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

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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.

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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.

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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 oxazyl) 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 noncovalant 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 AGGTCAnnnTGATCT (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 cystein 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 trancriptionally active state. The region 'D' has 37 aminoacids and function 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 has 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 intra-cellular 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 a immunophilin as it binds Forskolin (FK 506) and rafamycin 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 ~ 0.13μm in diameter, ~ 0.07μm 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 ~9 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 Radermencher, 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

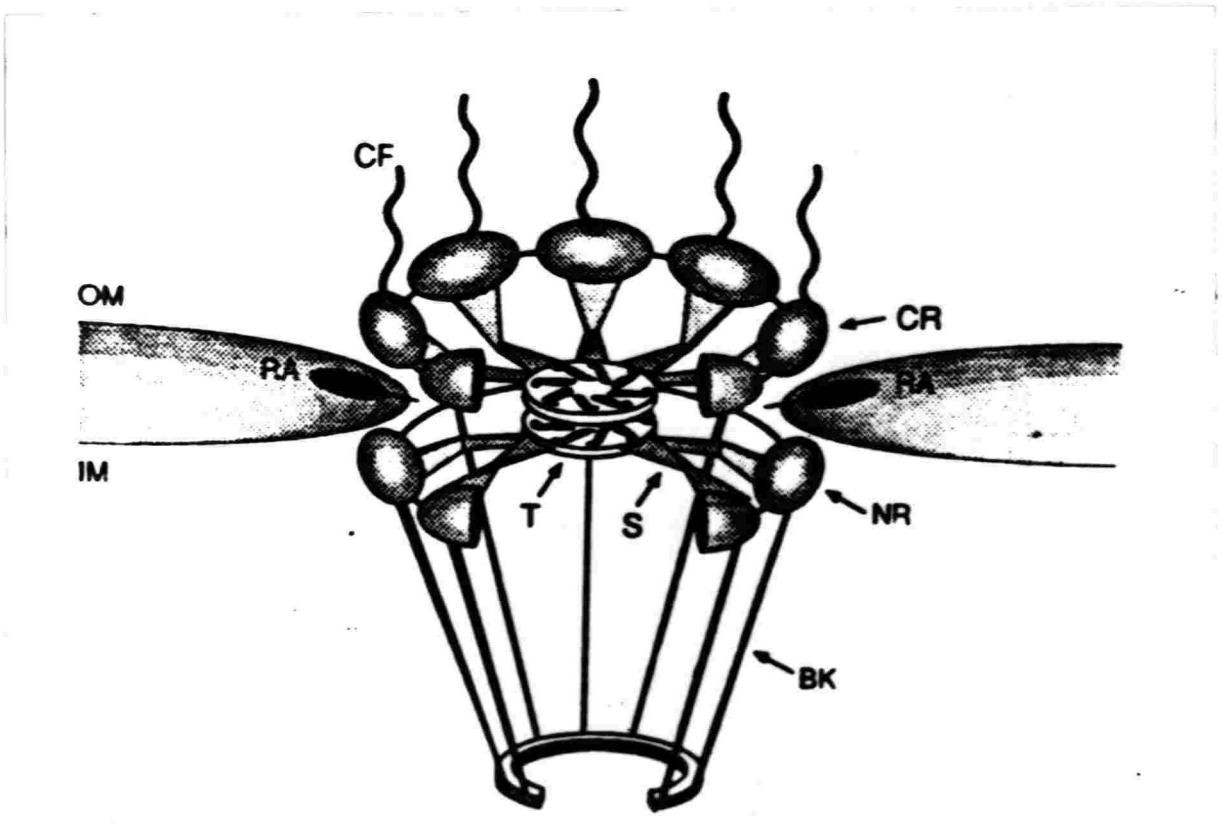
d) **a nucleoplasmic basket** (Fig.1.1)

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 ~ 360-380 A^0. Interspersed between the spokes are large 90 A^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 A^0 opening in the nuclear envelope, provide the 90 A^0 diffusion channel and support the smaller central transporter that regulates actual import and export.

b) **The central hub or transporter** (360-380 A^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 gl bular components of the cytoplasmic ring (CR) of the pore are connected to the spokes (S) as are the globular components of the nucleoplsmic ring (NR) The spokes are separated by 9 nm channels and support a central transporter (T) The transporter consists of two urises of 8 arms each Radial arms (RA) extend into the luminal space between the outer (ONI) and inner (IM) nuclear membranes Cytoplasmic f laments (CF) are shown extend ng from the cytoplasmic ring A basket like structure of filaments (BK) extends fr m the nucleoplasmic ring of the pore


CF
OM
RA
IM
CR
RA
T
S
NR
BK

open sequentially, each like the diaphrangm 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 Goldafarb., 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 (~33A^0 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 posses 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. Phoshphorylation 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:

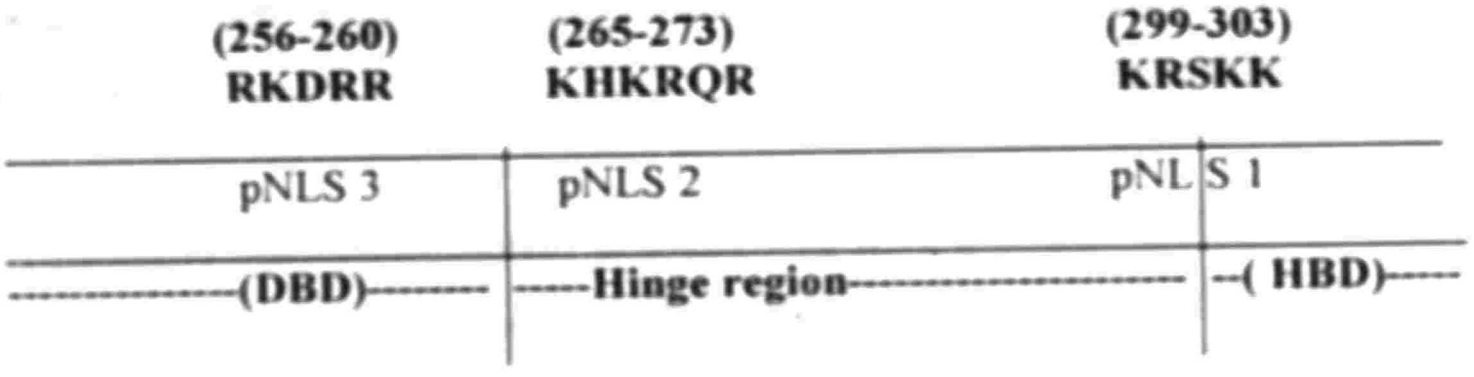
The NLSs of the members of the steroid/thyroid hormone receptor super family are located in the hinge region (region D) between the DNA binding and the hormone binding domains.

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 contest, 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


(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 sulphydral 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-permebilized 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.

Immunofluorescenc 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 electrone 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 *k*B (NF-*k*B) (Baeuerle and Baltimore, 1988a).

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

NF-*k*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 aminoterminus 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 (Veldschoste 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 (E_2) (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 pNLSs 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 roie 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-[^{3}H] estradiol 17β (sp.act, 101 Ci/mmol) was obtained from Amersham. Monoclonal anti-hsp25 (clone IAP-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 17β, Triton X-100, Nonidet P-40, Quercetin, CM-Sephadex, DEAE-Sepharose, 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 (aminoacid 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 4^0C. 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^{0}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 µl of the ER preparation was incubated overnight with 20 nM ^{3}H-estradiol (50µl). The unbound hormone was removed following adsorption of free hormone to dextran coated charcoal (100µl) (1% charcoal and 0.1% dextran in TEMN buffer). 200µl of ^{3}H-E_2-ER complexes (containing ~2µg) were incubated with goat uterine nuclei (250 µl) in the presence of 50 µl aliquot of the fractions containing the p55 for 30 minutes at 30^{0}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 (5µg) in the presence or absence of p55, p28 and p75 at 30^{0}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 4^0C. 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 4^0C. 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 4^0C 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 4^0C 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 vaccum. The final residue was lyophilized in order to remove traces of acetone. The acetone powder was obtained and stored at -70^{0}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

and ATP were added to final concentrations of 50 mM, 2mM and 1 mM respectively. The supernatant was stirred with a glass rod and left undisturbed for 2 hours. The KCl concentration of the supernatant was increased to 0.6 M and the stirring was continued for an additional one and a half hours. The polymerized actin was pelleted by centrifugation at 80,000 X g for 3 hours. The pellet was homogenized in TMC buffer containing 0.2 mM ATP and dialyzed for 3 days against the same buffer in order to depolymerise actin. The dialysate was centrifuged at 80,000 X g for 3 hours. The supernatant was collected and chromatographed over a column of Whatman DE-52, pre-equilibrated with imidazole buffer containing 100 mM KCl. The column was washed with the same buffer. Elution was carried out with 300 mM KCl in imidazole buffer. The eluate was dialyzed overnight against 2 liters of TMC buffer containing 50 mM KCl, 2mM $MgCl_2$ and 1 mM ATP to effect actin polymerization. The dialyste was centrifuged in order to pellet actin at 80,000 X g for 3 hours. The pellet was resuspended in TMC buffer containing 0.2 mM ATP and was dialyzed against the same buffer for two days. The dialyste was centrifuged at 80,000 X g for 3 hours. The supernatant which contained pure actin was lyophilized. Actin was dissolved in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M NaCl and coupled to CNBr activated Sepharose 4B as described above.

2.10 PREPARATION OF ESTROGEN RECEPTOR-SEPHAROSE.

The activated ER was purified form goat uterine cytosol following the procedure of Zafar and Thampan (1993). All the chromatographic procedures were performed using batch adsorptions and elutions. Cytosol was prepared in TEMN buffer. The cytosol was mixed with a Whatman DE-52 suspension equilibrated with TEMN buffer. The matrix was washed with TEMN buffer and eluted with

TEM buffer containing 0.2 M NaCl. The eluate was mixed with phosphocellulose. The phosphocellulose was washed with TEM buffer containing 0.2 M NaCl and eluted with TEM buffer containing 0.3 M NaCl. The NaCl concentration in the phosphocellulose eluate was diluted to 0.1 M using TEM buffer following which the eluate was mixed with ss-DNA-Sepharose equilibrated with TEMN buffer. DNA Sepharose was washed with TEMN buffer and was eluted with TEMN buffer containing 10 mM ATP. The ATP eluate contained the pure ER. The free ATP was dialyzed out against TEMN buffer and the dialysate was lyophilized. ER obtained from several independent preparations was pooled and about 10 mg of the ER was dissolved in 10 ml of 0.2 M $NaHCO_3$, pH 9.5 containing 0.5 M NaCl and coupled to CNBr activated Sepharose 4B as described before.

2.11 PREPARATION OF ER-NLS-SEPHAROSE.

The ER-NLS peptide, which includes pNLS1, pNLS2 and pNLS3 along with the intervening sequences (amino acids from 256-303; ***RKDRR***GGRM***LKHKRQRDD***GEGRGEVGSAGDMRAANLWPSPLMI***KRS KK***) was synthesised on hydromethyl tetraehylene glycol diacrylate-crosslinked polystyrene support using standard Fmoc.solid phase peptide synthesis protocol (Renil et al., 1994). The coupling reactions were carried out using dicyclohexyl carodimide/N-Hydroxybenzo traizole coupling procedure using 3 fold excess of Fmoc protected amino acid derivatives. The stepwise deprotection were carried out using 20% piperidine in NN'Dimethyl foramide. Finally, after the synthesis the peptide was cleaved from the support using trifluoro acetic acid: thioanisol: m-cresol (10:1:1) mixture at 40^0C for 6 hours. The peptide was purified by reprecipitating with diethylether and directly used for coupling reaction with CNBr activated Sepharose 4B.

2.12 PREPARATION OF ER-NLS (CONTROL)-SEPHAROSE.

The ER-NLS (control) peptide, which contains the same sequence as for the ER-NLS peptide but all the three NLSs were substituted with amino acid alanine residues. (Amino acid sequence *AAAAA*GGRM*AAAAAAAAA*GEGRGEVGSAG DMRAANLWPSPLMI*AAAAA*) was synthesized according to the procedure described by Renil et.al (1994).

2.13 PREPARATION OF ER-HBD-SEPHAROSE.

The ER-HBD peptide (amino acids 302-320;"***KK***NSLALSLTADQMV SALL") was synthesized according to the procedure described by Renil et.al(1994).10 mg of the synthetic peptide was directly coupled to CNBr activated Sepharose 4B.

2.14 PREPARATION OF p55-SEPHAROSE.

p55 was purified following the procedure of Nirmala and Thampan (1995a) with slight modifications. Cytosol was prepared in TEMN buffer. The cytosol was chromatographed over a 10 ml column of actin-Sepharose. The column was washed extensively with TEMN buffer and the p55 bound to the column was eluted with TEM buffer containing 0.8 M NaCl. There was no indication for the presence of the 48 kDa protein (which was often present in the procedure reported earlier) in my preparation. The NaCl eluate contained highly purified p55. The salt was dialyzed out following which the dialysate was lyophilized. About 5 mg of p55 was coupled to 7 ml of CNBr activated Sepharose 4B as described above.

