

2.26 ESTIMATION OF INORGANIC PHOSPHATE (Pi).

Estimation of Inorganic Phosphate (Pi) was performed following the procedure of Fiske and Subbarow (1925) with some modifications.

Reagents used:

- 1) 20 µg/ml potassium phosphate solution (for the standard) in 0.05 N hydrochloric acid.
- 2) 0.05 N hydrochloric acid.
- 3) Acid molybdate: 1.25% ammonium molybdate in 2.5 N sulphuric acid.
- 4) Fiske-Subbarow reagent: 7.312 g sodium metabisulphate, 400 mg 1-amino-2-naphthol-4-sulphoic acid (ANSA) and 250 mg sodium sulphite were ground well together in a porcelain crucible. This powder was stored in an amber-colored bottle. One g of this powder was dissolved in 25 ml double distilled water. The reagent was prepared immediately before use.

Standard phosphate solution was diluted with 0.05 N HCl to different concentrations (2 µg to 20 µg/ml). To the phosphate solution, 1 ml acid molybdate was added and mixed. This was followed by the addition of 250 µl of the Fiske-Subbarow reagent. The mixture was left at room temperature for 10 minutes. The blue color developed was measured at 660 nm against a blank containing 500 µl 0.05 N HCl that was treated similarly. An absorbance of 0.76 for 10 µg phosphate was taken as the standard value.

2.27 ATPase ASSAY:

The proteins p55 and p12 were incubated independently with 6 mM ATP in TMg buffer at 30°C for 30 mins with different protein concentrations. The volume

of the incubation mixture was 250 μ l. The incubation was terminated with the addition of equal volume of ice cold 20% TCA. The tubes were left on ice for an hour for optimal precipitation of the proteins. The supernatants were isolated following centrifugation in the cold at 10,000 X g for 20 minutes. The Pi present in the supernatants was estimated as described above. The concentration of the Pi released was calculated from the standard graph and the value was converted to its molar concentration (20 μ g/ml is 0.65 mmol/l). The ATPase activity was expressed as μ moles Pi released/hour.

2.28. ESTIMATION OF PROTEINS:

Proteins were estimated following the procedure of Bradford (1976). 100 μ l of the test sample was mixed with 1 ml of Bradford's reagent (10 mg of Coomassie Brilliant Blue G-250 was dissolved in 5 ml of ethanol. To this 10 ml of 85% (w/v) phosphoric acid was added. The resulting solution was made upto 100 ml with DDW and was filtered through Whatman No.1 filter paper). After 5 minutes the color developed was measured at 595 nm. BSA was used as the protein standard.

**CYTOSOLIC PROTEINS THAT REGULATE THE
NUCLEAR ENTRY OF THE GOAT UTERINE
ESTROGEN RECEPTOR.**

The mechanism of nuclear transport of estrogen receptor has been shown to be mediated by a 55 kDa cytosolic protein, p55, which apparently recognizes the nuclear localization signal (NLS) on the ER and facilitates its movement to the nuclear membrane (Nirmala and Thampan, 1995 a & b).

The control of nuclear uptake provides a powerful means to regulate the activity of transcription factors and other proteins with gene regulatory functions. The various strategies for inhibition of nuclear uptake seem to center around the access of nuclear localization signals of proteins to the receptors involved in protein delivery to the nucleus.

3.1 CHROMATOGRAPHY OF GOAT UTERINE CYTOSOL ON ESTROGEN RECEPTOR-SEPHAROSE:

In order to study the factors that regulate the nuclear entry of estrogen receptor, it was assumed that the regulator might bind to the NLS, mask it and make it unavailable for the binding of the transport receptor (p55). Thus, the matrix of the first obvious choice for the identification and isolation of the regulator was estrogen receptor-Sepharose. It was felt that this matrix should prove useful in the isolation of the regulatory factor as it could bind to the NLS on the immobilized ER. For this purpose, the ER purification protocol developed by Zafar and Thampan (1993) was of exceptional advantage as this procedure gave the highest yield of ER among all the available procedures for ER isolation. ER was purified from the goat uterine cytosol and coupled to CNBr activated Sepharose 4B as described in 'methods'.

3.8 ESTRADIOL RELEASES p73 FROM THE HBD OF ER.

In order to test the possibility that p73 will be released from the HBD-Sepharose under the influence of estradiol (E_2), the goat uterine cytosol was chromatographed over ER-HBD Sepharose column. The column was washed extensively with TEMN buffer and elution was achieved using 20 nM estradiol in TEMN buffer. 1 ml fractions were collected and absorbance was measured at 280 nm. The proteins were eluted from the column as a single major peak as soon as the elution was started (Fig. 3.7A). The peak fractions were subjected to SDS-PAGE. Upon silver staining the fractions displayed all three proteins viz p73, p55 and p28 (Fig. 3.7B). The above result does indicate the possibility that p73 was released from the HBD as soon as the estradiol is applied and facilitating the release of p28 from the NLS sequence. Whereas the release of p55 from the NLS is not reflecting the true sense since estradiol will not dissociate the ER-p55 complex in the estradiol labeled ER nuclear transport assay systems otherwise the ER would not been entered into the nucleus (Nirmala and Thampan, 1995).

To demonstrate whether the release of p73 and p28 in the presence of estradiol was specific, the following experiment was conducted. Goat uterine cytosol was chromatographed over HBD-Sepharose column and elution was achieved by using 20 nM progesterone, testosterone and dexamethasone in TEMN buffer. No proteins were found eluted from the column (data not shown)

3.9 p55 AND p28 CROSS REACT WITH MONOCLONAL anti hsp70 AND anti hsp 25 ANTIBODIES.

To test whether p73, p55 and p28 are related to the heat shock family proteins hsp 70 and hsp 25, western blot analysis was performed by using

monoclonal antibodies raised against hsp 70 and hsp 25. Goat uterine cytosol was chromatographed over ER-Sepharose column. The column was washed with TEMN and the proteins bound to the column were eluted using 1M NaCl. The eluate contained p73, p55 and p28. This mixture of proteins was resolved on SDS-PAGE in and transferred subsequently to nitrocellulose membranes in duplicates. One of the blots was exposed to monoclonal anti-hsp 25 antibody (clone NO.IAP-28) and the other to monoclonal anti-hsp-70 antibody (clone BRM-22). The blots were re-exposed to alkaline phosphate coupled antimouse IgG and stained with BCIP/NBT. Both the anti hsp-25 and anti hsp-70 antibody cross-reacted with p55 and p28. No cross reactivity between p73 and the antibodies was detected in any of the blots examined (Fig 3.8).

3.10 IS p28 A PROTEOLYTIC DERIVATIVE OF p55?

Since both p55 and p28 bind to the ER-Sepharose and the ER-NLS sequence and shows cross reactivity with monoclonal antibodies against hsp 25 and hsp 70 it may be argued that the p28 is a proteolytic product of the p55.

p55 was purified following chromatography of the uterine cytosol on a column of actin-Sepharose in a single step procedure as described by Nirmala and Thampan (1995a). If p28 is the degraded product of the p55, that retained its actin-binding domain, one would expect its presence in the fractions eluted from the actin-Sepharose column. To test this the following experiment was carried out

Goat uterine cytosol was chromatographed on a column of actin-Sepharose. The column was washed with TEMN and elution was achieved using an increasing salt gradient (50 mM-1M NaCl). The eluate was collected as 1.5 ml fractions and the absorbance was measured at 280 nm. A single peak of protein was eluted at

0.6 M NaCl (Fig 3.10A). The peak fractions were subjected to SDS-PAGE. Upon silver staining a single band of molecular mass 55 kDa was observed (Fig 3.10B). p28 was totally absent in these gels.

p12 is a nuclear membrane/pore complex associated protein and helps in the recognition of ER-p55 transport complex at the nuclear membrane periphery and subsequently leading the ER to enter the nucleus. Detailed description about p12 and of the experiment is given in section 4.4. It has been observed from these experiments that only p55 was bound to the p12-Sepharose column and not p28. This means that only p55 is involved in the actual transport of ER while the p28 acts as a regulator in the cytoplasm. The results, until the full structure of p28 and p55 is made known, indicate a possibility that p28 may be a proteolysed p55. The p55 may have lost the actin-binding and the p12-binding domains during the process. The net result is that p28 is left incapable of transporting ER to the nucleus. The transport, therefore, will require dissociation of p28 from the NLS site on the ER.

3.11 COMPETITION OF FREE-NLS PEPTIDE WITH ER FOR BINDING TO THE p55: POSSIBILITY THAT p55 IS THE NLSBP:

To test the assumption that NLS of ER is actually involved in the interaction with p55, the following experiment was designed.

The nuclear transport assay mixture containing the ^3H -E₂-ER complexes (2 μg ER), p55 (0.5 μg) and goat uterine nuclei was incubated with increasing concentrations of free NLS-peptide (0 to 6 μg) at 30°C for 30 min. The maximal inhibition of the ER nuclear transport was seen with 2.5 μg of the free peptide (Fig

3.11A). The NT assay was repeated using a system that contained 2.5 µg of free NLS-peptide, a 10-fold excess of ER and nuclei in the presence or absence of p55. A control set without the peptide was also incubated under the same conditions. There was no transport of ER into the nucleus noted in the presence of a 5-fold excess of free NLS-peptide. This inhibition was not overcome by the addition either of excess ER or of nuclei but only by the addition of excess p55 (Fig 3.11B). The free NLS peptide bound to the p55 and made it unavailable for the transport of ER into the nucleus. This also suggested that the site on the p55, which recognized the ER, is the NLS.

3.12 FLUORESCENCE ASSAY OF THE NUCLEAR TRANSPORT OF THE ER MEDIATED BY p55, p73 and p28:

The nuclear transport assay involving the use of ³H-estradiol fails to distinguish between binding of ER to the nuclear membrane and the full transport of ER into the nucleus. To confirm the actual transport of ER into the nucleus, the fluorescence assay for the ER transport was performed as described in the 'methods'.

Goat uterine ER was purified and conjugated with FITC following the procedure of Chard et al., (1987) Goat uterine nuclei (with intact membranes) were incubated with FITC-ER either in the presence or absence of p55. The transport of the ER into the nucleus was found to be absolutely dependent on the presence of the p55 in the assay medium (Fig 3.12C) No transport was observed in the absence of p55 (3 12 B)

Goat uterine nuclei were incubated with FITC-ER either in the presence or absence of the p55, p28 and p73. In the presence of saturating amounts of p28, p55 remained incapable of transporting ER into the nucleus (Fig 3.12D). Addition of the p73 to the incubation medium reversed this inhibition and facilitated the ER transport (Fig 3.12 E).

DISCUSSION

A mechanism of regulated entry of estrogen receptor under the influence of estradiol from the uterine cytoplasm to the nucleus has been identified. The nuclear transport of ER has been found to be mediated by a cytosolic 55 kDa protein. p55 recognizes the NLS on the ER, binds to it and transports it to the nucleus. Within the cytosol, a 28 kDa protein, the p28 antagonizes the ER-p55 interaction, apparently through the masking of the NLS on the ER. p28 binds to the NLS of the ER, masks it and make it less available for binding by the p55. ER remaining in a complex with p28 may be an internal regulatory mechanism that necessitates an estrogen-dependent, controlled entry of the ER into the nucleus. The p55-mediated ER transport, under these conditions, can take place only in the presence of p73.

It is clear from the early observations that the fractions collected from the ER-Sepharose column, which contains both p55 and p28, are not able to transport the ER into the nucleus. On the other hand a peak of activity was observed in the fractions where p73 was specifically eluted in addition to p55 and p28. Therefore, it is apparent that the presence of p73 is very much necessary for the reversal of the inhibitory effect of p28. The p73 was observed to be associated with the hormone-binding domain of the ER through chromatography on HBD peptide-

Sepharose. The elution pattern was the same as that observed with the ER-Sepharose column. The peptide sequence contained two lysine residues in it which corresponded to the p-NLS1 in association with the HBD. The presence of two lysine residues might determine the specificity for the binding of p55 and p28 to the peptide-Sepharose. In the SV 40 large T-antigen NLS (pro.lys.lys(128).lys.arg.lys.Val.gly) a point mutation resulting in the substitution of threonine for lysine at the codon 128 position abolishes its ability to localize to the nucleus (Kalderon et al., 1984). Therefore, it may be argued that the position and presence of lysine residues are very important for the NLS function. From the HBD peptide-Sepharose column alone, it was possible to elute all the three proteins which were obtained earlier following the use of the estrogen receptor-Sepharose column. From these results, it is imperative that p73 binds to the HBD and brings about the nuclear transport of estrogen receptor in a regulated mechanism. Since estradiol is effective in releasing p73 and p28 from the HBD-Sepharose column it is possible to hypothesize that under hormonal control the masking of NLS by p28 will be released and make the site free for p55 binding. The model presented (Fig. 3.13) serves to explain the hypothesis. The presence of p55 in the estradiol eluted fraction derived from the ER-HBD-peptide Sepharose column could be due to the non-specific association that this protein might have with either p28 or p73.

The transcription factor NF- κ B is found to be associated with the cytoplasmic retention factor I- κ B in unstimulated B-lymphocytes (Baeuerle & Baltimore, 1988). Phosphorylation disrupts the I κ B-NF- κ B complex and NF- κ B enters the nucleus. Glucocorticoid receptor is cytoplasmic in location in the absence of the hormone and also while remaining in complex with hsp 90 hsp 90 masks the NLS sequence in the receptor. Hormonal stimulation disrupts the

complex and NLS becomes exposed. Subsequently the receptor enters the nucleus (Sanchez et al., 1985). Yeast transcription factor SW15, during S, G2 and M phase of the cell cycle, is cytoplasmic in cellular location. However it enters the nucleus during the G1 phase. The nuclear translocation of SW15 remains is inversely correlated with the cell cycle-dependent activity of the CDC-28 kinase. When all the three serine residues in the proximity of the NLS are phosphorylated by the CDC28 kinase, SW15 remains cytoplasmic in nature. When the serine residues are dephosphorylated, SW15 achieves nuclear entry (Moll et al., 1991).

What appears significant is that no single common system can take care of the various nuclear transport processes involving nuclear proteins. May be that each system is individualistic. The p73-p28-p55 mechanism underscores this possibility and reflects a physiological necessity (eg. presence and absence of the hormone).

The p55 binds to the ER, the NLS sequence in the ER, actin and the p12 (nuclear Membrane /pore complex protein). p28 binds to the ER and the ER-NLS sequence, but not to the p12 or actin. It maybe argued that the p28 is a proteolytic product of p55. Under these circumstances one would anticipate the detection of the other p28 fragment that retains the actin or the p12 binding domain. It has not been possible to identify this fragment following chromatographic analysis of unfractionated cytosol over actin-Sepharose or p12-Sepharose columns. This would indicate one of the two alternatives: 1) The p28 is an a independent protein and not a fragment of the p55.

2) the non-NLS binding region of the p55 has undergone extensive degradation so that the protein fragment bearing the actin/p12 binding domain

does not appear as a specific entity. Under any of these conditions, p28 would provide as a powerful regulatory factor for the nuclear entry of ER under hormonal control. The very observation that p55, but not p28 interacts with actin is a clear indication to the possibility that actin has a functional role in the nuclear transport of the ER.

Both p55 and p28 were shown to have immunological cross reactivity with monoclonal antibodies against hsp 25 and hsp 70 showing that both p55 and p28 might have some common antigenic motif in their primary structure. The amino acid homology may suggest an evolutionary conservation of domains critical to the function of the protein (recognition of nuclear localization sequence). The cross reactivity with the monoclonal antibodies is again indicative of the high sequence homology of the two proteins with both hsp 25 and hsp 70, giving a clue to their function as molecular chaperones. Faucher et al. (1993) have reported the immunological identity between the 28 kDa protein of human mammary adenocarcinoma MCF-7 cells and the estrogen-regulated "24-kDa" protein. Partial amino acid sequencing of the 28-kDa protein revealed identity with both the 24-kDa protein and the mammalian hsp 27. Unless the amino acid sequence is made known, one will not be in a position to confirm the potential similarity that p28 may have with the molecules mentioned above.

The competition between the free NLS peptide and the ER for binding to the p55 and also the observation that the peptide was capable of inhibiting the p55 mediated nuclear transport of ER indicated that the p55 is the nuclear localization signal binding protein of ER.

Figure 3.1. Chromatography of goat uterine cytosol on estrogen receptor-Sepharose.

(A) Goat uterine cytosol was chromatographed on an estrogen receptor-Sepharose column (10 ml). The column was washed with TEMN buffer and eluted with a linear NaCl gradient (50 mM to 1M). Fractions (2 ml) collected were subjected to the nuclear transport assay. The ^3H -E₂-ER complexes transported to the nucleus were measured. The NaCl concentration in the fractions was also estimated (broken line).

(B) The protein in fractions from number 4 to 16 was precipitated with TCA and was subjected to SDS-PAGE in 10% gels. The lane numbers depicted are the corresponding fraction numbers from Figure 1A.