2.15 PREPARATION OF p12-SEPHAROSE.

The purification of p12 was described in section 4.2. About 10 mg of p12 was coupled to 7 ml of CNBr activated Sepharose 4B.

2.16 PREPARATION OF WHEAT GERM AGGLUTININ (WGA)-SEPHAROSE.

WGA was purified according to the procedure described by Nagata and Burger (1974). Wheat germ was finely powdered and stirred in 10 volumes of 0.05 N HCl for one hour at room temperature. The suspension was centrifuged at 4000 X g for 10 minutes. To the supernatant solid ammonium sulphate was added to 35% saturation and the mixture was stirred for one hour at 4^0C. The precipitated proteins were collected by centrifugation at 9000 X g for 15 minutes. The precipitate was dissolved in 0.05 N HCl and butanol was added dropwise with constant stirring at room temperature to a final concentration of 20% (v/v). The stirring was continued for another one hour and the preparation was centrifuged at 5000 X g for 30 minutes at 4^0C. The aqueous phase was collected and was subjected to the butanol extraction twice again. The aqueous phase was dialyzed overnight against two changes of 0.05 N HCl. Ammonium sulfate was added at 35% saturation to this extract as described above. The precipitate was resuspended in a small volume of 0.05 N HCl and was dialyzed overnight against 0.01 M Tris-HCl, pH 8.5. The dialysate was centrifuged at 10,000 X g for 15 min and was chromatographed on a DE-52 column equilibrated with 0.01 M Tris-HCl, pH 8.5. Elution of WGA from the column was achieved using the same buffer. WGA appeared in the flow through fraction of the DE-52 column.10 ml fractions were collected and the absorbance was measured at 280 nm. The peak absorbance fractions were pooled, dialyzed overnight against distilled water and lyophilized.

The lyophilized material was dissolved in minimum volume of 0.05 N HCl, neutralized carefully with NaOH and finally centrifuged at 5,000 X g for 10 min. Crystals appeared after 1or 2 days at 4^0C. The crystals were harvested and rinsed with distilled water by low speed centrifugation and were recrystallized as above. WGA thus obtained was tested for homogeneity by SDS-PAGE and about 10 mg of the purified protein was coupled to CNBr activated Sepharose 4B.

2.17PREPARATION OF CONCANAVALIN-A(CON-A) SEPHAROSE.

10 mg of commercially available Con A was coupled to CNBr activated Sepharose 4B following the procedure of March et al. (1974).

2.18 FITC- LABELLING OF ER, p55 and p12.

Goat uterine estrogen receptor was purified following the procedure described by Zafar and Thampan (1993). The ER was labeled with FITC following the procedure of Chard (1987). One mg of ER was dissolved in 250 µl of 0.15 M $Na_2PO4.2H_2O$ pH 9.5. 100 µl of the FITC solution (1 mg/ml) in the same buffer was added to the protein solution. The pH of the mixture was adjusted to 9.5 with 1M Tris and the mixture was incubated overnight at 4^0C. The unbound FITC was removed following chromatography of the mixture on a column of Sephadex G-25.

Goat uterine p55 was purified following the procedure of Nirmala and Thampan (1995a). The p55 was labeled with FITC following the procedure of Chard (1987) as described above.

P12 was purified from goat uterine nuclei (described in section 4.2) and labeled with FITC as described above.

2.19 IMMUNIZATION PROTOCOL.

The following immunization protocol was followed. The protein under study was dissolved in 10 mM Tris buffer pH 7.6 and was mixed with Freund's complete adjuvant (in the ratio 1:1). The mixing was done thoroughly to make a thick colloidal suspension. Two ml of the suspension was injected into five different subcutaneous locations and also into the foot pad of a New Zealand white rabbit. The second injection (first booster) was given 15 days after the first. Protein for the booster injections was prepared by mixing the protein solution with Freund's incomplete adjuvant in the ratio 1:1. The second and the third boosters were given at 15 days intervals.The rabbits were bled a week after the second booster. The serum was collected and tested for the presence of antibodies by western blotting analysis. The rabbits were given another booster injection and bled from the ear vein a week later. The serum was isolated, aliquoted and stored at -20^{0}C.

Antibodies were raised against goat uterine estrogen receptor, p55 and p12.

2.20 ISOLATION OF IgG FROM THE ANTISERUM.

The IgG was purified following chromatography of the serum on a column of protein A-Sepharose as described by Sambrook et al.(1989). The antiserum was mixed with 0.1 volume of 1 M Tris-HCl pH 8.0 and was chromatographed on a column of protein A-Sepharose (3 ml), equilibrated with 0.1 M Tris- HCl pH 8.0. The column was washed with ten volumes of 0.1 M Tris-HCl pH 8.0 followed by ten volumes of 10 mM Tris-HCl, pH 8.0. Elution was achieved using 100 mM glycine-HCl, pH 3.0. The fractions (500µl) were collected in tubes containing 50µl of 1 M Tris-HCl pH 8.0. The absorbance of the fractions at 280 nm was

monitored. The IgG was eluted as a single peak of absorbance at 280 nm. The fractions containing the IgG were pooled and dialyzed extensively against 10 mM Tris-HCl pH 8.0 in order to remove glycine.

2.21 PREPARATION OF PROTEINS FOR SDS-PAGE.

To the protein solution 100% TCA was added to give a final TCA concentration of 30%. The solution was mixed thoroughly and left on ice for a minimum of two hours. The samples were transferred to clean Eppendorf tubes and were centrifuged at 12,000 X g for 20 min. The supernatant was decanted and the tubes were inverted over a blotting paper to drain off traces of TCA. The precipitate was then washed twice with distilled ethanol. The final precipitate was dried in air to remove alcohol and was dissolved in 20µl of SDS sample buffer. The samples were denatured following heating in a boiling water bath for 3 mins.

2.22 SDS-PAGE.

SDS-PAGE was performed as described by Laemmli (1970). Proteins were resolved either on standard gels (15cm X 13 cm X 0.1 cm) or mini gels (7.5cm X 8cm X 0.1cm) for rapid analysis.

Solutions used:

1) Acrylamide solution: 30 g acrylamide and 800 mg methylene bis-acrylamide were dissolved in double distilled water and the volume was adjusted to 100 ml. The Acrylamide solution was filtered through Whatman No.1 filter paper and stored in amber colored bottles at 4^0C.

2) Lower Tris (4X)(Separating gel buffer)(1.5 M Tris-HCl pH 8.8 with 0.4% SDS); 18.17 g Tris was dissolved in double distilled water. To this 4 ml 10% SDS was added and the pH of the solution was adjusted to pH 8.8 with 12 N HCl. The

volume was then made up to 100 ml. The lower Tris was filtered through Whatman No.1 filter paper and was stored in glass bottles at 4^0C.

3) Upper Tris (4X)(Stacking gel buffer)(0.5 M Tris-HCl pH 6.8 with 0.4% SDS): 6.06 g Tris was dissolved in double distilled water. To this 4 ml 10% SDS was added following which the pH of the mixture was adjusted to 6.8 with 12 N HCl. The final volume was then made up to 100 ml. The upper Tris was filtered and stored at 4^0C as indicated above.

4) Reservoir buffer(8X): 24 g Tris and 115.2 g glycine were dissolved in double distilled water and the volume was made up to a liter. The buffer was stored at 4^0C and diluted just before use.

5) Reservoir buffer(X): 125 ml of 8X reservoir buffer and 10 ml 10% SDS was diluted to 1000 ml with double distilled water.

6) 10% SDS: 10 g SDS was dissolved in double distilled water and the volume was made up to 100 ml and stored at room temperature.

7) 10%APS: 1 g APS (ammonium persulphate) was dissolved in 10 ml double distilled water. This solution was always prepared fresh before use.

8)Sample buffer: 1.0 ml glycerol, 5.0 ml β-mercaptoethanol, 3.0 ml 10% SDS and 1.25 ml upper Tris (4X) were mixed and the volume of the mixture was made up to 10 ml using double distilled water. 10 μg bromophenol blue was added to the sample buffer. The sample buffer was stored at -20^0C as 1.0 ml aliquots.

Electrophoresis: Discontinuous standard slab gels or mini slab gels were polymerized just before use. Gels of 1 mm thickness were routinely used as 1 mm gels since they were easy to handle and were of the optimal thickness for silver staining. 5 to 15% gels were prepared as given in the table shown in the nest page (the volumes of the solutions are given in milli litres).

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(µl)	20	10	10	10	10

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

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 and100 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 µl 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 µl of 37% formaldehyde was added to this solution immediately before use.

5) Developer: 12 gm of anhydrous sodium carbonate and 100 µl 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 $MgCl_2$ in the case of alkaline phosphatase-IgG.

2.25 GEL FILTRATION ANALYSSIS.

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 µl of this solution was applied carefully onto the column. Fractions collected were subjected to measurement of absorbance at 280 nm. The kd (distribution coefficient) of the protein was calculated and a standard graph of kd versus R_s of the marker proteins was made.

$$kd = \frac{Ve-Vo}{Vt-Vo}$$

where,

Vo = Volume of buffer used to elute blue dextran,

Vt = Volume of buffer used to elute glycine,

Ve = Volume of buffer used to elute a given protein Ve_{BSA}, Ve_{OV}, Ve_{CYT-C} etc.

The kd 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^{0}C for 30 mins with different protein concentrations. The volume

of the incubation mixture was 250 µl. 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 concentrantion (20 µg/ml is 0.65 mmol/l). The ATPase activity was expressed as µmoles Pi released/hour.

2.28. ESTIMATION OF PROTEINS:

Proteins were estimated following the procedure of Bradford (1976). 100 µl 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'.

Goat uterine cytosol was prepared as described in 'methods'. The cytosol was chromatographed over a column of estrogen receptor-Sepharose, which was pre-equilibrated with the TEMN buffer. The column flowthrough fraction was recycled onto the column in order to optimize the binding of proteins to estrogen receptor-Sepharose. The column was extensively washed with TEMN buffer and elution was achieved using a linear gradient of 0-1 M NaCl in TEM buffer. Fractions (2 ml) collected from the column were subjected to the nuclear transport assay. All the isolation steps were carried out at 4^0C.

The active fractions which displayed the ER transport-function were eluted from the estrogen receptor-Sepharose as a single peak of activity with 0.5-0.6 M NaCl (Fig.3.1A). The protein in the gradient fractions was precipitated with TCA and subjected to SDS-PAGE. Upon silver staining three major protein bands were visualized, of molecular mass 73, 55 & 28 kDa (Fig 3.1B). There was an extra band observed below p73 in a few fractions. Its recovery was not reproducible always and therefore it is taken to be a proteolytic product of p73.

It was striking to note that the nuclear transport activity was detected only in those fractions which contained a 73 kDa protein, p73 in addition to the p55. The p55 was recovered in several other fractions, along with a 28 kDa protein, the p28. It was clear that the presence of p28 in these fractions caused an inhibitory effect on the p55 mediated nuclear entry of the ER. In the fractions which displayed the transport function the presence of p73 seemed to have made the difference in spite of the presence of p28. This observation raised the possibility that p73 possessed the capacity to reverse the p28-mediated inhibition of the nuclear transport of ER.

3.2 SEPARATION OF THREE ER-ASSOCIATED PROTEINS ON SEPAHDEX G-100 COLUMN:

In order to test the possibility mentioned above it was essential to isolate the three ER-associated proteins in pure form. Goat uterine cytosol was chromatographed over a column of estrogen receptor-Sepharose. The column (10ml) was washed extensively with TEMN and eluted with 1M NaCl in TEM buffer. The protein in the eluate was precipitated with solid ammonium sulphate at 0.7 saturation. The precipitate was redissolved in a small volume of the buffer. It was dialyzed against two changes (500 volumes) of the buffer and the macromolecules were concentrated through ultrafiltration. The concentrated material was chromatographed over a column of Sephadex G-100 equilibrated with TEMN buffer containing 0.3 M NaCl. Fractions (2 ml) collected were analysed for their absorbance at 280 nm. Three clear peaks were observed which represented the proteins of molecular mass 73, 55 & 28 kDa (Fig 3.2A). SDS-PAGE analysis was performed in order to confirm this inference and also the purity of the proteins. The three proteins appeared as single bands in silver stained SDS gels (Fig 3.2B). The protein fractions were pooled separately, dialyzed extensively against 10 mM Tris-HCl pH 7.6, and finally concentrated through ultrafiltration for use in further studies.

3.3 INHIBITORY EFFECT OF p28 ON p55:

The nuclear transport assay mixture consisting the ^{3}H-E_2-ER complexes (containing ~2 μg ER), p55 (0.5 μg) and goat uterine nuclei were incubated with increasing concentrations of p28 (0 to 0.1μg). The incubation was carried out at $30^{0}C$ for 30 minutes in a water bath. The ^{3}H-E_2-ER that bound to the nuclei was

quantitated. It was observed that the nuclear transport decreased along with the increase in the amount of p28 added to the medium (Fig.3.3A).