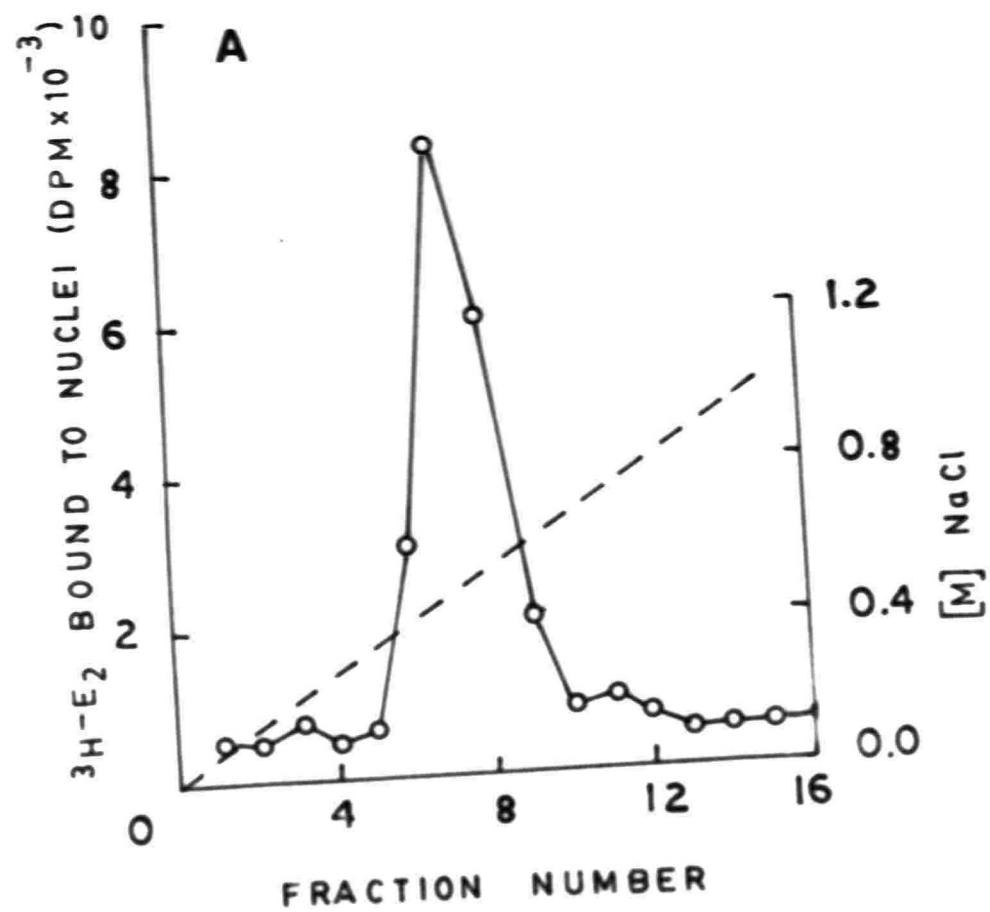


Figure 3.2. (A) Chromatographic Separ

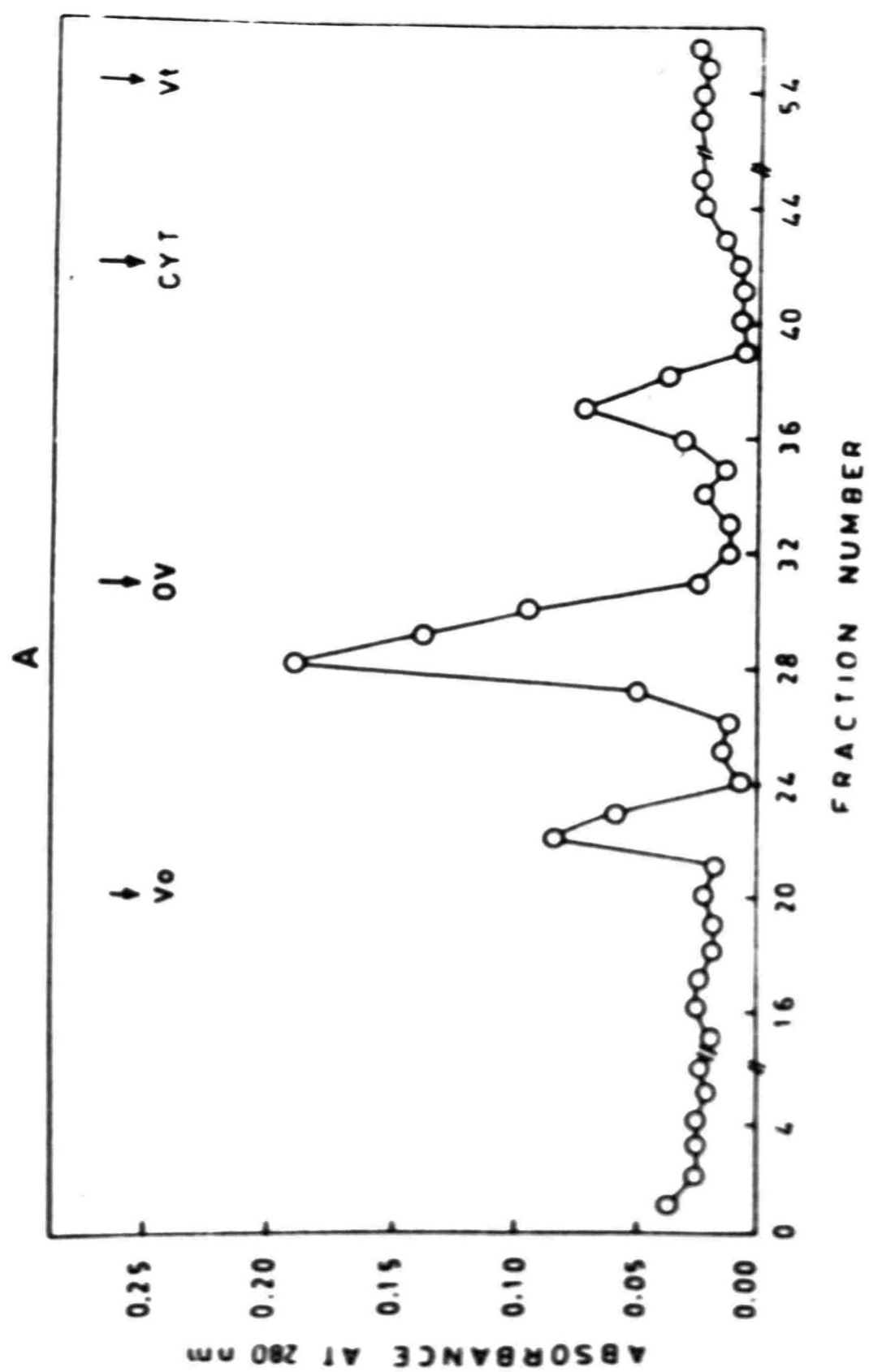


Figure 3.3A. The nuclear transport of ER

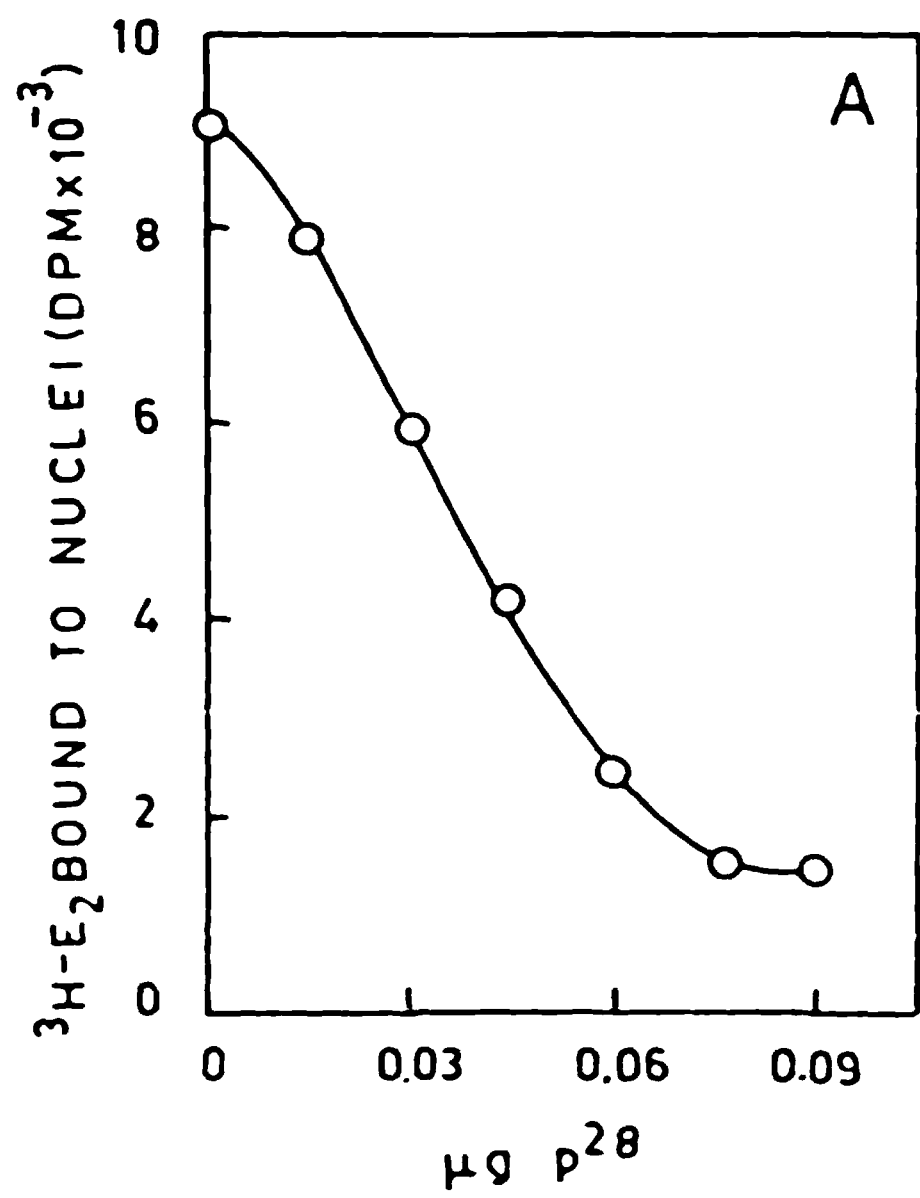
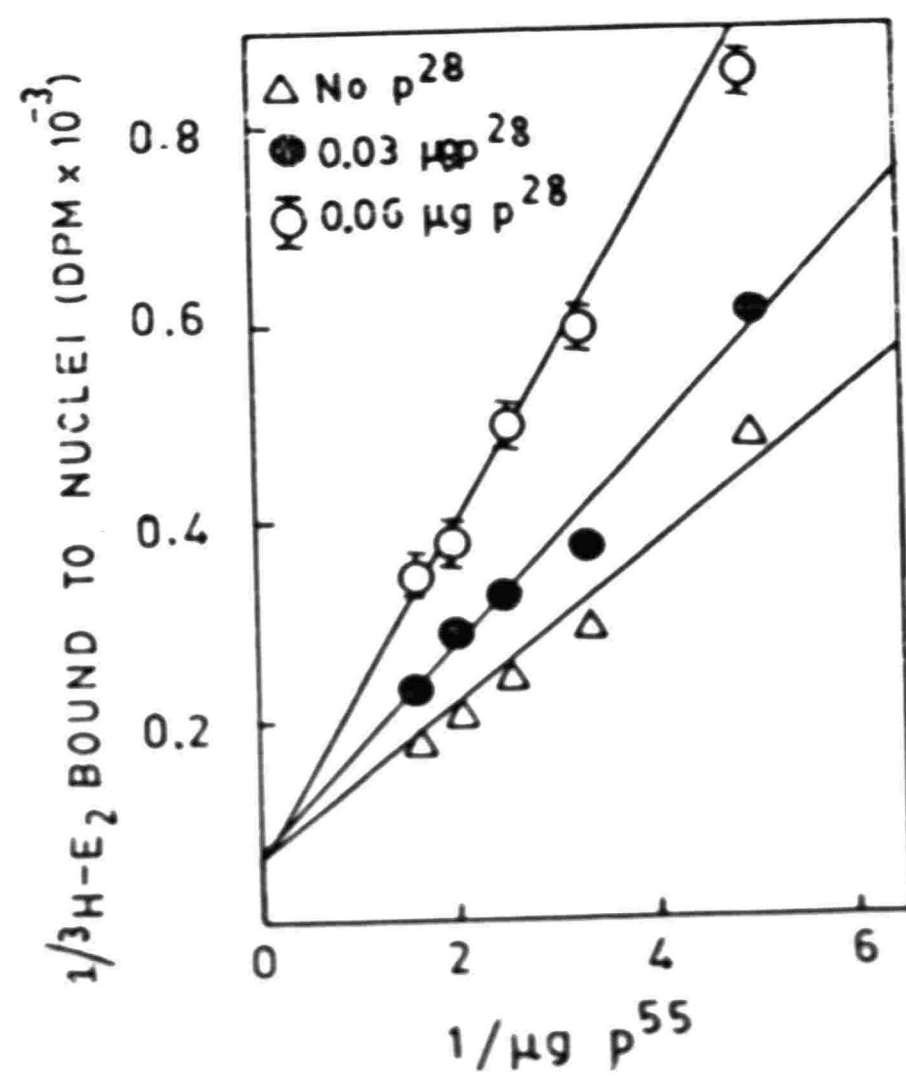


Figure 3



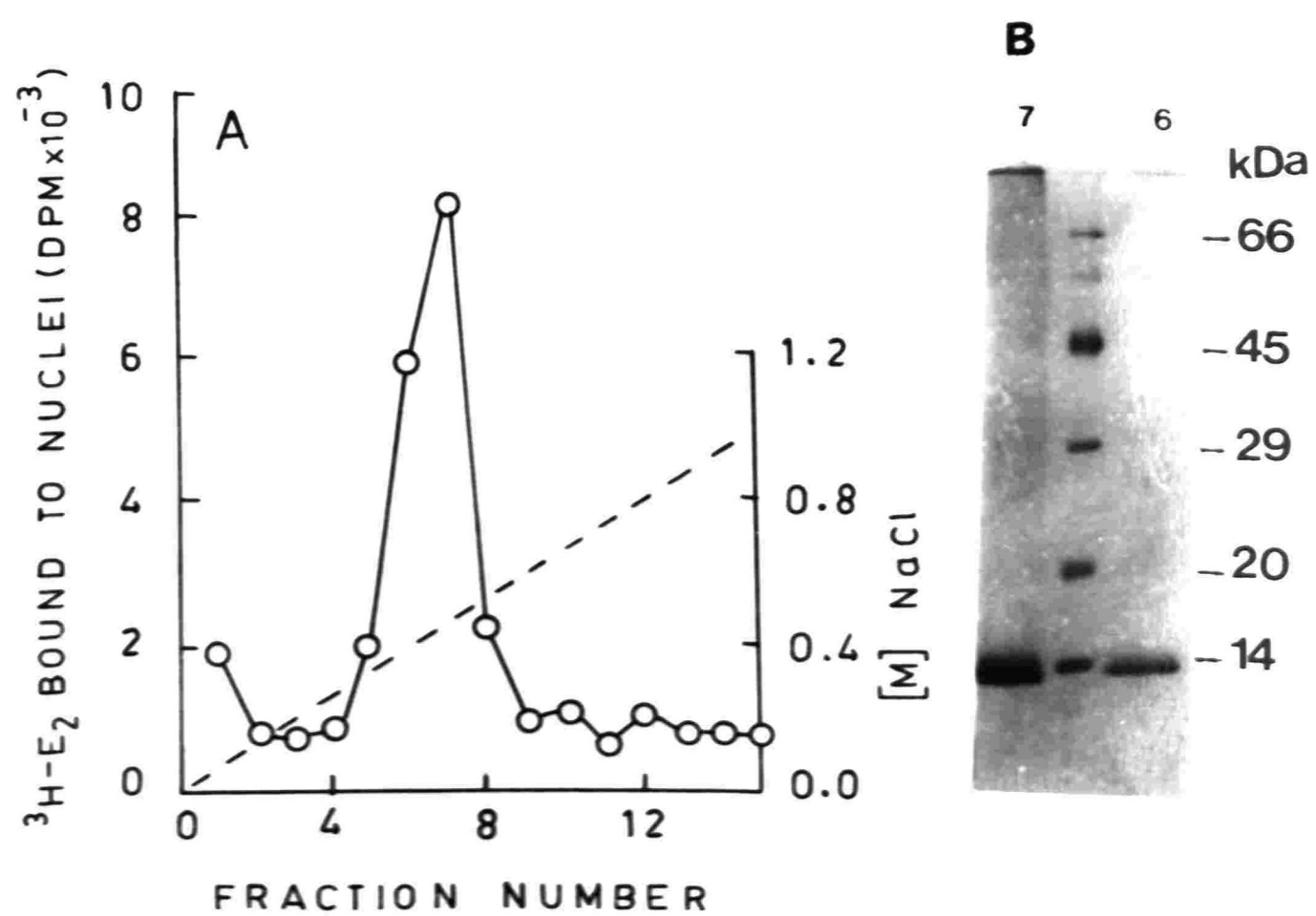
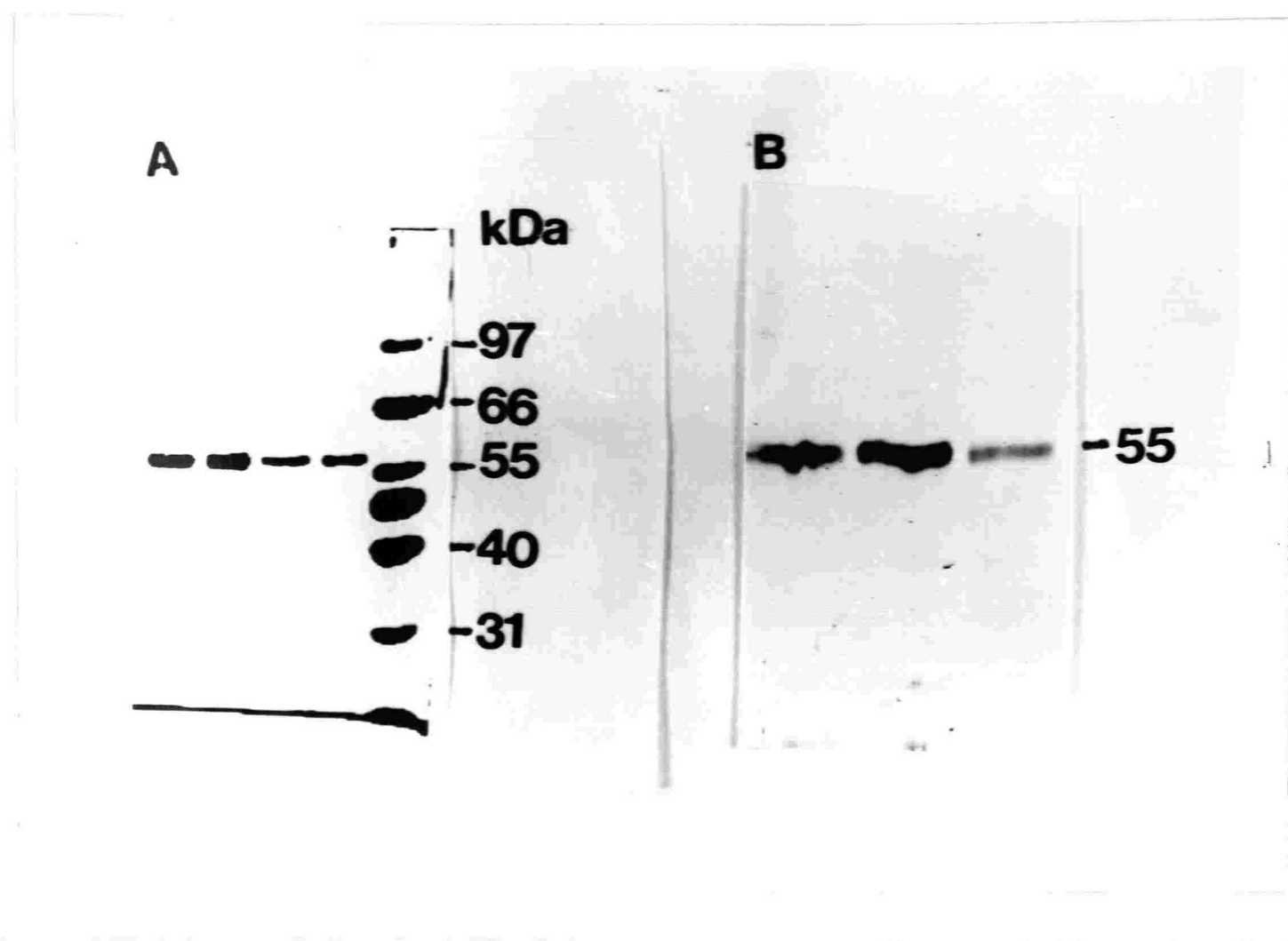


Figure 4.7. Interdependence of p55 and p12 for the nuclear transport of ER from cytoplasm to nucleus.

(A) 0 to 1.0 μg of p55 was incubated with $^3\text{H-E}_2\text{-ER}$ complexes and intact nuclei at 30°C for 30 minutes and the assay was performed as described in methods (●). The other set of incubation (control) was carried out using detergent treated nuclei under identical conditions as mentioned above (○). The $^3\text{H-E}_2\text{-ER}$ transported into the nuclei was extracted with ethanol and the radioactivity associated with ethanol was measured. The nuclear transport activity was expressed as $^3\text{H-estradiol}$ bound to nuclei.

(B) 0 to 0.5 μg of p12 was incubated with $^3\text{H-E}_2\text{-ER}$ complexes, 0.5 μg of p55 and detergent treated nuclei (without membrane) at 30°C for 30 min (●) and the assay was performed as described previously. Control set was incubated without p55 in the assay medium (○).