3.4 REVERSAL OF p28-MEDIATED TRANSPORT INHIBITION BY p73:

The possibility that p73 can reverse the p28-mediated inhibition of the ER transport was examined using an assay system that contained p55 (0.5 μg), p28 (0.1 μg) and increasing concentrations of p73 (0 to 0.1 μg) in the presence of ^{3}H-E_2-ER and nuclei. The incubation was carried out at 30^{0}C for 30 minutes. The presence of 0.1 μg of p28 had a total inhibitory effect on the p55-mediated ER transport (Sec 3.3). Addition of p73 from 0 to 0.1 μg to the nuclear transport assay mixture was seen to effect a total reversal of this inhibition produced by p28 (Fig 3.3B).

3.5 p55 AND p28 COMPETE FOR THE SAME SITE ON THE ER:

Since p28 inhibited the p55-mediated nuclear transport of the ER it was of interest to find out whether the p28 competes with p55 for the same site on the receptor. Varying concentrations of p55 were incubated with the nuclear transport system in the presence or absence of a fixed quantity of p28.

^{3}H-E_2-ER complexes (2 μg) were incubated with goat uterine nuclei and varying molar concentrations of p55. Two different concentrations of p28 (0 03 and 0.06 μg) were added to the reaction media. The transport assay was carried out as described previously. The results were presented in the form of a Lineweaver-Burke plot (Fig 3.4). The results apparently indicated that p28

competed with p55 for the same site on the ER. Though there is no direct evidence to demonstrate that the p55 binds to the NLSs of the ER, there are several indirect evidences to suggest this possibility from the studies carried out by Nirmala and Thampan (1995a). The possibility existed that p28 could also interact with the NLS site on the ER. This assumption was put to test in another experiment.

The estrogen receptor consists of three constitutive NLSs (termed proto-NLSs) and one hormone inducible NLS in its structure between the aminoacids 256-320. The three pNLSs 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 (HBD), which on its own is not sufficient for ensuring efficient nuclear accumulation, but can co-operate with the constitutive p-NLSs. This inducible p-NLS apparently contributes to the nuclear accumulation of the wild type receptor in the presence of the hormone (Ylikomi et al., 1992).

Three types of nuclear localization signal peptides were synthesized in our laboratory. One consisted of amino acids from 256-303 with all three p-NLSs. The second one consisted of the same sequence but the three NLSs were substituted with poly alanine, thereby serving as a control sequence devoid of NLS. The third one represented amino acids from 302-320, which is the hormone binding domain (HBD) of the ER. p-NLS1 overlaps with the HBD of ER. The three synthetic peptides were coupled to CNBr activated Sepharose 4B The affinity columns were designated as NLS peptide-Sepharose column, NLS peptide (control)-Sepharose column and HBD peptide-Sepharose column respectively.

3.6 DIRECT EVIDENCE FOR THE BINDING OF p55 AND p28 TO THE NLS OF ER:

Goat uterine cytosol was chromatographed over a column of ER NLS peptide-Sepharose column. The flowthrough fraction from this column was recycled on the column for maximal binding. The NLS peptide-Sepharose was washed extensively with TEMN. Elution of the bound proteins was achieved with a linear salt gradient (0-1 M NaCl in TEM). The eluate was collected as 2 ml fractions. All the fractions were examined for their absorbance at 280 nm. The proteins eluted from the NLS-peptide column as a single peak in the presence of 0.5- 0.6 M NaCl (Fig 3.5A). The peak fractions were subjected to SDS-PAGE. The fractions displayed only 2 proteins, of molecular mass 55 and 28 kDa (Fig 3.5B). The above result was also indicative of the possibility that the p55 and p28 are the only proteins in the uterine cytosol which will bind to the NLS of ER. Both have almost the same affinity for the ER-NLS since both were eluted from the column using media containing the same salt concentration. The reason for the predominant nuclear localization of ER irrespective of the hormone could be the higher quantity of the number of copies of p55 per cell when compared to that of p28. So the chances of interaction between ER and p55 would be more when compared to the interaction between ER and p28.

In the control experiment the affinity column used contained the amino acids 256-303 of the ER in which the pNLS sequences were replaced by poly alanine. Goat uterine cytosol was chromatographed over this control column and it was observed that no proteins remained bound to the column (data not shown) This was a confirmatory evidence for the assumption that the p55 and the p28 recognized the pNLS(s) in the ER.

In another experiment p55 and p28 were chromatographed independently over the NLS Sepharose. It was observed that both the proteins were eluted from the column using buffers containing the same salt concentration (data not shown).

3.7 p73 BINDS TO THE HBD OF ER:

Results of the experiment mentioned above indicate that p73 was not bound to the NLS peptide column. This was also indicative of the possibility that the p73 binding to the ER is direct and not mediated either by p55 or by p28. This question was put to test through chromatography of the pure p73 on ER-Sepharose. The column was washed extensively with TEMN buffer and the protein bound to the column was eluted with 1M NaCl. The eluate was concentrated through ultrafiltration and subjected to SDS-PAGE. p73 was visualized upon silver staining. In order to confirm the binding of p73 to the specific part of ER and to test the possibility of binding of p73 to the HBD of ER, the HBD peptide was synthesized and coupled to Sepharose-4B.

Goat uterine cytosol was chromatographed over an ER-HBD peptide-Sepharose column. The column was washed extensively with TEMN and subsequently eluted with a linear gradient of 0-1M NaCl in TEM. 2 ml fractions were collected. Individual fractions were analysed for their absorbance at 280 nm (Fig.3.6A). The peak fractions were subjected to SDS-PAGE. Upon silver staining three major proteins of molecular weight 73, 55 and 28 kDa were visualized (Fig 3.6B). The elution pattern was the same as that observed with the ER-Sepharose column.

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 ^{3}H-E_2-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^{0}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 ^{3}H-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-*k*B is found to be associated with the cytoplasmic retention factor I-*k*B in unstimulated B-lymphocytes (Baeuerle & Baltimore,1988) Phosphorylation disrupts the I*k*B-NF-*k*Bcomplex and NF-kB 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

doesnot 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.

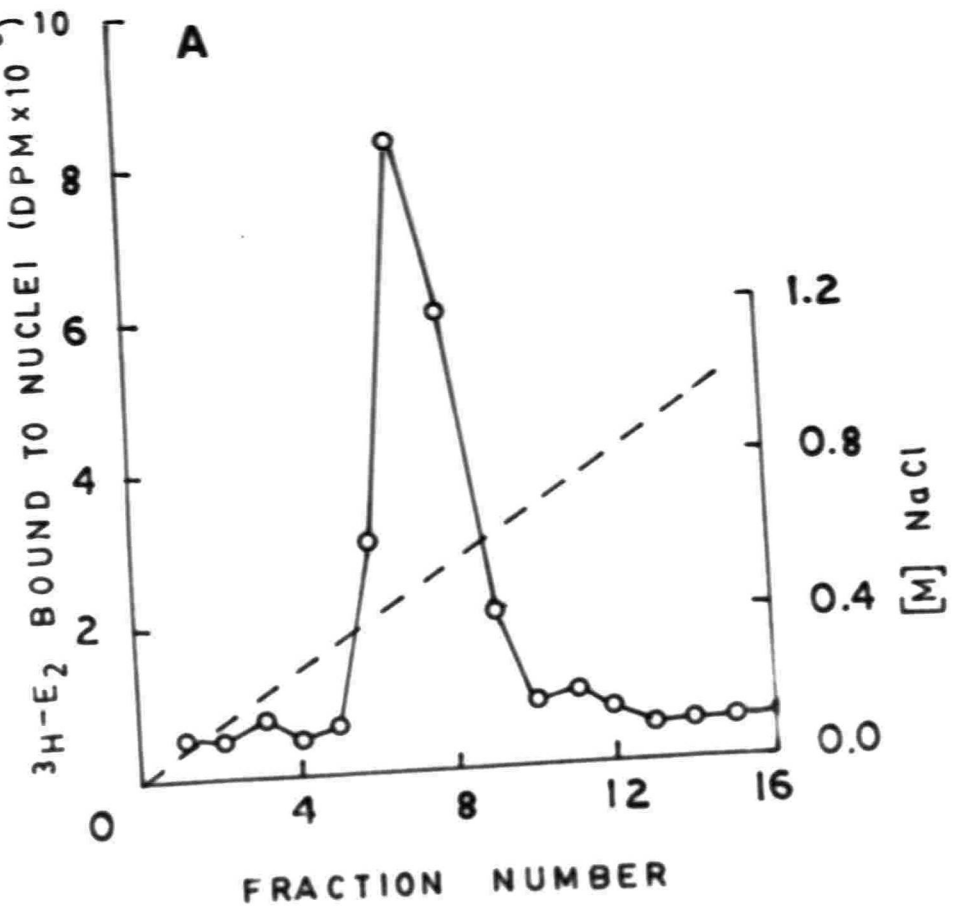
Both p55 and p28 were shown to have immunological cross reactivity with monoclonal antibodies against hsp 25 and hsp 70 showing that both p55 and p28 might have some common antigenic motif in their primary structure. The amino acid homology may suggest an evolutionary conservation of domains critical 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 ^{3}H-E_2-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


A
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
10
8
6
4
2
0
4
8
12
16
FRACTION NUMBER
1.2
0.8
0.4
0.0
[M] NaCl

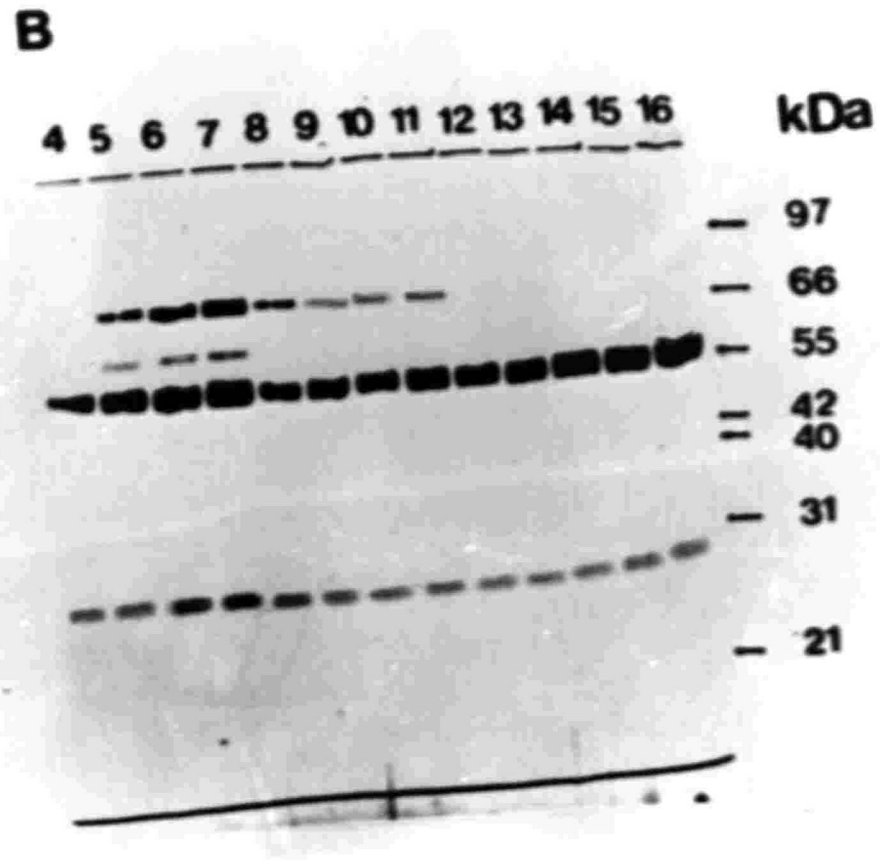

B
4 5 6 7 8 9 10 11 12 13 14 15 16
kDa
97
66
55
42
40
31
21

Figure 3.2. (A) Chromatographic Separation of the ER-transport-associated proteins through gel filtration on Sephadex G-100.

Goat uterine cytosol was chromatographed over an estrogen receptor-Sepharose column Protein in the 1M NaCl eluate from the ER-Sepharose column was precipitated with ammonium sulphate at 0 7 saturation The precipitate was dialyzed extensively and was concentrated through ultrafiltration. The protein mixture was chromatographed on a precalibrated column of Sephadex G-100. The column was developed with TEM buffer containing 0 3 M NaCl Two millilitre fractions collected were analysed for their absorbance at 280 nm

(B) SDS-PAGE analysis of the three proteins.

The fractions belonging to the three peaks from the Sephadex G-100 column were pooled individually and subjected to SDS-PAGE on a 12% gel The gel was stained with silver nitrate

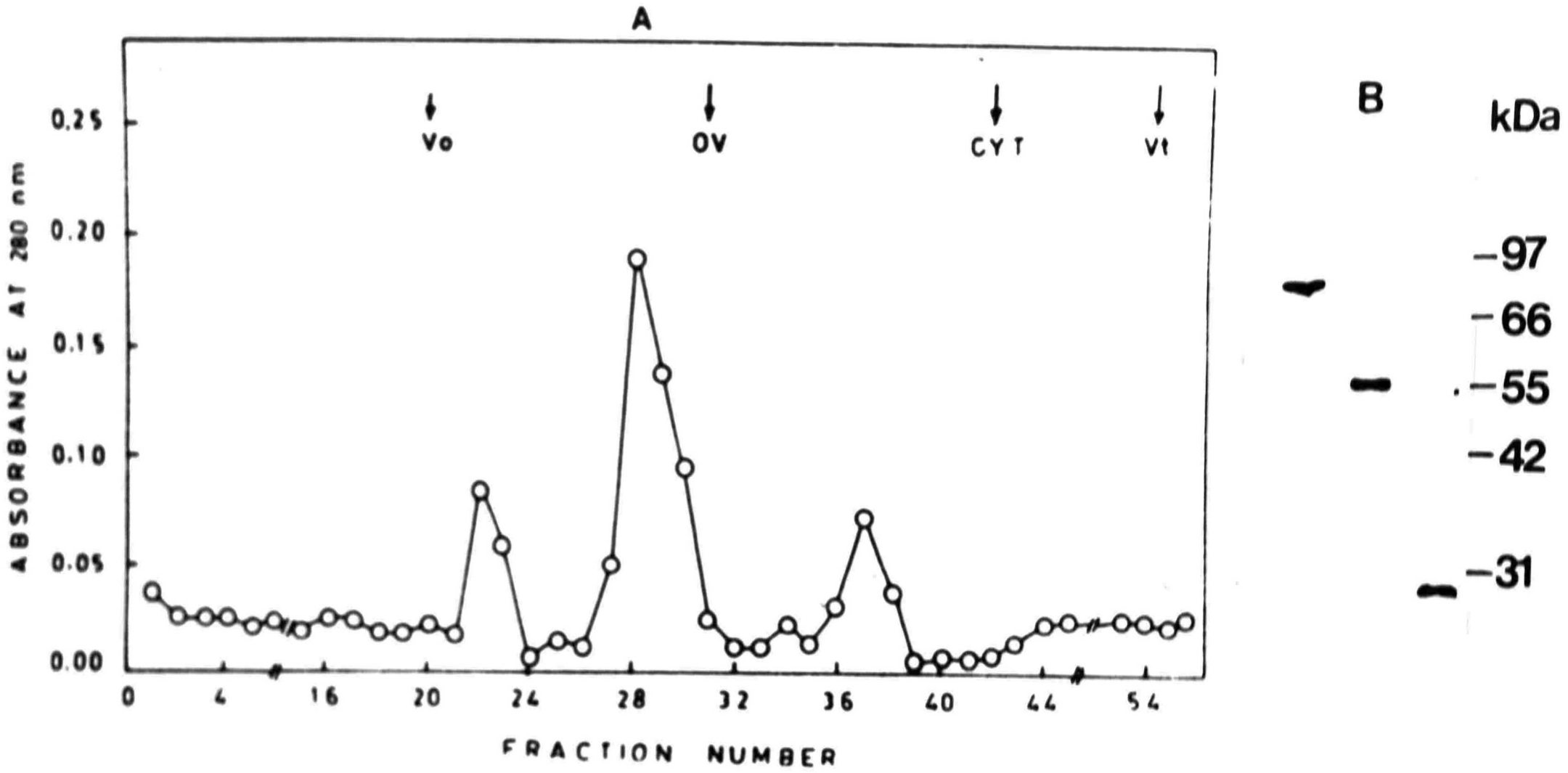

A
Vo
OV
CYT
Vt
0.25
0.20
0.15
0.10
0.05
0.00
ABSORBANCE AT 280 nm
0
4
16
20
24
28
32
36
40
44
54
FRACTION NUMBER
B
kDa
97
66
55
42
31

Figure 3.3A. The nuclear transport of ER mediated by p55 is inhibited by p28.

p55 (0.5 μg) was incubated with ^{3}H-E_2-ER complexes, nuclei and 0 to 0.1 μg of p28 at 30^{0}C for 30 minutes and the transport assay was performed as described in 'methods'. The ^{3}H-E_2 bound to the nucleus was extracted with ethanol The radioactivity in the ethanol extract was measured. The nuclear transport activity was expressed as ^{3}H-estradiol bound to the nuclei

B) p73 reverses the inhibition by p28 of the p55 mediated ER transport.

^{3}H-E_2-ER complexes were incubated with 0 5 μg of p55, 0 1 μg of p28, nuclei and increasing concentrations (0 to 0 1μg) of p73 at 30 C for 30 minutes The assay was performed as described in the 'methods' The ^{3}H-E_2-ER transported into the nucleus was measured following extraction of the bound hormone with ethanol The radioactivity in the ethanol extract was analysed The nuclear transport activity was expressed as ^{3}H-estradiol bound to the nuclei

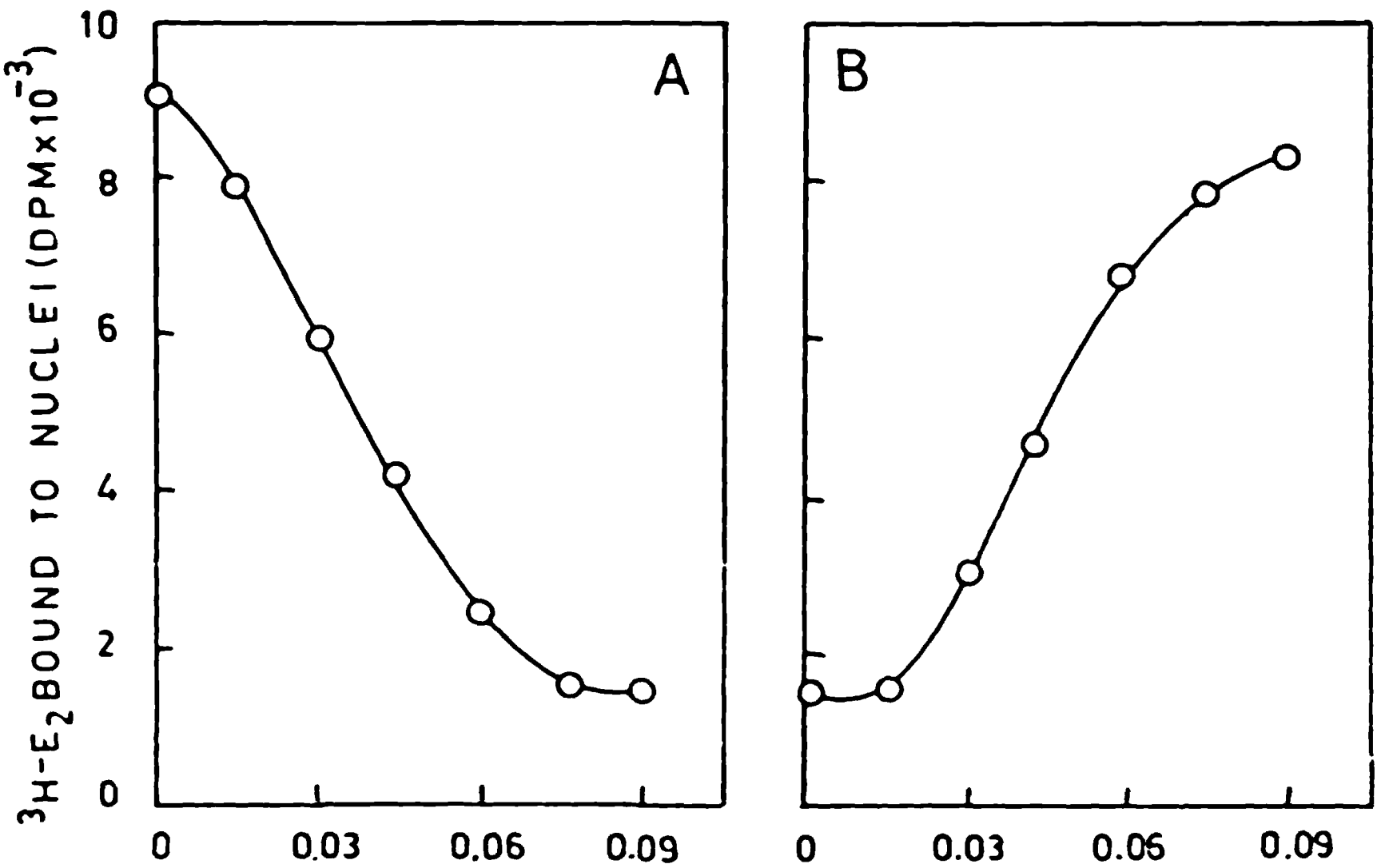

A
B
^3H-E_2 BOUND TO NUCLEI (DPM×10^{-3})
10
8
6
4
2
0
0
0.03
0.06
0.09
0
0.03
0.06
0.09
μg P^{28}
μg P^{73}

Figure 3.4. Competition between p55 and p28 for binding to the ER.

Nuclei were incubated with ^{3}H-E_2-ER in the presence of varying molar concentrations of p55 either in the presence or absence of p28, employed at two different concentrations. 30 C for 30 min The ^{3}H-E_2-ER transported into the nuclei was measured as described before The activity, expressed as ^{3}H-estradiol bound to the nuclei, was represented in the form of a Lineweaver-Burke plot

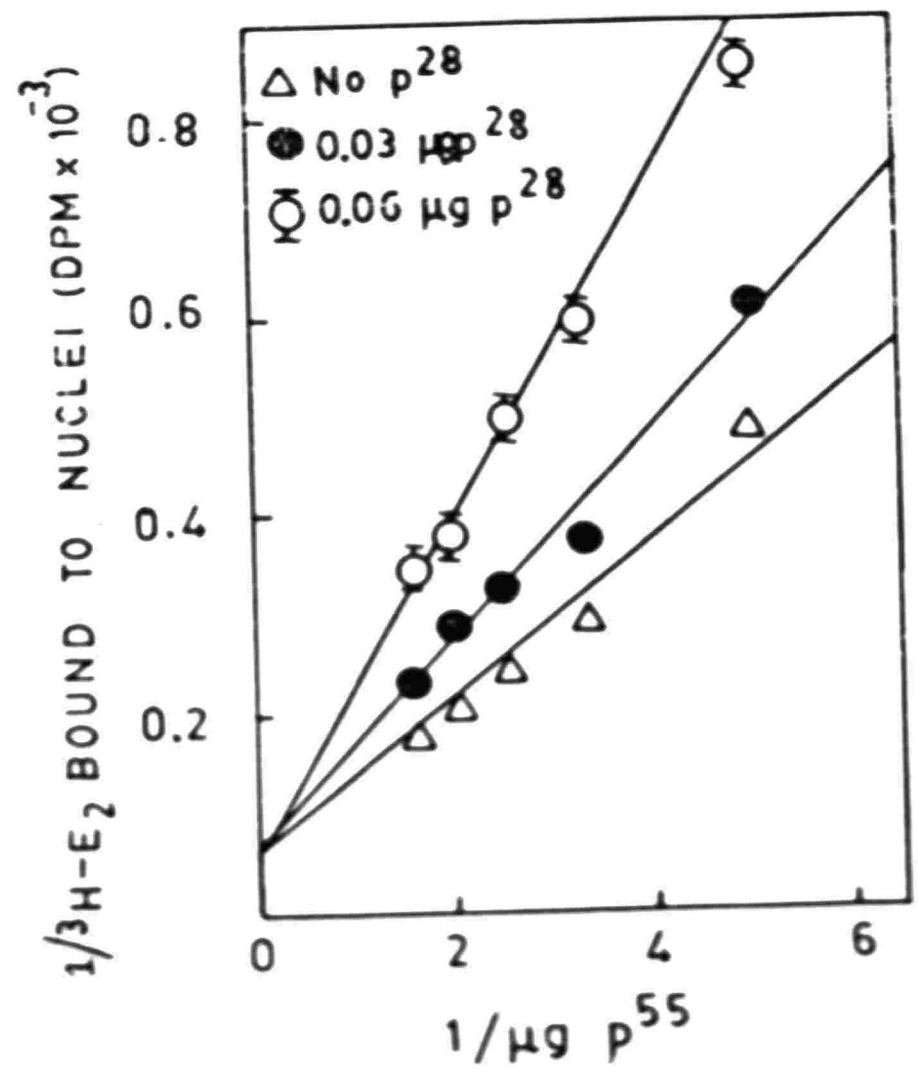

△ No p28
● 0.03 µg p28
○ 0.06 µg p28
0.8
0.6
0.4
0.2
0
2
4
6
1/3H-E2 BOUND TO NUCLEI (DPM x 10-3)
1/µg p55

Figure 3.5 Chromatography of goat uterine cytosol on ER-NLS peptide-Sepharose.