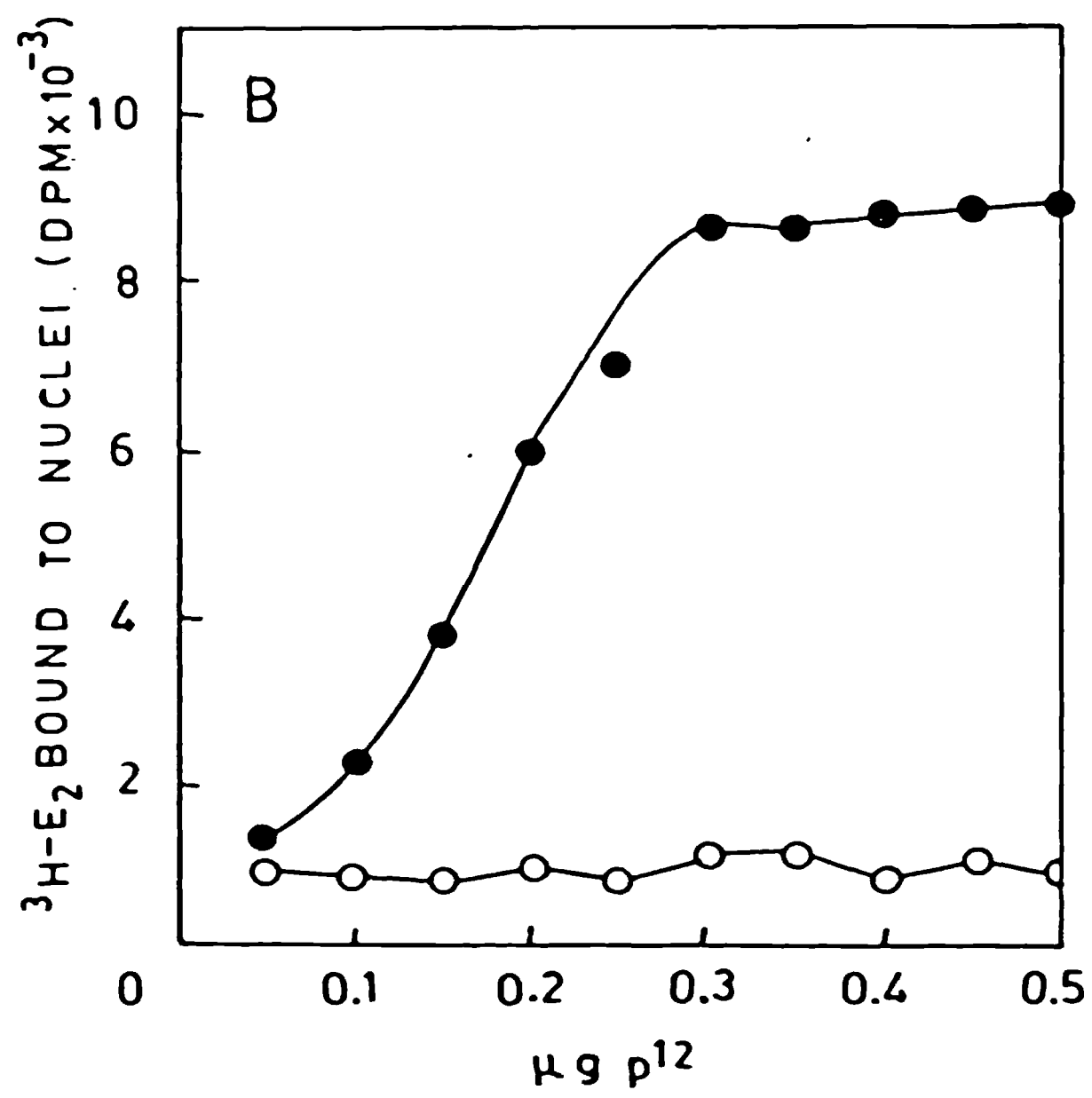
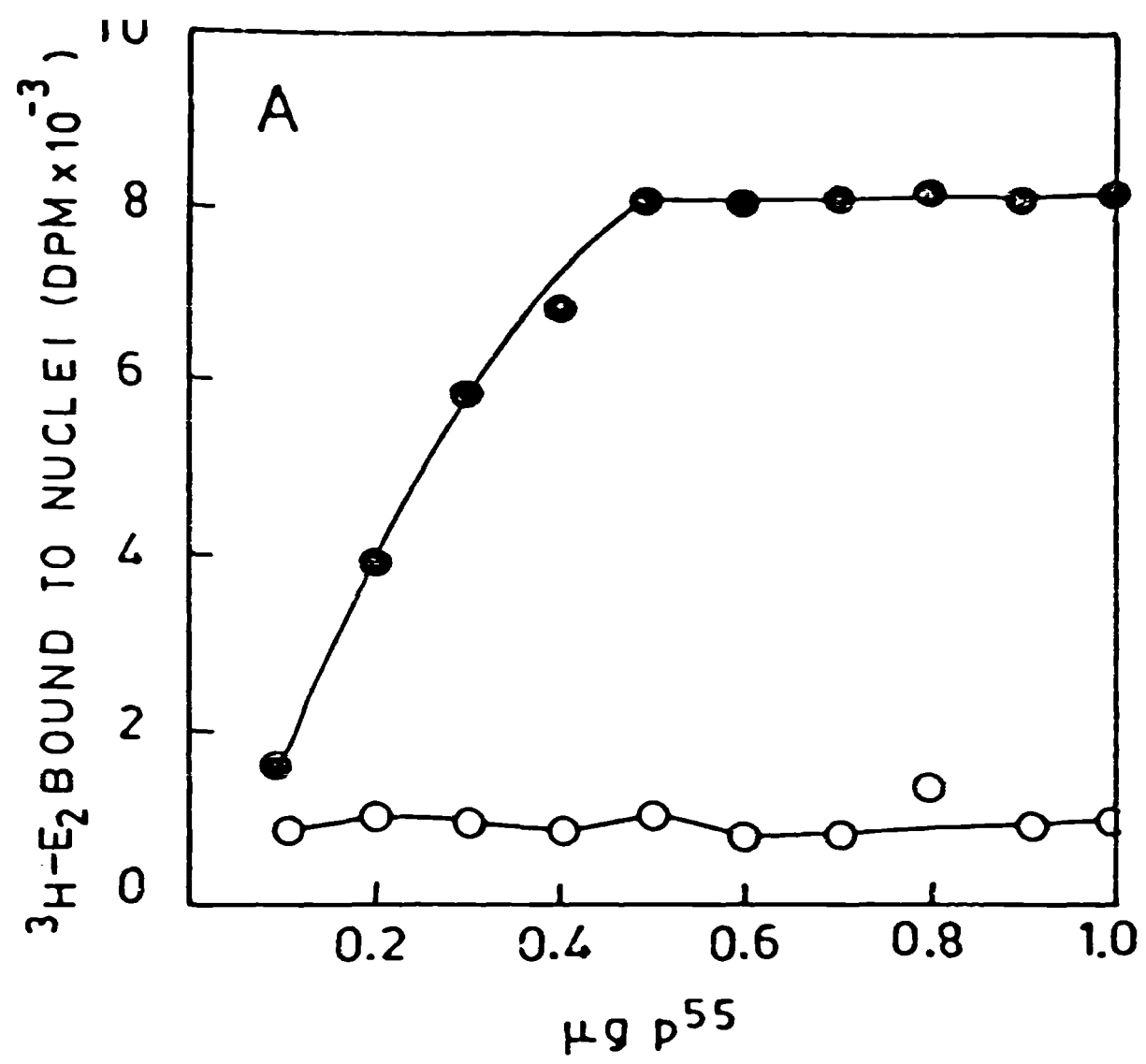


Figure 4.8. Time dependence of the transport of ER into the nucleus.

(A) Detergent treated nuclei (without membranes) were incubated with $^3\text{H-E}_2$ -ER complexes, 0.5 μg p55 and 0.3 μg of p12 at 30°C for different time intervals (●). The control incubation was performed without adding p12 to the incubation medium (○). The assay was performed as described in the methods. The incubated nuclei were extracted with ethanol and the nuclear transport activity was expressed as $^3\text{H-estradiol}$ bound to nuclei.

(B) Control nuclei (with membranes) were incubated with $^3\text{H-E}_2$ -ER complexes, 0.5 μg of p55 either in presence or absence of additional p12 at 30°C for different time intervals. The $^3\text{H-E}_2$ -ER transported into the nuclei were measured as described before. The activity was expressed as $^3\text{H-estradiol}$ bound to the nuclei. (●) without added p12; (○) with exogenously added p12.

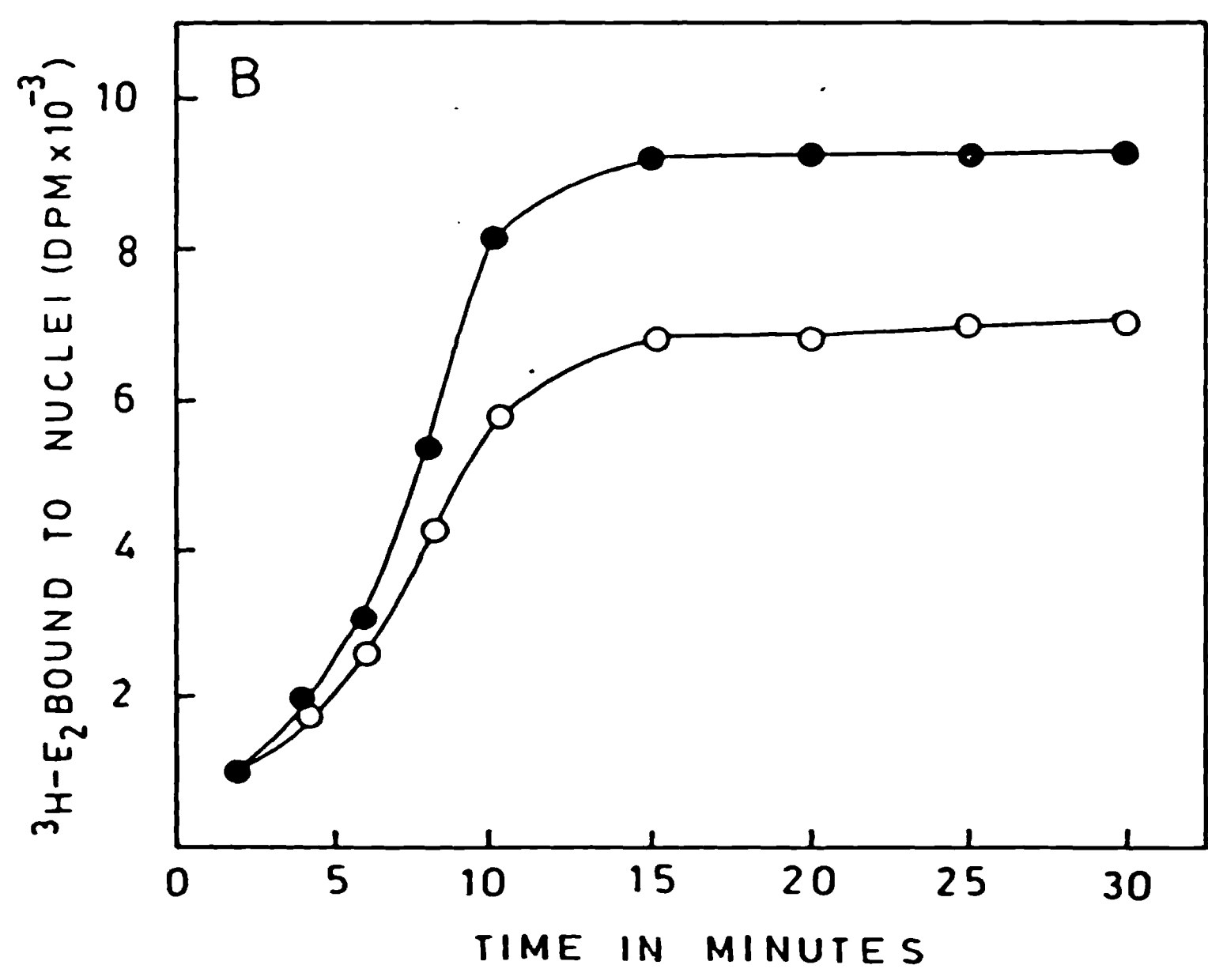
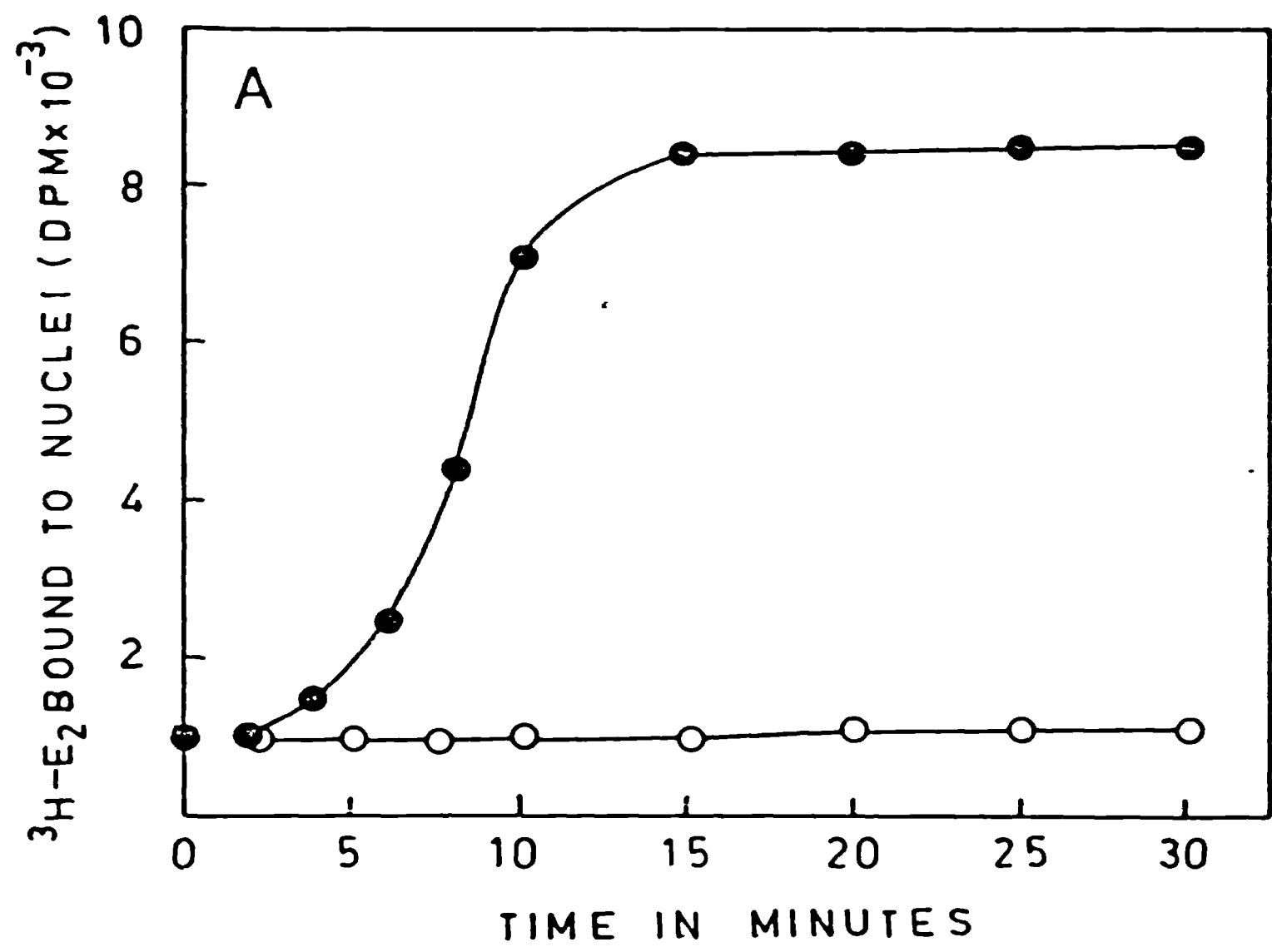


Figure 4.9. ATP-dependence of the transport of ER into the nuclei.

(A) Detergent treated nuclei were incubated with $^3\text{H-E}_2\text{-ER}$ complexes, 0.5 μg of p55 and 0.3 μg of p12 in the presence of 0 to 8 mM ATP. The incubation was carried out at 30°C for 30 minutes. The $^3\text{H-E}_2\text{-ER}$ transported into the nuclei were measured and the activity was expressed as $^3\text{H-estradiol}$ bound to the nuclei.

(B) Control nuclei (not detergent-treated) were incubated with $^3\text{H-E}_2\text{-ER}$ complexes and 0.5 μg of p55 in the presence of 0 to 8 mM ATP. The assay was performed as described above.

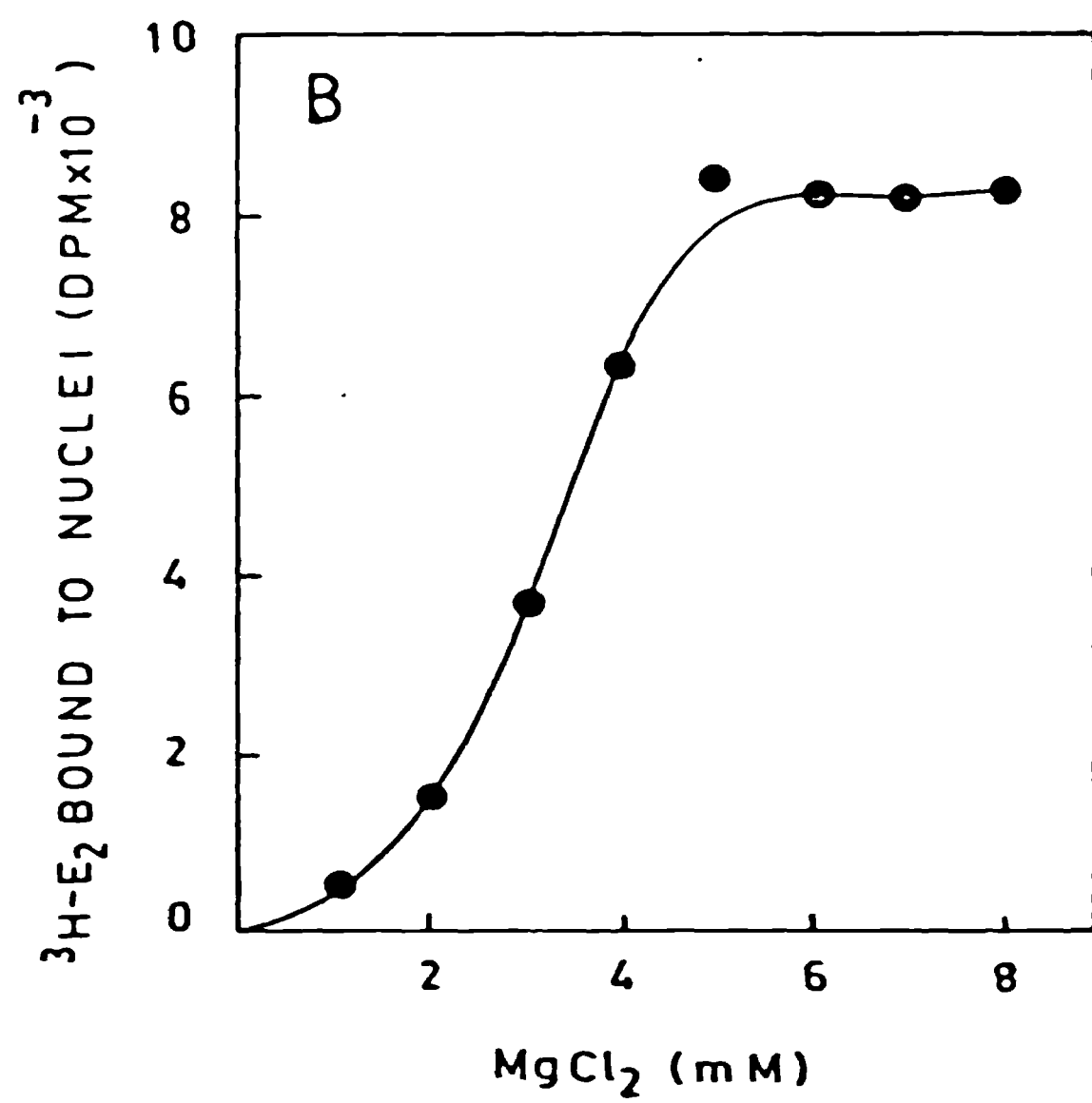
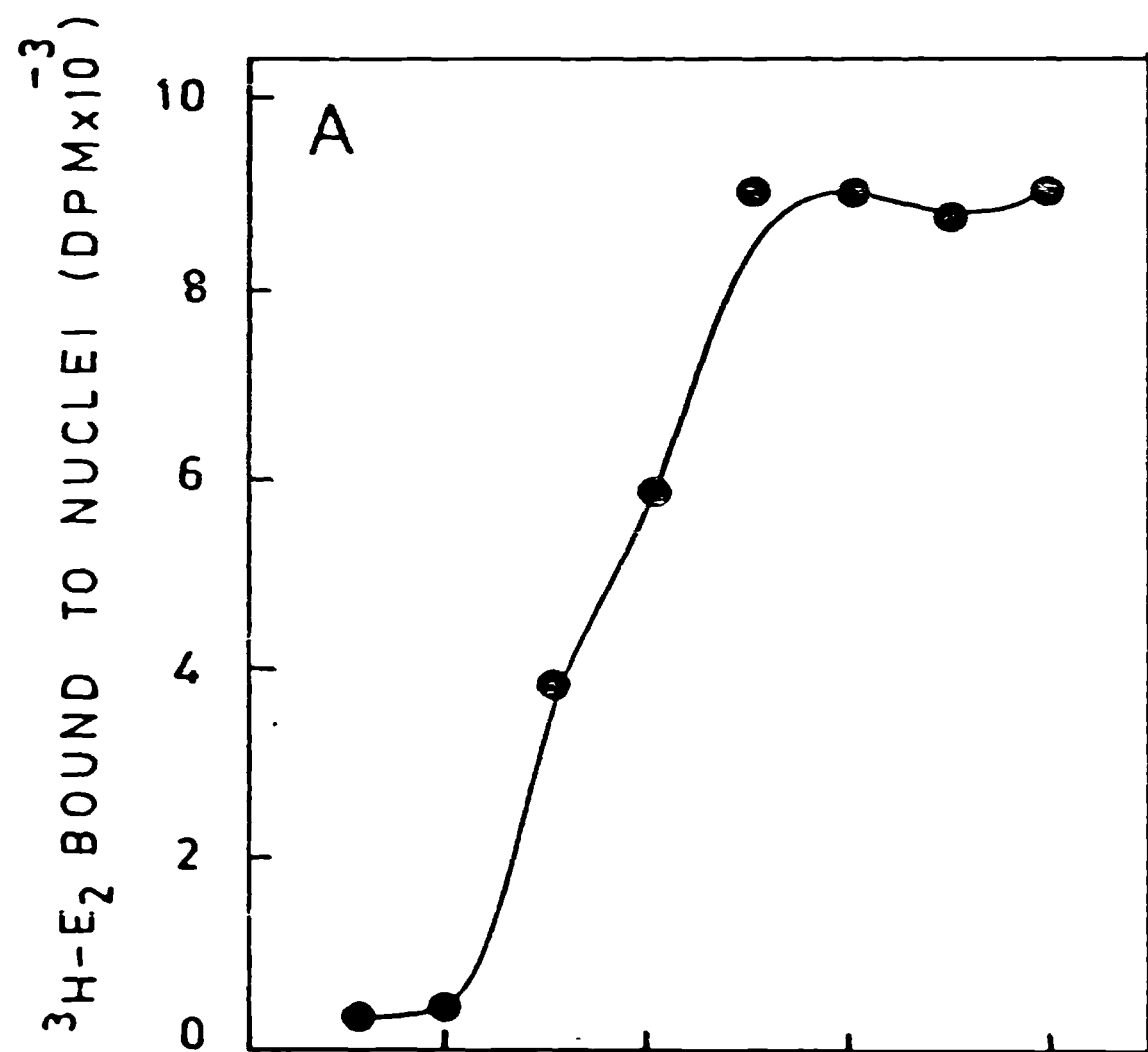


Figure 4.11. ATPase activity of p55 and p12.

Both p55 and p12 (0 to 2.0 μ g) were incubated independently with 6 mM ATP at 30⁰C for 30 minutes as described in methods. The incubation was terminated by the addition of trichloro acetic acid (TCA). The inorganic phosphate (Pi) released into the TCA soluble supernatant was estimated as described in the 'methods'. The Pi released is expressed here as μ moles Pi released /hr.