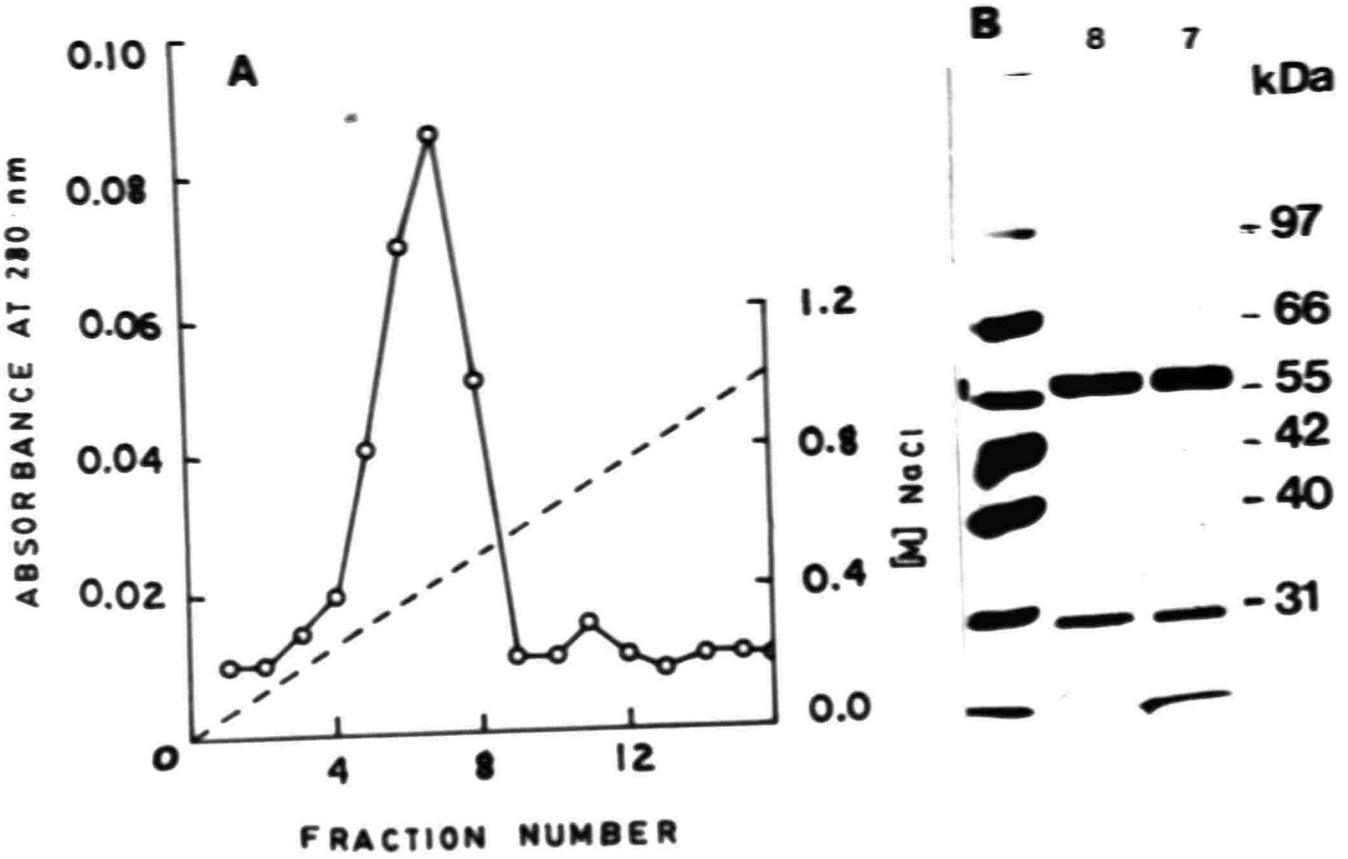
(A)Goat uterine cytosol was chromatographed on ER-NLS peptide Sepharose (peptide with pNLS1, pNLS2, pNLS3 amino acids from 256- 303; *RKDRR*GGRM*LKHKRQRDD*GEGRGEVGSAGDMRAANLWPSP LMI*KRSKK*), equilibrated with TEMN buffer The column was washed with the same buffer and eluted with a linear NaCl gradient (50 mM to 1M NaCl in TEM) The eluate was collected in 2 ml fractions. The absorbance was measured at 280 nm

(B) The fractions showing maximum absorbance were subjected to SDS-PAGE in 10% gels The gel was stained with silver nitrate The number on the lanes corresponds to the fraction numbers in (A)

Figure 3.6 Chromatography of goat uterine cytosol on ER-HBD peptide-Sepharose.

(A) Goat uterine cytosol was chromatographed on ER-HBD peptide-Sepharose (peptide contains a pNLS1 and HBD, amino acids 302-320, "KANSLALSLTADQMVSALL") The column was washed with TEMN and eluted with a linear NaCl gradient (50 mM to 1M NaCl in TEM) The eluate was collected in 2 ml fractions The absorbance was measured at 280 nm

(B) The fractions showing maximum absorbance were subjected to SDS-PAGE on a 12% gel The gel was stained with silver nitrate The lane numbers depicted correspond to the fraction numbers specified in Fig A


A
0.10
0.08
0.06
0.04
0.02
0
ABSORBANCE AT 280 nm
1.2
0.8
0.4
0.0
[M] NaCl
4
8
12
FRACTION NUMBER
B
8
7
kDa
97
66
55
42
40
31

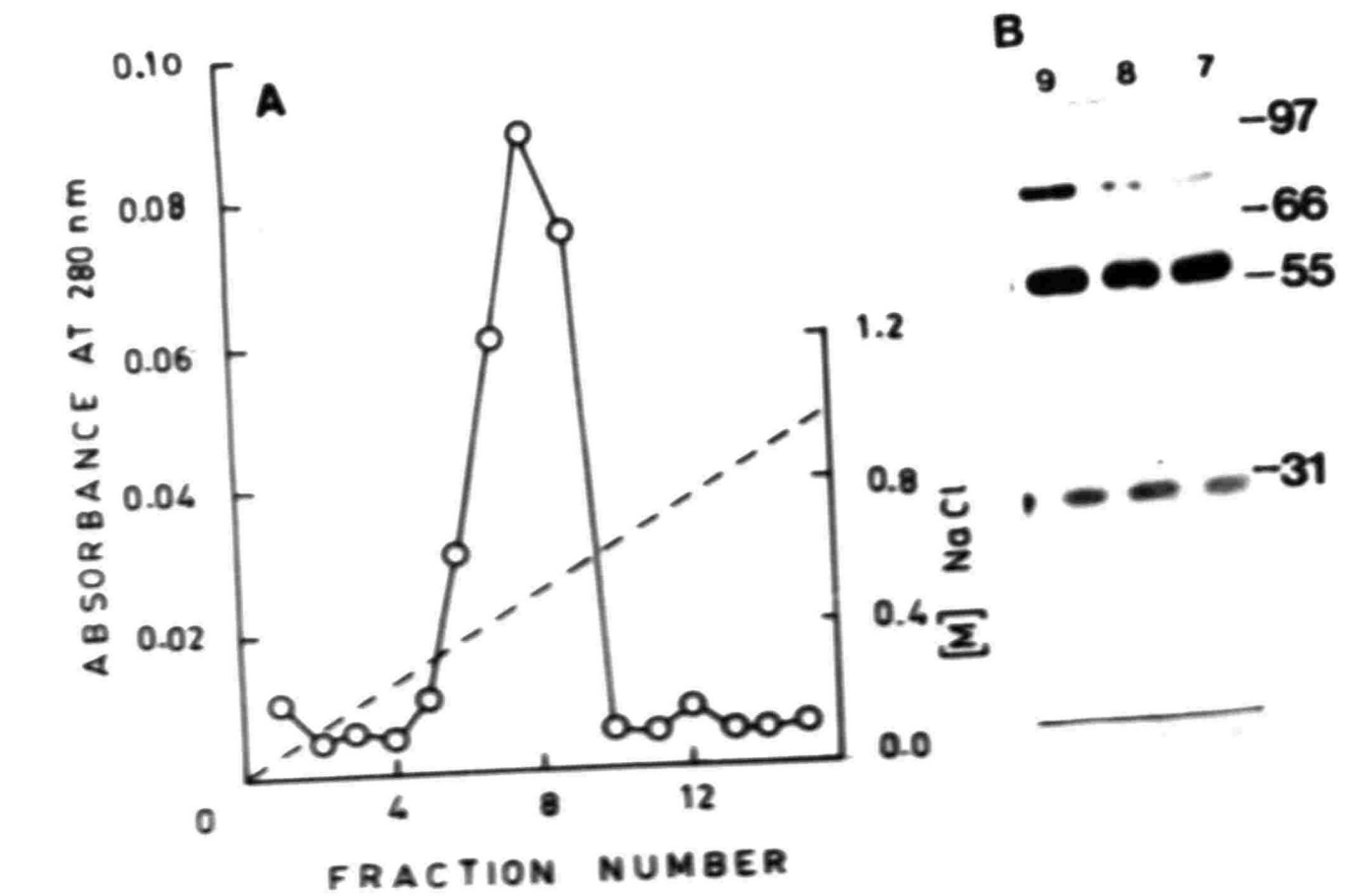

A
0.10
0.08
0.06
0.04
0.02
0
ABSORBANCE AT 280 nm
1.2
0.8
0.4
0.0
[M] NaCl
4
8
12
FRACTION NUMBER
B
9
8
7
97
66
55
31

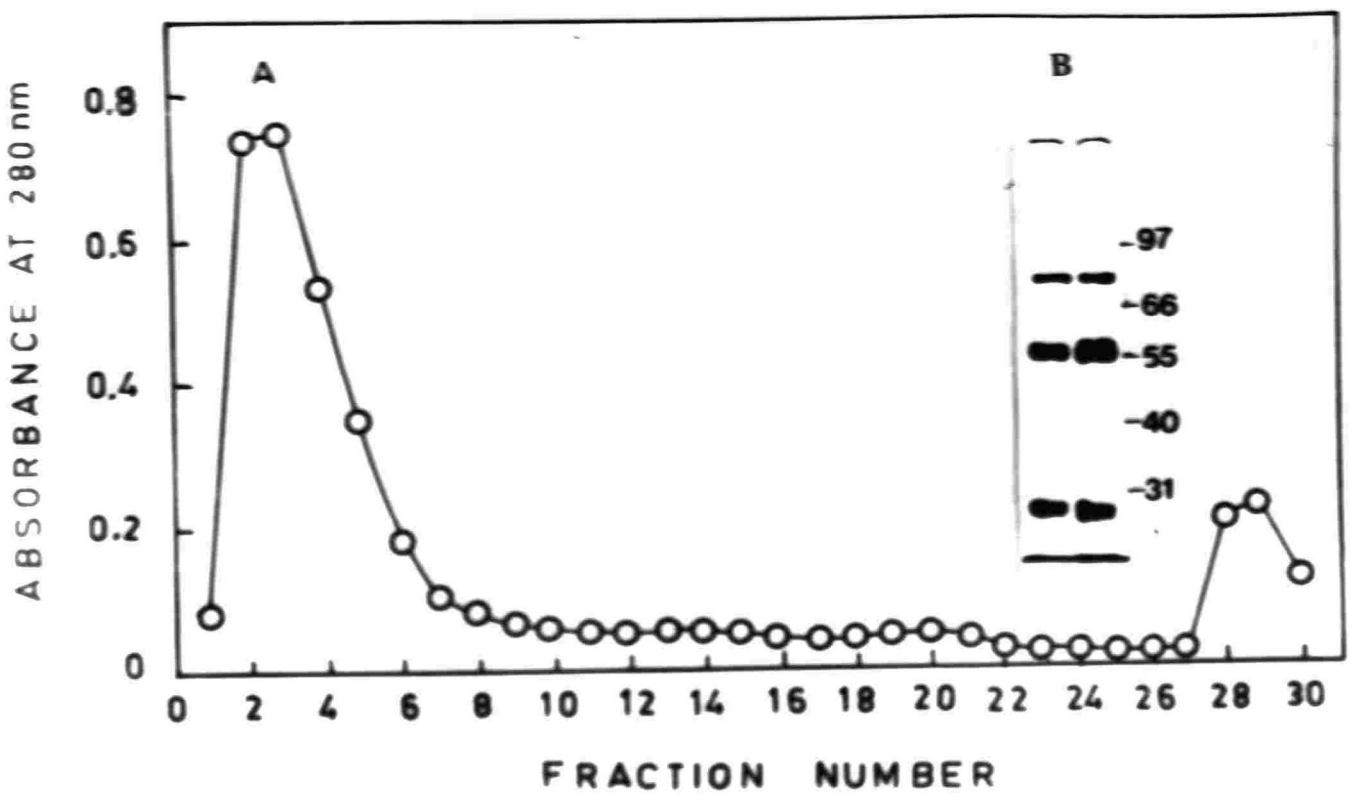
Figure 3.7 Estradiol releases p73 from the HBD of ER

(A) Goat uterine cytosol was chromatographed over ER-HBD peptide-Sepharose. The column was washed with TEMN and eluted with 20 nM estradiol in TEMN buffer. The eluate was collected as 1 ml fractions. The absorbance was measured at 280 nm.