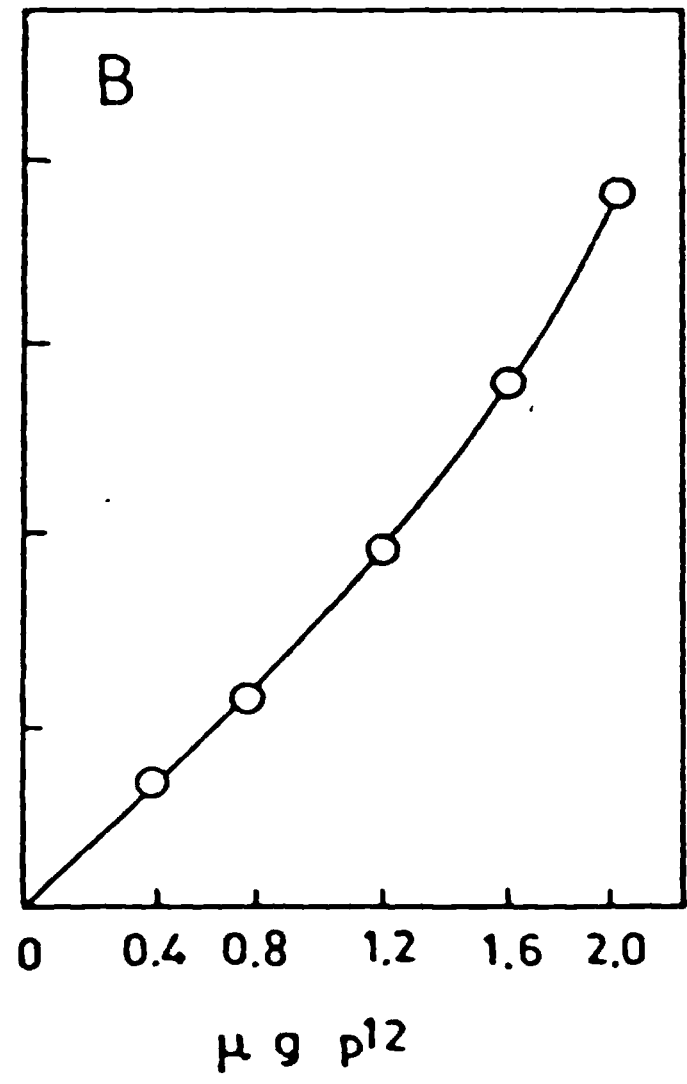
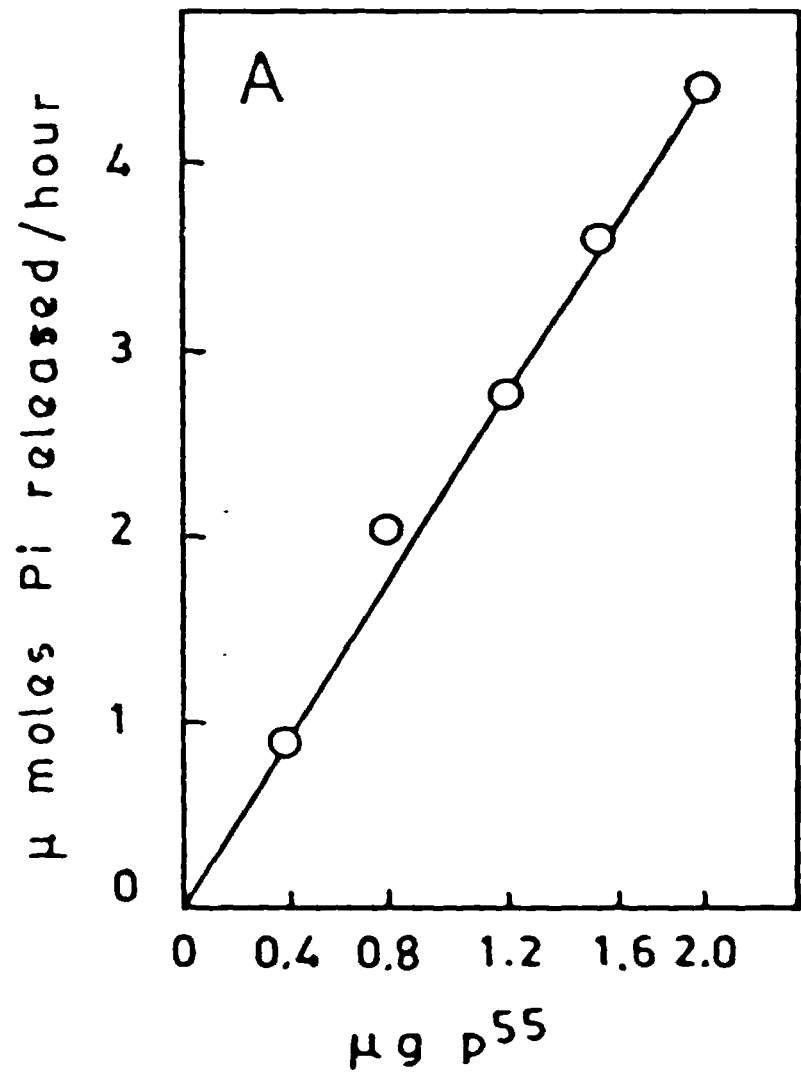


Figure 4.12. Enhanced ATPase activity introduced by interaction between p55 and p12.

A mixture containing equal protein concentrations (1:1) of p55 and p12 was incubated with 6 mM ATP at 30⁰C for 30 min. In another set only p12 was incubated in the same protein concentration range, with 6 mM ATP at 30⁰C for 30 minutes. The incubation was terminated by the addition of TCA. The Pi released into the TCA soluble supernatant was estimated and is expressed as μ moles/Hour.

- (●) 1:1 mixture of p55 and p12
- (O) p12 alone.

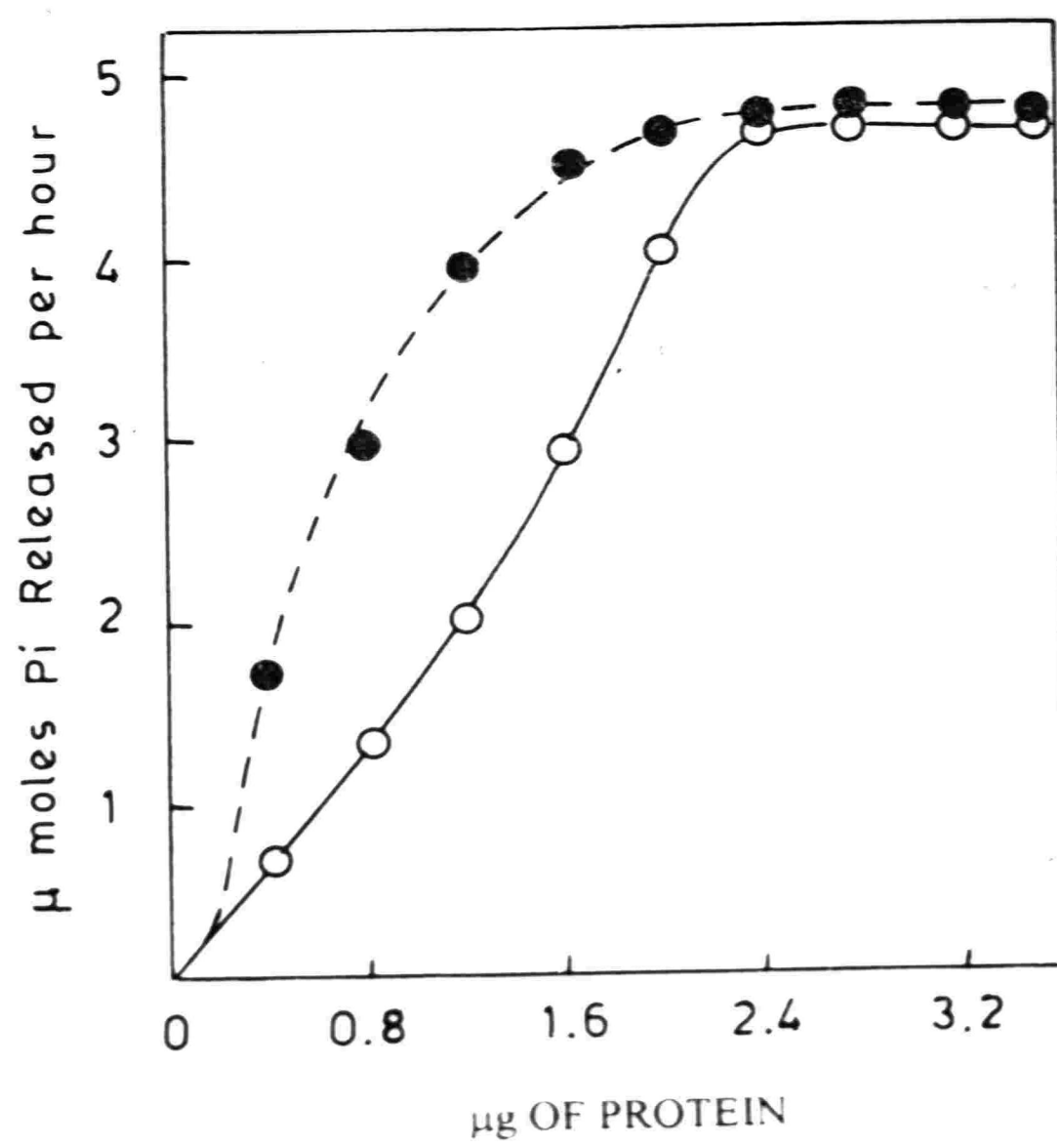


Figure 4.13. p55 and p12 are magnesium and calcium dependent ATPases.

p55 (2 μ g) and p12 (2 μ g) were incubated independently in the presence of varying molar concentrations of (A) MgCl_2 or (B) CaCl_2 at 30°C for 30 minutes. The incubation was terminated by the addition of TCA. The P_i released into the TCA soluble supernatant was estimated as described in the methods. The P_i released is expressed here as $\mu\text{moles Pi released/ hour}$.

(●) p55; (○) p12.

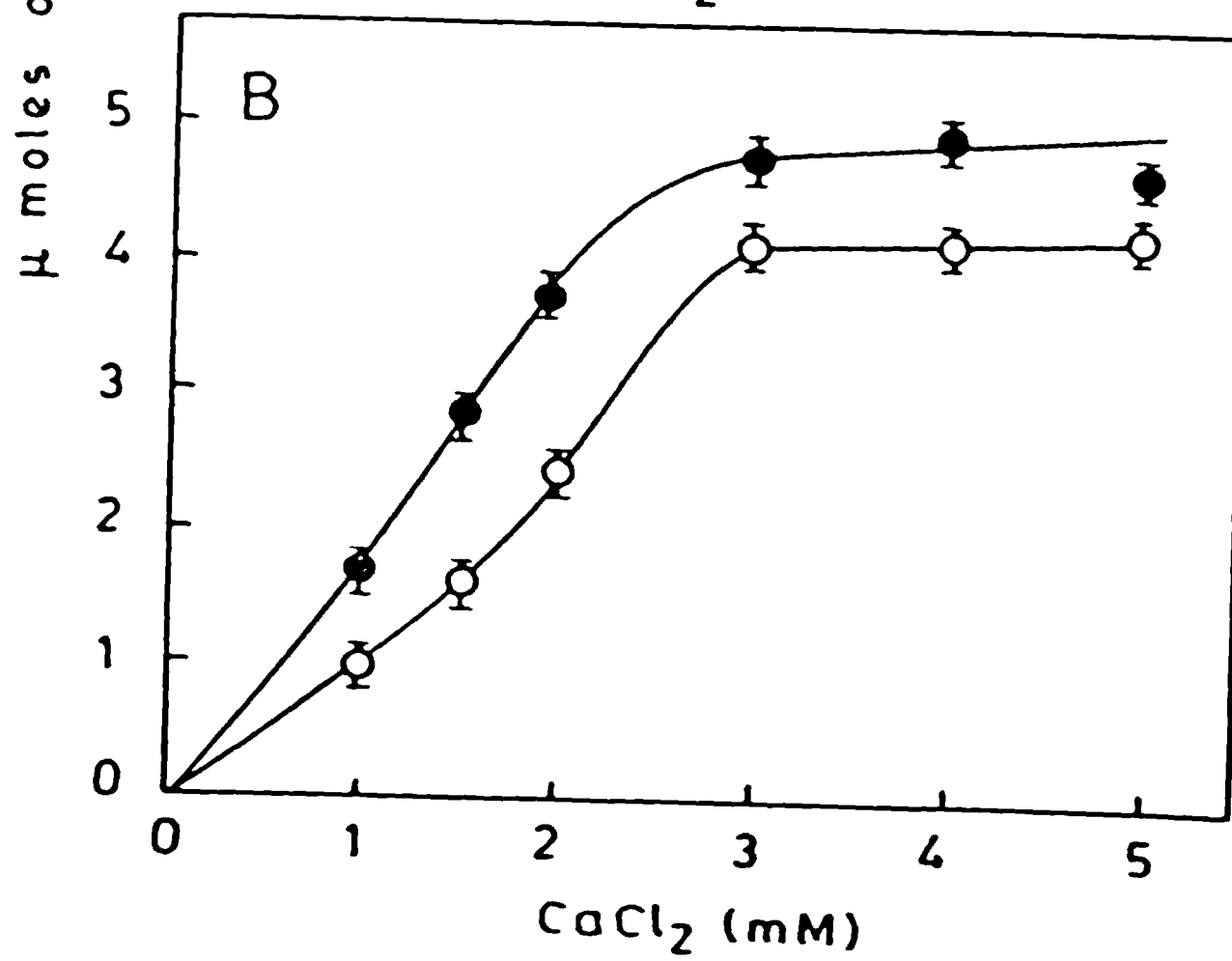
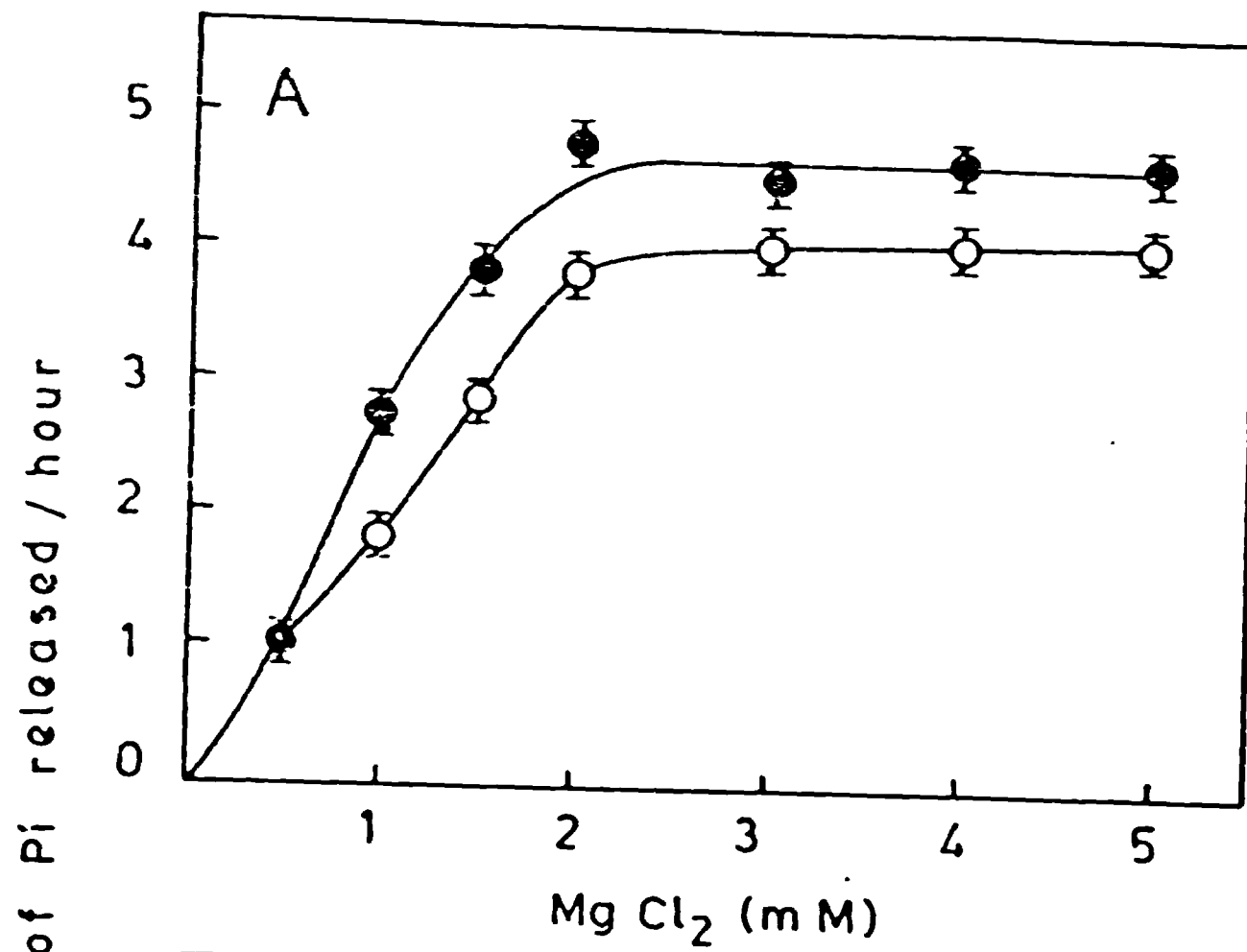


Figure 4.14 Quercetin inhibits the nuclear transport of ER.

(A) Detergent treated nuclei (without membranes) were incubated with 0.5 μg p55, 0.3 μg p12 and ^3H -E₂-ER complexes in the presence of varying concentrations (0 to 20 $\mu\text{g}/\text{ml}$) of quercetin at 30⁰C for 30 minutes. The assay was performed as described in the methods. The ^3H -E₂-ER transported into the nucleus was measured. The activity was expressed as ^3H -estradiol bound to nuclei.

(B) Control nuclei (not detergent treated) were incubated with 0.5 μg p55, ^3H -E₂-ER complexes and 0 to 20 $\mu\text{g}/\text{ml}$ quercetin. The incubation was carried out at 30⁰C for 30 min. The ^3H -E₂-ER transported into the nucleus was measured.

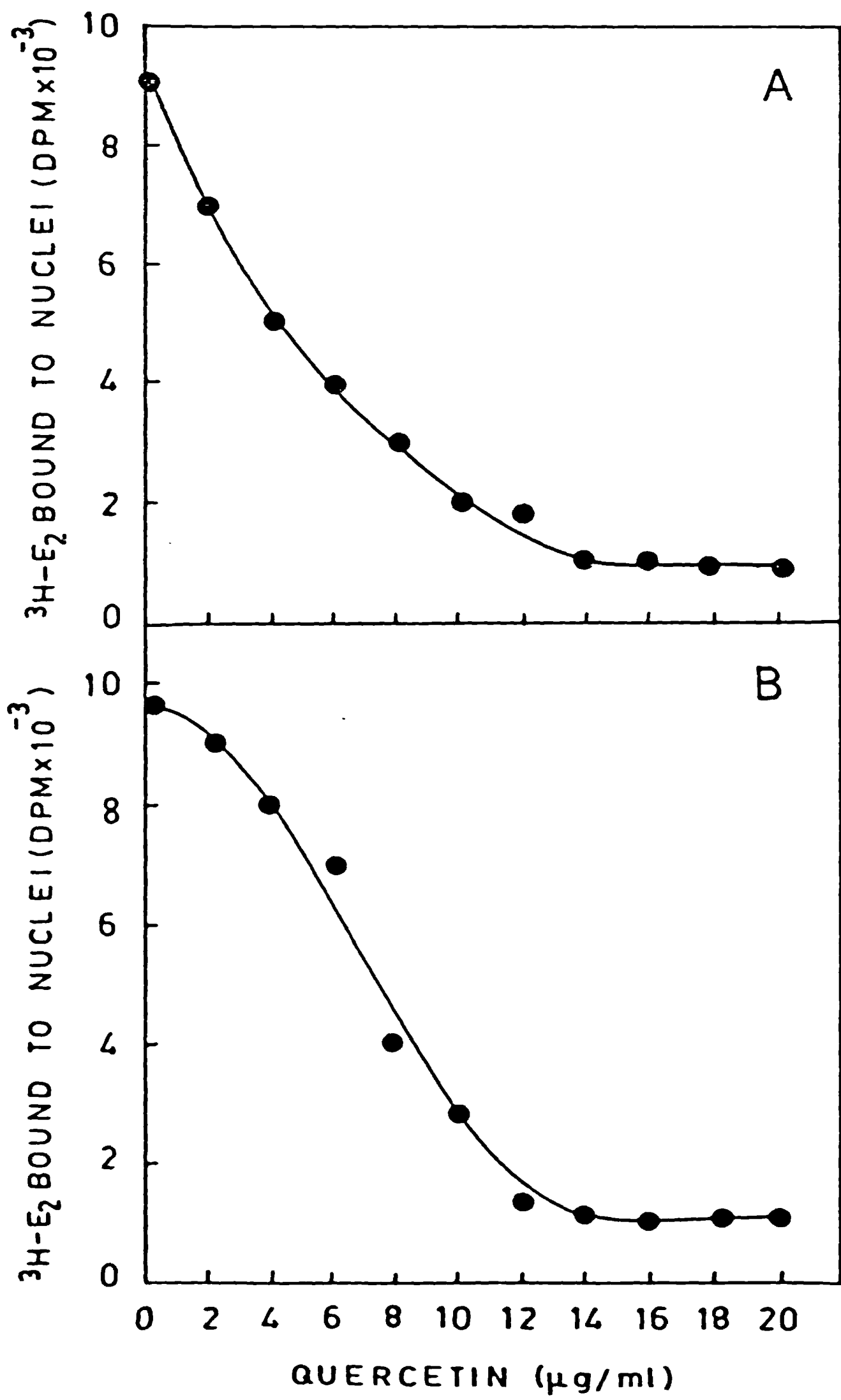


Figure 4.15 Fluorescence assay for the ER nuclear transport.

Goat uterine nuclei, treated with Triton X-100, were suspended in the nuclear transport assay buffer containing 4 mM ATP and were incubated with FITC-ER (5 μ g) at 30⁰C

- (A) Detergent treated nuclei incubated in the absence of p55 (100X).
- (B) Detergent treated nuclei incubated with 0.5 μ g of p55, in the absence of p12 (100X).
- (C) Phase contrast micrograph of B (100X).
- (D) Nucleus incubated with 0.5 μ g p55 and 0.5 μ g of p12 (100X).
- (F) Goat uterine nuclei (with membranes) were suspended in the assay buffer without ATP and were incubated with FITC-ER (5 μ g) and p55 (0.5 μ g) at 30⁰C for 30 min. (100X).
- (E) Phase contrast micrograph of the nucleus presented in (F).
- (G) To the above incubation mixture ATP was added to a final concentration of 4 mM and the material was examined under the fluorescence microscope after 5 minutes (100X).
- (H) Phase contrast photograph of the nucleus presented in (I).
- (I) To a similar incubation medium as described in (F), ATP (4mM) and quercetin (10 μ g/ml) were added simultaneously to the medium and visualized after 30 minutes (100X).

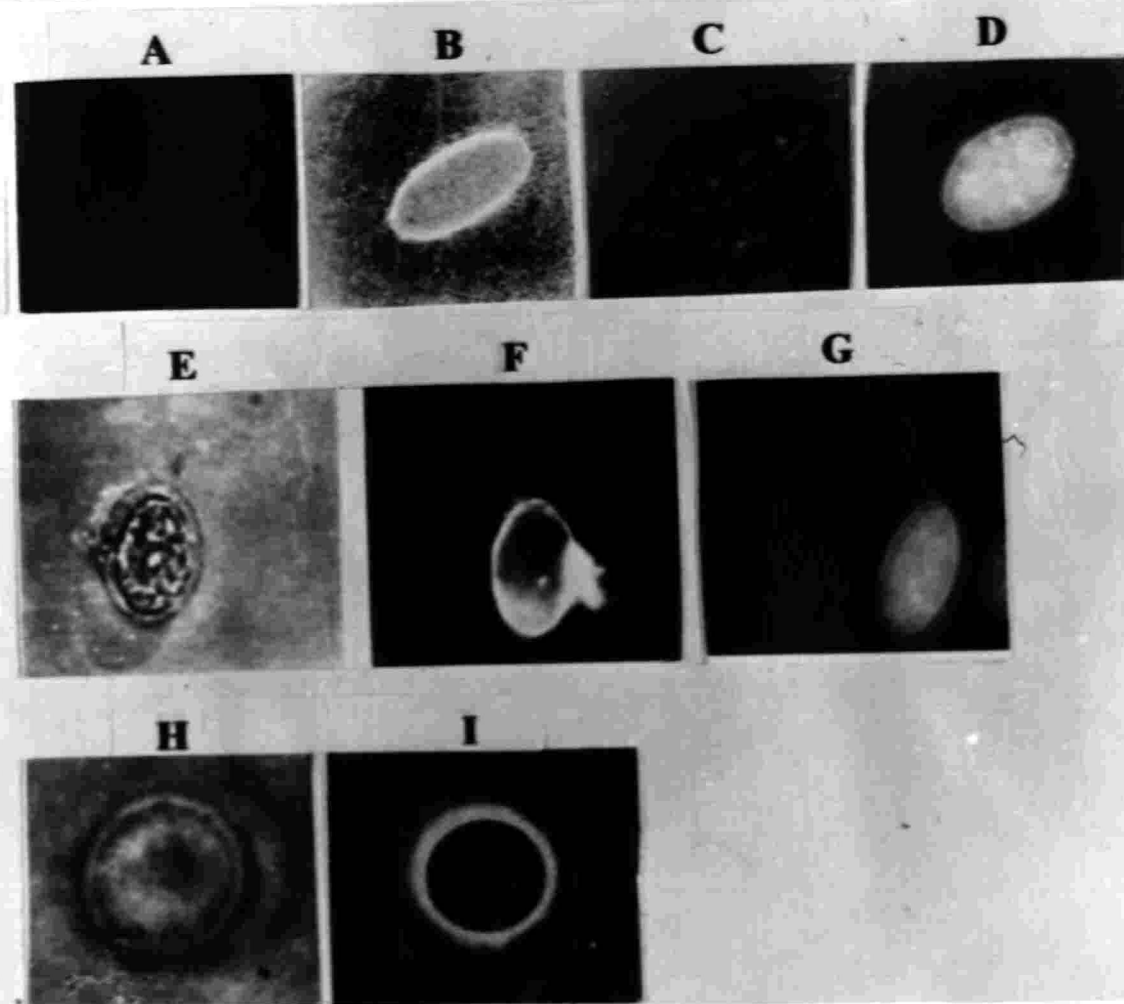


Figure 4.16 Fluorescence assay of ER transport with FITC-p55 and FITC-p12.

Goat uterine nuclei (with membranes) were incubated with 0.5 μg of FITC-p55 in the presence of unlabelled ER and 4 mM ATP. The incubation was carried out at 30°C for 30 minutes.

(A) phase contrast micrograph of the nuclei (100X).

(B) Fluorescence micrograph of the same (100X).

Goat uterine nuclei were treated with Triton X-100 and incubated with unlabelled ER (5 μg), unlabelled p55 (1 μg) and about 0.5 μg of FITC-p12. The incubation was carried at 30°C for 30 minutes.

(C & E) phase contrast micrographs of the nuclei (100X).

(D & F) fluorescence micrographs of C & E respectively (100X).