(B) The fractions showing maximum absorbance (2 and 3) were subjected to SDS-PAGE on a 10% gel The gel was stained with silver nitrate.

Figure 3.8 Recognition of p55 and p28 by monoclonal anti hsp 25 and anti hsp 70 antibodies.

p73, p55 and p28 recovered from an ER-Sepharose column were subjected to SDS-PAGE The proteins were transferred to two nitrocellulose membranes One of the blots carrying both the proteins was incubated with anti-hsp70 antibody and the other was incubated with anti-hsp 25 antibody The blots were re-exposed to alkaline phosphatase coupled anti-mouse IgG and were stained with BCIP/NBT solution Panel (A) indicates cross reactivity with anti-hsp 70 and panel (B) with anti-hsp 25 antibody The numbers indicate molecular mass in kDa


A
B
ABSORBANCE AT 280 nm
0.8
0.6
0.4
0.2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER
-97
-66
-55
-40
-31

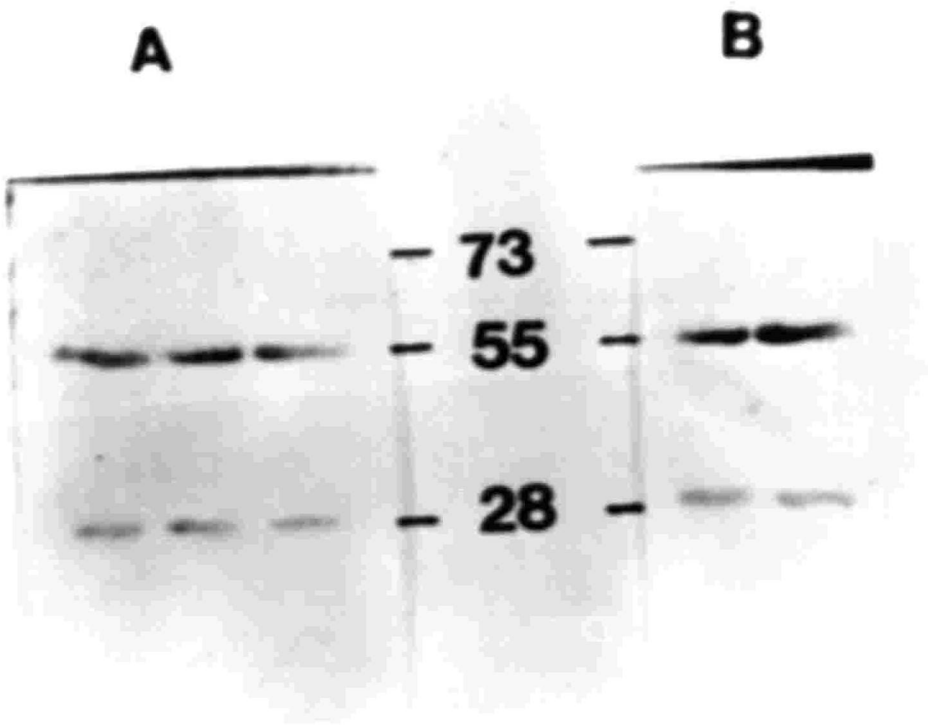

A
B
73
55
28

Figure 3.9A. SDS-PAGE analysis of purified actin.

Actin was purified as described in the methods. The protein was subjected to SDS-PAGE on a 12% gel. The gel was subjected to silver staining.

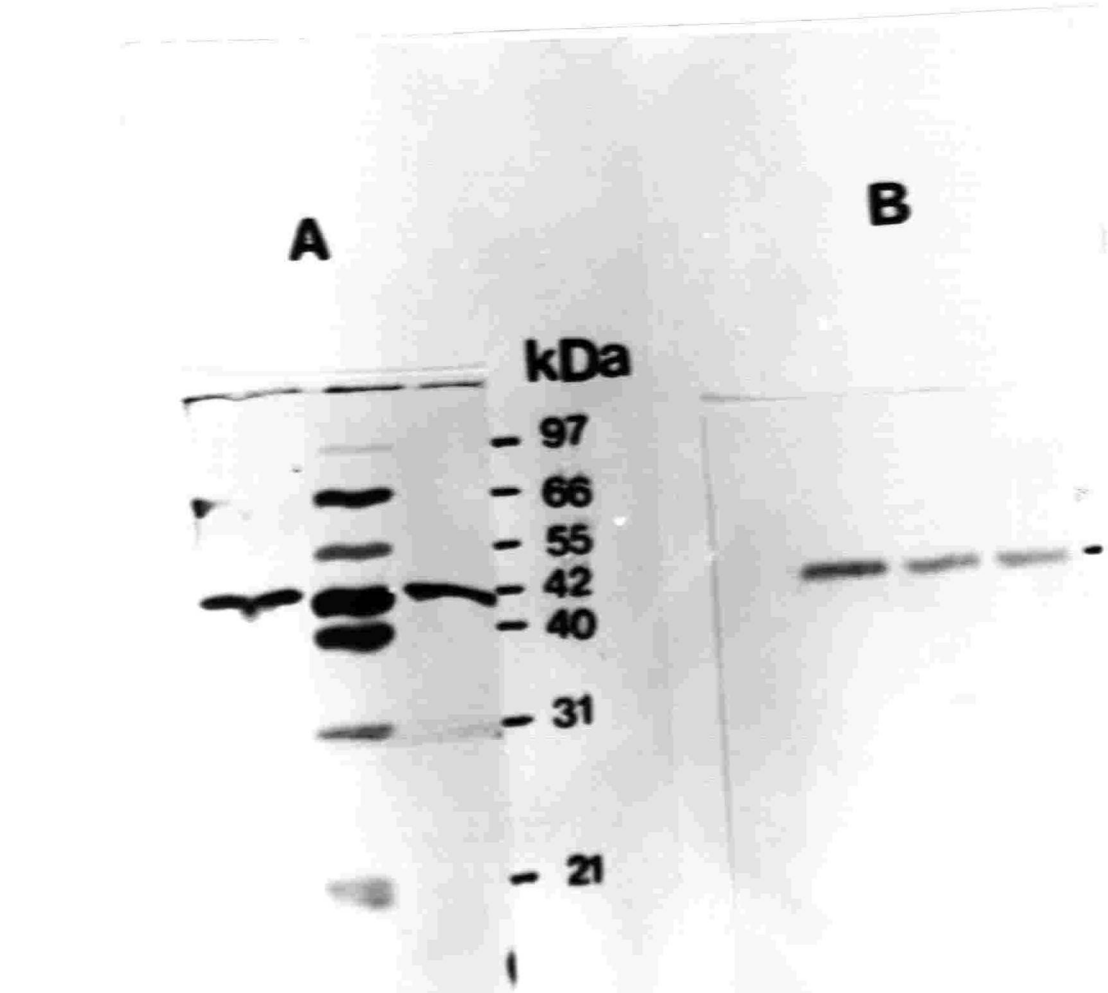
(B)Western blotting analysis of actin

Purified actin was subjected to SDS-PAGE on a 12% gel and the protein in the gel was transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated overnight with anti actin IgG (1:50). The membranes were re-exposed to HRPO-coupled anti-rabbit IgG (1:500). The blots were stained with 4-chloro-1-naphthol and H_2O_2

Figure 3.10. Chromatography of goat uterine cytosol on actin-Sepharose and purification of p55.

(A) Cytosol was chromatographed over a 10 ml column of actin-Sepharose The column was washed with TEMN and eluted with a linear NaCl gradient (50 mM to 1M NaCl in TEM) The fractions (1 5 ml) collected were subjected to the nuclear transport assay

(B) The fractions with ER transport activity eluted from actin-Sepharose were subjected to SDS-PAGE on a 12% gel The lane numbers are those corresponding to the fraction numbers in the figure 3 9A The gels were subjected to silver staining


A
B
kDa
97
66
55
42
40
31
21

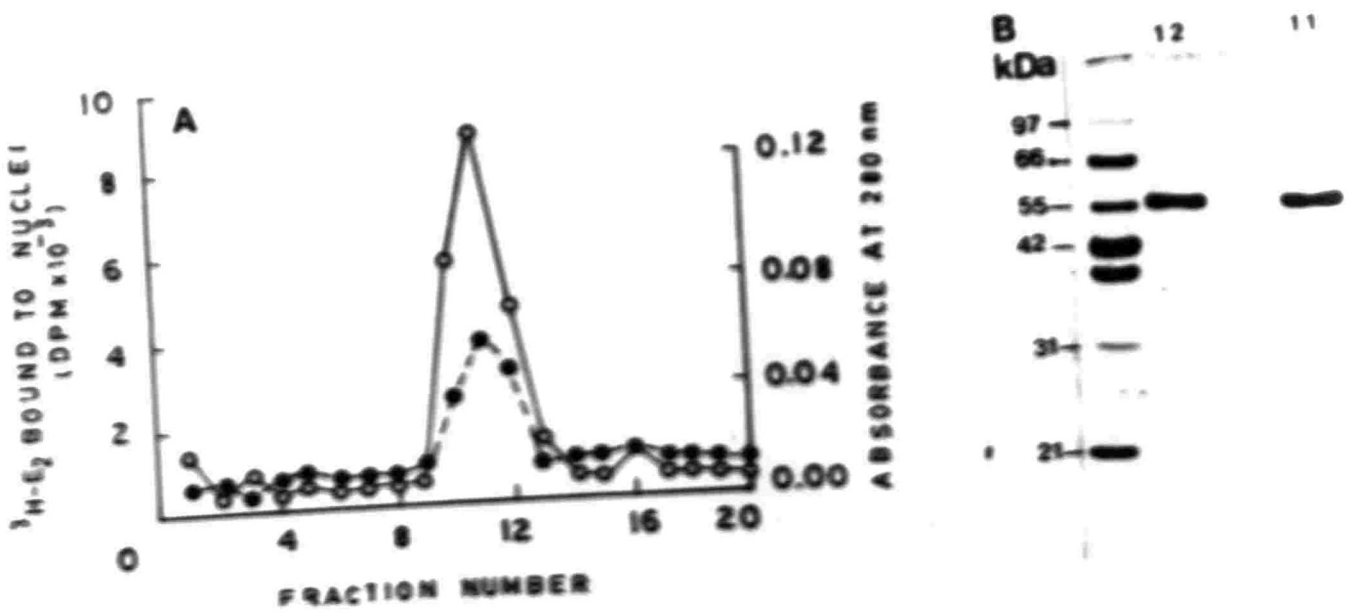
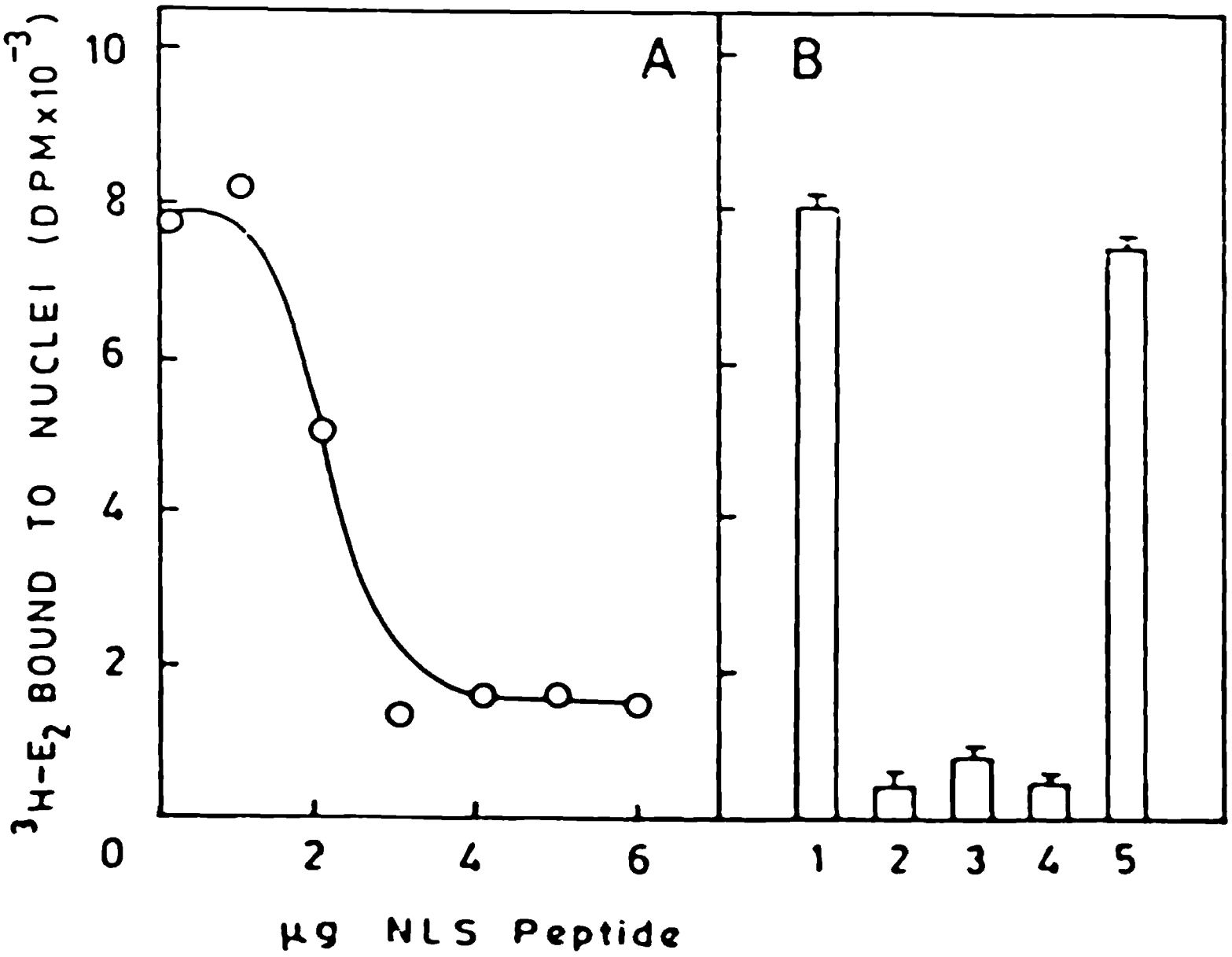

A
10
8
6
4
2
0
4
8
12
16
20
FRACTION NUMBER
0.12
0.08
0.04
0.00
B
kDa
12
11
97
66
55
42
31
21

Figure 3.11. Free NLS peptide blocks nuclear transport of the ER.

(A) The NT assay mixture consisting of ^{3}H-E_2-ER complexes (2 μg), nuclei and p55 (0.5 μg) was incubated with increasing concentrations of free NLS-peptide (0 to 6 μg) and the assay was performed as described under methods. The ^{3}H-E_2-ER complexes transported to the nucleus were measured and the transport function was expressed as ^{3}H-estradiol bound to nuclei.

(B) The nuclear transport assay was performed as described under methods: (1) control without exogenous NLS peptide. (2) to the assay mixture free NLS-peptide was added in the ratio 1:10. (3) the incubation mixture was same as in 2) but contained 10-fold excess of ER. (4) the incubation conditions were same as in 2) but the mixture contained 10 fold excess of nuclei (5) the incubation was performed as in 2) but the medium contained 10-fold excess of p55



μg NLS Peptide

Figure 3.12. Fluorescence assay for the ER nuclear transport.

Goat uterine nuclei were suspended in the nuclear transport assay buffer containing 4 mM ATP and were incubated with FITC labeled ER (5 μg) at room temperature for 30 minutes.

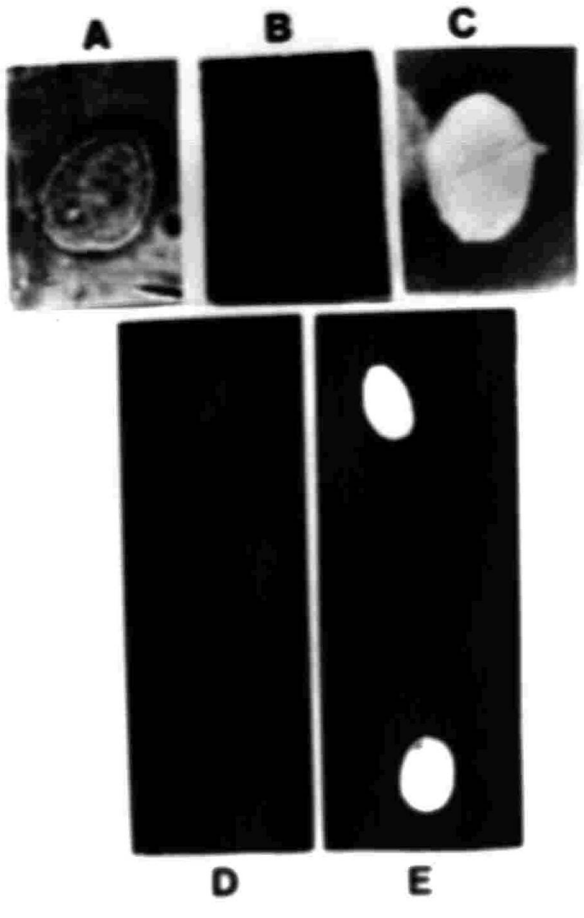
A) Phase contrast picture of nucleus 30 minutes after incubation without p55 (100X)

B) Fluorescence micrograph of (A)

C) Effect of addition of 0 5 μg of p55 on the nuclear transport of ER into the same nucleus

D) Goat uterine nuclei were suspended in the assay buffer and incubated with FITC labeled ER (5μg) at 30 C in the presence of 0 5 μg p55 and 0.1 μg of p28 for 30 minutes

E) to the above incubation mixture was added 0 1 μg of p73 and the nuclei were visualized 10 minutes later


A
B
C
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PURIFICATION AND CHARACTERISATION OF A NUCLEAR MEMBRANE/PORE COMPLEX – ASSOCIATED PROTEIN INVOLVED IN THE NUCLEAR TRANSPORT OF THE GOAT UTERINE ESTROGEN RECEPTOR.

It was observed from the studies reported by Nirmala and Thampan (1995a) that the transport of ER into the nucleus is mediated by an interaction between the 55 kDa cytosolic protein (p55) with a 12-14 kDa nuclear membrane/pore complex protein. Transport of the ER into the nucleus can be divided into two (a) peripheral binding, the p55-mediated binding of ER to the nuclear periphery. This step did not require ATP. (b) Translocation of ER into the nucleus. This step was found to be ATP dependent and also mediated by the 12-14 kDa protein(s) associated with the nuclear membrane/pore complex. Nirmala and Thampan succeeded in purifying the p55 to homogeneity (Nirmala and Thampan, 1995a). The p12-14 remained in a partially purified form in their studies.

The work presented in this chapter describes the procedure adopted for the purification of this low molecular weight protein and some aspects of the mechanism of nuclear transport of ER mediated by the nuclear membrane pore complex protein.

Only nuclei with intact membranes remained competent in transporting ER. The nuclei treated with Triton X-100 were incapable of transporting ER in the presence of p55 (Nirmala and Thampan, 1995a). Such a requirement of intact membrane for nuclear transport was also demonstrated independently by two groups (Imamoto-Sonobe et al., 1988; Zimmer et al., 1988). Nuclear pore complexes retain their structural integrity even after treatment of isolated rat liver nuclei with non-ionic detergent Triton X-100 (Aaronson and Blobel 1974; Gerace et al., 1982). The pore complexes remain attached to the nucleus, presumably through their association with the lamina.

The 12-14 kDa protein(s) fraction isolated and partially purified from the Triton X-100 solubilised material could restore the p55-mediated nuclear transport of ER when added to a medium containing Triton X-100 treated nuclei (Nirmala and Thampan 1995a). It appeared that that this 12-14 kDa protein(s) might be localized on the nuclear membrane close to the nuclear pore complex, the transport apparatus of the nuclei. The 12-14 kDa protein probably binds to the p55-ER complex, docks them to the NPC and presents the complex to the NPC transporter and translocates the ER into the nucleus. An important question that needed to be addressed here is "does p55 enter the nucleus along with the ER?"

4.1 PURIFICATION OF THE MEMBRANE/PORE COMPLEX ASSOCIATED PROTEIN:

In order to purify the membrane protein, chromatography of the detergent extract on various conventional matrices was attempted. Neither anion exchange resins (DE-52) nor cation exchange resins (phospho and CM-celluloses) proved as useful matrices because the proteins failed to bind to any of these columns.

The next attempt was made using lectin columns (Concavalin A and Wheat germ agglutinin), since lectins have been found to interact with membrane glycoproteins and thereby block the nuclear transport (Gerace et al., 1982: Finlay et al., 1987 Newmeyer and Forbes, 1988; Richardson et al., 1988).

If the nuclear membrane/pore complex protein involved in nuclear transport of ER is a glycoprotein, the lectin columns should have proved useful in its isolation. For this purpose WGA was purified from unprocessed wheat germ following the procedure of Nagata and Burger (1974). A single protein band of

molecular mass of 17 kDa was observed in the silver stained polyacrylamide gel of the protein fraction. WGA was coupled to Sepharose-4B as described in 'methods'. WGA has specificity towards the n-acetyl glucosamine residues. Chromatography of Triton X-100 extracted nuclear proteins was performed on WGA-Sepharose. It was observed that the p12-14 kDa protein(s) did not bind to the WGA-Sepharose column (data not shown).

In another experiment chromatography of the detergent extract was performed using a column of concanavalin-A-Sepharose. Con-A has the specificity for α-D-methyl glucopyranoside. This column was also found ineffective in the isolation of the nuclear membrane proteins (data not shown).

The above two experiments gave an indirect information related to a structural feature of the 12-14 kDa protein(s) that it is not a glycoprotein like many other nuclear membrane proteins. There is, however, no general agreement in that all the nuclear membrane or pore complex proteins are glycoproteins in nature.

Since the conventional chromatography and lectin affinity chromatography proved ineffective the alternative method would have been the use of the protein affinity column, p55-Sepharose. Making available p55-Sepharose in large quantities was not practical in view of its low yield and uncertainty about the half life of the column. Finally it was decided to rely upon the gel filtration technique involving Sephadex G-100.

4.2 CHROMATOGRAPHY OF TRITON X-100 EXTRACT ON SEPHADEX G-100:

Goat uterine nuclei were suspended in TCKM-sucrose buffer containing 0.05% Triton X-100 and the detergent solubilised material was dialyzed extensively against TEMN buffer. The dialysate was chromatographed over a column of DE-52 in a batch wise fashion and the flowthrough fraction was collected and concentrated. About 300 µl of the material was applied carefully onto the Sephadex G-100 column, which was preequilibrated with TEM buffer containing 0.3 M NaCl. Sodium chloride was added to the buffer to prevent non-specific adsorption of proteins to the Sephadex beads and self-aggregation of proteins resulting in the formation of large complexes. The flowrate of the column was maintained at 10 ml/hr. 2 ml fractions were collected and absorbance of the fractions at 280 nm was measured. All the fractions collected were analysed for the nuclear transport activity using a marginally modified nuclear transport assay system described below.

Goat uterine nuclei were isolated and suspended in TCKM-sucrose buffer containing 0.05 % Triton X-100. The nuclei were washed again to remove the detergent. ^{3}H-E_2-ER complexes were incubated with the detergent treated nuclei in the presence of pure p55 and the serial fractions eluted from the Sephadex G-100 column. A protein fraction that eluted from the column was seen to enhance the binding of the ER-p55 complex to the detergent treated nuclei. These fractions were subjected to SDS-PAGE using a 15% gel. Upon silver staining a 12 kDa molecular weight protein band was observed along with some other high molecular weight contaminants (Fig 4.2).

In order to remove the high molecular weight contaminants a second gel filtration step was performed. The active fractions from the first G-100 column were pooled, dialyzed in order to remove NaCl and concentrated through ultrafiltration. The concentrated material (around 300 µl) was re-chromatographed on a column of Sephadex G-100 that was also developed with TEM buffer containing 0.3 M NaCl. The fractions (2 ml) collected were assayed for the activity (Fig. 4.1) The active fractions were pooled and subjected to SDS-PAGE in a 12% gel. Upon silver staining a single band of molecular mass 12 kDa was observed. The high molecular weight contaminants were completely eliminated in the earlier fractions and they did not show any activity.

The p12 thus purified was used in further analysis.

4.3 CALUCULATION OF THE STOKES RADIUS:

The Stokes radius of the p12 was calculated by performing gel filtration analysis as described in the methods. Pure p12 was concentrated to a small volume and about 300 µl was chromatographed on a column of Sephadex G-100 (75 x 1.5 cm). The column was developed with TEM buffer containing 0.3 M NaCl. 2 ml fractions were collected and subjected to the nuclear transport assay involving detergent treated nuclei. The nuclear transport activity was found associated with a single peak eluted with 68-72 ml of the buffer (Fig.4.3)

The Sephadex G-100 column was precalibrated as described in the methods with proteins of known Stokes radii. Thus a standard graph of distribution coefficient (kd) versus Stokes radius was obtained for the column. The distribution coefficient (kd) of the p12 was calculated as below.

$$kd = \frac{Ve-Vo}{Vt-Vo}$$

Where Ve=volume with which p12 was eluted

$$\text{The p12 kd} = \frac{72-40}{108-40} = 0.47$$

The corresponding Stokes radius for the kd of 0.47 is 23 A^0. Thus the Stokes radius of p12 was determined to be 23 A^0.