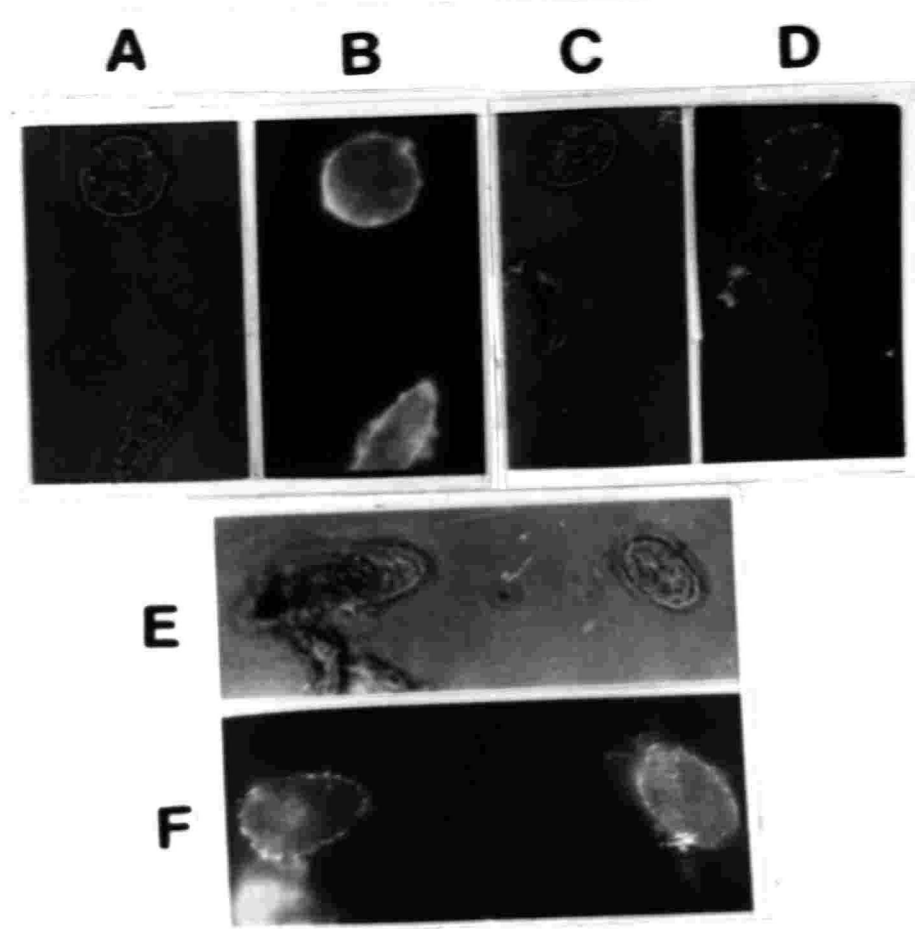


Figure 3.13. A model explaining the protein-protein interactions that precede the nuclear migration of the estrogen receptor. The proteins involved are estrogen receptor (ER), p28, p55 and p73. The hormone binding domain (hbd) and the nuclear localization signal (NLS) on the estrogen receptor are indicated.

(A) In the absence of estradiol (E_2) bound to the ER, p28 remains bound to the NLS and the p73 to the hbd of the ER. The p28 bound to the NLS prevents the association between p55 and the NLS.

(B) Estradiol binding to the ER causes dissociation of p73 from the ER-hbd. p73 binds to p28 which results in the dissociation of p28 from the NLS.

(C) p55 occupies the NLS that has been vacated by p28.

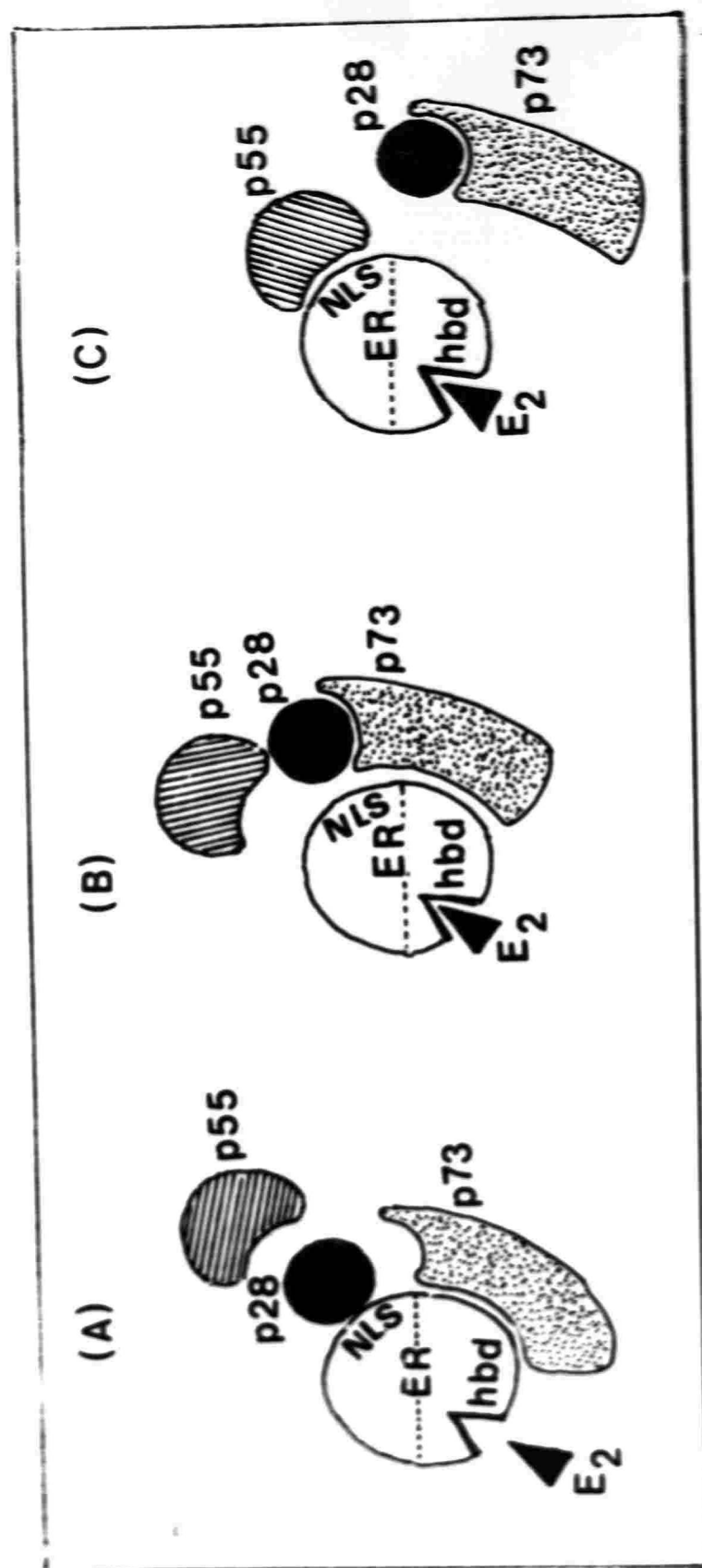
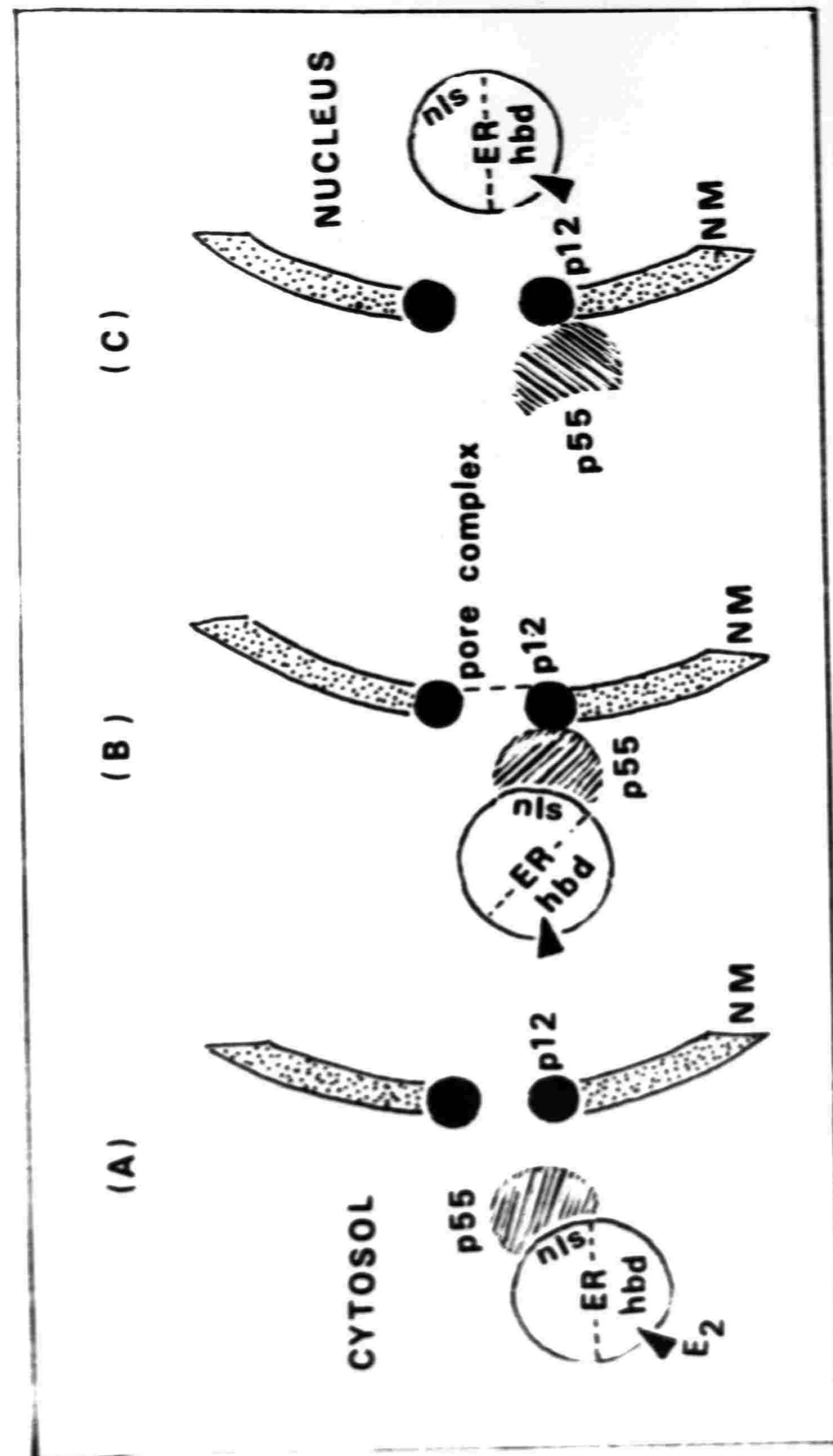


Figure 4.17. A model that attempts to explain the regulatory mechanisms that precede the nuclear entry of the estrogen receptor (ER). hbd and nls represent the hormone binding domain and the nuclear localization signal respectively. The major proteins involved are the cytosolic p55 and the p12 of the nuclear membrane (NM) or pore complex origin.

- (A) p55 binds to the nuclear localization signal on the ER
- (B) The p55-ER complex 'docks' at the nuclear periphery with the p55 interacting with p12.
- (C) ER enters the nucleus while the p55 is left behind.



CONCLUSIONS

Transport of IR from cytoplasm to the nuclear membrane is mediated by a complex of β -tubulin and α -tubulin.

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CONCLUSIONS

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1. The transport of ER from cytoplasm to the nuclear membrane is mediated by a 55 kDa cytosolic protein, p55.
2. The receptor entry into the nucleus is highly regulated. A 28 kDa protein, p28 antagonizes the ER-p55 interaction, apparently through the masking of the NLS on the ER.
3. This inhibition is reversed by yet another protein, p73. The p73 binds to the HBD of the ER and under the influence of estradiol probably it gets in touch with the p28. This interaction apparently facilitates the dissociation of p28 from the NLS on the ER.
4. p55 recognizes the NLS and binds to the ER. The ER-p55 complex gets docked at the pore complex.
5. Both p55 and p28 cross react with monoclonal anti hsp 70 and anti hsp 25 antibodies suggesting an evolutionary conservation of domains, critical to the function of the proteins (recognition of the NLS).
6. The possibility that p28 may have derived from p55 cannot be ignored. The p55-p28 conversion apparently resulted in the loss of actin and p12 binding domains on p55. This transformation has made it totally unfit to transport the ER into the nucleus

7. Free ER NLS-peptide inhibits the nuclear transport of the estrogen receptor by competing with ER for binding to p55. p55 also binds to the ER NLS-peptide-Sepharose column clearly showing that p55 could be the nuclear localization signal binding protein (NLSBP) of the ER.
8. p55-ER complex is recognized by a secondary site represented by a 12 kDa protein, p12 present in the nuclear membrane close to the nuclear pore complex. This interaction is highly specific. That the p12-Sepharose could recognize only free p55 and the ER-p55 complex from among the total cytosolic proteins and also that the p55-Sepharose column could recognize only p12 from among the total nuclear membrane extract indicated the high specificity that characterized this interaction.
9. Both p55 and p12 display ATPase activities. The ATPase activity gets enhanced when the two proteins come together. These are the Mg^{+2} - Ca^{+2} -dependent ATPases.
10. Quercetin, a Mg^{+2} -dependent-ATPase inhibitor, inhibits the translocation of ER into the nucleus. Energy released upon ATP hydrolysis is required in the translocation step

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