4.4 p12 IS AN A INTEGRAL MEMBRANE PROTEIN:

It was of interest to know whether the 12 kDa protein is peripheral or integral in nature before proceeding with further studies.

The nuclei were isolated as described previously. The nuclear pellet was washed with buffer A (TCKM+250 mM sucrose) and the pellet was suspended in buffer A containing 150 mM NaCl or in buffer B (TCKM+250 mM sucrose+0.05% Triton X-100). The suspension was kept for about 10 minutes on ice. The nuclei were sedimented and the supernatant was subjected to SDS-PAGE. Upon silver staining the 12 kDa protein was observed only in the Triton wash but not in the NaCl wash. If the protein is loosely attached to the membrane by electrostatic interactions or is peripheral in nature it should have appeared in the NaCl wash. The results showed that the protein was extracted only by the detergent. Therefore it is reasonable to assume that p12 is an a integral nuclear membrane protein.

4.5 INTERACTION OF p55-ER COMPLEX WITH p12 AT THE NUCLEAR MEMBRANE.

Earlier experiments had demonstrated that addition of p12 to the assay system containing detergent treated nuclei restored the transport function. The ER-p55 complex can bind to the nuclear periphery resulting in the subsequent entry of the ER in the presence of exogeneously added p12. This is possibly due to the recognition of ER-p55 complex by p12 and the subsequent presentation of the same to the nuclear pore complex. The p12, therefore, acts as a docking protein in the nuclear transport. In order to examine the interaction more closely, p12-Sepharose column was made as described in methods and the following experiment was carried out. Goat uterine cytosol was chromatographed over a column of p12-Sepharose. The column was washed extensively with TEMN buffer and eluted with TEMN buffer containing 10 mM ATP. The column was washed with TEMN in order to remove ATP and elution was carried out once again using a 50 mM to 1M NaCl gradient. 2 ml fractions were collected which were assayed for their effect on the nuclear transport of ER (Fig 4.4A). The ATP eluate and all the salt eluted fractions were subjected to SDS-PAGE. Upon silver staining two protein bands of molecular mass 66 and 55 kDa, appeared in the ATP eluate while p55 alone appeared in the gradient fractions (Fig 4.4B). The result shows that with ATP elution all of the 66 kDa ER- p55 complex eluted in the first phase thereby showing a clear interaction of ER-p55 complex with p12 at the nuclear periphery. With the salt elution the remaining tightly bound p55 eluted from the column. This again shows a clear interaction between p55 and p12 at the nuclear membrane/pore complex.

To confirm the possibility that the 66 kDa and the 55 kDa bands represented the ER and the p55 respectively western blot analysis was performed. The ATP eluate and sodium chloride eluates were subjected independently to SDS-PAGE. The proteins were transferred to two nitrocellulose membranes. The ATP eluted proteins were incubated with anti ER antibody and the salt eluted proteins were incubated with anti p55 antibody. The blots were reexposed to HRPO coupled anti-rabbit IgG and stained with 4-chloro-1-naphthol and H_2O_2. Cross-reactivity was observed in both the blots confirming that the 66 kDa and the 55 kDa proteins represented ER and p55 respectively (Fig 4.4 C, a & b).

4.6 INTERACTION OF p12 WITH p55-SEPHAROSE:

In the experiments mentioned earlier, it was shown that direct interaction existed between the ER-p55 complex and the p12 immobilized on Sepharose. It was felt necessary to show the interaction between p12 and immobilized p55. This experiment was carried out to demonstrate the interaction of free p12 with p55 immobilized on Sepharose.

p55 was purified to homogeneity through chromatography of cytosol on a column of actin-Sepharose (Fig.4.5). Highly purified p55 was coupled to CNBr activated Sepharose. The nuclear Triton X-100 extract was dialyzed extensively to remove the detergent and was chromatographed over a p55-Sepharose column. The column was washed extensively with TEMN buffer. Elution was achieved using a linear gradient of 50 mM-1M NaCl. The fractions (2 ml) collected were analysed for the p12 activity using an assay system containing detergent-treated nuclei. A single peak of activity was observed that was eluted with 0.7-0.8 M NaCl (Fig 4.6 A). The fractions belonging to the peak activity were subjected to

SDS-PAGE. Silver staining revealed a single band of molecular mass 12 kDa (Fig 4.6B). That there were no other proteins bound to the column was indicative of the high specificity that characterized this interaction.

The mechanism of transport of karyophilic proteins into the nucleus can be separated into two major steps. The first is the recognition of the NLSs on the nuclear proteins by the NLS binding proteins (NLSBP) and the targeting of this complex to the nuclear pore complex. The second step is the translocation of the protein into the nucleus through the nuclear pore complex in a temperature and ATP dependent manner (Newmeyer and Forbes, 1988; Richardson et al., 1988).

The in vitro assay system can be subjected to easy manipulations and is free of other cytoplasmic proteins which can compete for the transport. By the removal and addition of various factors under examination, the mechanism of ER transport into the nucleus has been elucidated to a considerable extent.

4.7 INTERDEPENDENCE OF p55 AND p12 FOR THE NUCLEAR TRANSPORT OF ER INTO THE NUCLEUS:

The nuclear transport was performed in two ways to show the requirement of the 2 factors in an isolated nuclear system.

For the first assay system, intact nuclei (with membrane) were incubated with increasing concentrations of highly purified p55 (0-1μg). The p55 and ^{3}H-E_2-ER complexes were incubated with nuclei in the assay system at $30^{0}C$ for 30 mins. The ^{3}H-E_2-ER complexes transported into the nuclei were measured as described in the 'methods'. The control system contains the detergent treated nuclei under

identical conditions. There was a near linear increase in the transport observed in the presence of p55 upto a concentration of 0.5 μg per medium. The transport was found to reach the saturating limit in the presence of 0.5μg p55. No further increase in the transport was noticed in the presence of p55 concentrations beyond this level (Fig 4.7A). There was no transport detected in the control media, where p12 was absent.

The second set of experiment was performed using detergent treated nuclei and exogeneously added p12. ^{3}H-E_2-ER complexes were incubated with 1 μg of p55, detergent treated nuclei and increasing concentrations of highly purified p12 (0-0.5 μg) in the assay buffer at $30^{0}C$ for 30 minutes. The ^{3}H-E_2-ER complexes transported into the nucleus were measured. Control test was performed in the absence of p55 in the assay system. A near-linear increase in the transport was observed. The transport was found to reach the saturation limit in the presence of 0.3 μg. No further increase was observed in the transport beyond this level. Also there was no ER-transport observed in the control samples which did not contain p55.

From the results of the experiments mentioned above it is possible to draw 3 conclusions

1) both p55 and p12 are unavoidable requirements for the nuclear transport of ER.

2) the transport is saturable in both the cases when one or the other factor (either p55 or p12) is in limited quantity, suggesting that one protein is acting as a limiting factor for the other.

3) there is a clear indication for the interdependence of p55 and p12 for the nuclear entry of ER.

4.8 TRANSPORT OF ER INTO THE NUCLEUS IS RAPID

The nuclear transport of ER was studied using ^{3}H-E_2-ER complex, 1μg of p55 and either intact or detergent treated nuclei. For the first set of experiment, ^{3}H-E_2-ER complexes were incubated with p55 and detergent treated nuclei either in the presence or absence of p12 (0.3 μg) for different time intervals. The transport was terminated after 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 minutes of incubation. The nuclei were washed with the assay buffer and the radioactivity associated with the nuclei was measured following extraction of the nuclei-bound estradiol with ethanol. The transport of ER into the nucleus was observed to be very rapid. About half of the ER present in the medium was transported within ten minutes and almost the entire transport process was completed within 15 mins (Fig 4.8A).

In another set of experiment, the assay medium containing the ^{3}H-E_2-ER complexes, p55 (0.5μg) and control nuclei were incubated either in the presence or absence of p12 (0.3 μg). The transport was terminated after 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 minutes of incubation. The ^{3}H-E_2-ER complex that was bound to the nuclei was measured. The ER was transported into the nuclei within 15 minutes as in the case mentioned above. There was no marked difference observed in the time taken for the nuclear transport between the two experiments suggesting that the exogeneously added p12 to the detergent treated nuclei could fulfill the entire requirement of an intact nuclear membrane during the transport (Fig 4.8B).

In another experiment it was observed that when p12 added exogeneously to the control nuclei, decreased the nuclear transport of ER to a considerable extent. This could be due to the binding of free p12 to p55 in the medium thereby making it unavailable for interaction with the p12 associated with the nuclear

membrane. A similar observation was reported by Tachibana et al., (1996) who observed that cytoplasmically injected p10/NTF2 strongly inhibited the nuclear import of NLS-containing substrates in a dose-dependent manner.

4.9 TRANSPORT OF ER INTO THE NUCLEUS IS ATP DEPENDENT:

The nuclear transport assay was performed as described before but the assay media were prepared with different concentrations of ATP added to them.

The transport of ER in the presence of p55 was performed in the presence of 1, 2, 3, 4, 6 or 8 mM ATP in the assay media. A control set was incubated with ATP-free media. The experiment was performed using both intact nuclei and detergent treated nuclei, the latter having been supplemented with exogeneously added p12.

There was no transport of ER observed in the absence of ATP and an almost linear increase in the ER transport was observed in the presence of different concentrations of ATP, leading upto 4 mM. Further increase in ATP concentration did not cause a corresponding increase in the transport (Fig 4.9 A & B).

4.10 TRANSPORT OF THE ER INTO THE NUCLEUS IS MAGNESIUM DEPENDENT:

The transport assay was performed as described before but the assay media contained different concentrations of $MgCl_2$.

The transport of ER in the presence of p55 was performed with 1 to 8 mM concentrations of $MgCl_2$ in the assay buffer. The control medium did not contain $MgCl_2$. The experiment was performed in two sets. One set with control nuclei and p55 and the other set with detergent treated nuclei, p55 and p12. There was a linear increase in the ER transport observed in the presence of increasing concentrations of $MgCl_2$ reaching a plateau in the presence of 5 mM $MgCl_2$. No transport was found to take place in the absence of $MgCl_2$ (Fig 4.10 A & B).

4.11 ATPase ACTIVITY:

The p55 mediated transport of ER is ATP-dependent and it has also been shown that p55 has an inherent ATPase activity (Nirmala and Thampan, 1995b). It was of interest to examine if the p12 also possessed any ATPase activity. Increasing concentrations of p55 (0.2-2 μg) or p12 (0.2- 2 μg) were incubated with 6 mM ATP at 30^0C for 30 minutes as described in the methods. The inorganic phosphate (Pi) released into the supernatant as a result of the enzyme action was estimated. A linear increase in ATPase activity was observed depending on the concentration of the protein added. The activity was directly proportional to the amount of either p55 or p12 added to the medium (Fig 4.11 A & B). The results confirmed the assumption that both the p55 and p12 are ATPases. The energy released as a result of ATP hydrolysis is probably required for the transport of ER into the nucleus.

4.12 ENHANCED ATPase ACTIVITY DURING THE COMBINED PRESENCE OF p55 AND p12:

It was of interest to see if the simultaneous presence of the two proteins in the assay medium caused an enhancement in the total enzyme activity.

Increasing concentrations of p12 and p55 were incubated in a 1:1 ratio (0-4 μg) with 6 mM ATP at 30^0C for 30 minutes. A control set contained increasing concentrations of p12 in the presence of 6 mM ATP. The Pi released into the supernatant was calculated. The ATPase activity associated with the p12 increased rapidly till the protein concentration in the medium reached 2.5 μg and plateaued off subsequently. Under identical conditions the ATPase activity of the p12-p55 complex displayed a marked enhancement (Fig 4.12). The reaction rate plateaued off in the presence of a lower concentration of the enzyme. The enhanced activity of the protein mixture was found to use up the substrate in the medium in a very fast manner probably indicating that the interaction between p12 and p55 brought about an added sensitivity to the ATPase function of the two proteins.

4.13 ATPase ACTIVITY IN THE PRESENCE OF MONOVALENT AND DIVALENT CATIONS:

The ATPase activity associated with the nuclear pore complex (NPC) or the nuclear membrane is a Mg^{++}-activated ATPase and plays an important role in RNA transport through the NPC (Clawson et al., 1984; 85). The nuclear ATPase was found to be inhibited by Quercetin, a flavonoid inhibitor of Mg^{++}-dependent ATPase (Thampan, 1988). It was therefore of interest to see the metal ion dependency of the ATPase activity associated with p55 and p12.

Two micrograms of p55 and 2 μg of p12 were incubated independently with increasing concentrations of $MgCl_2$ or $CaCl_2$ (0-5 mM). The incubation was carried out at 30^0C for 30 minutes in the presence of 6 mM ATP. The reaction was terminated following the addition of 20% TCA. The inorganic phosphate released into the TCA soluble supernatant was estimated. The ATPase activity associated

with both p55 and p12 was found to increase in a linear fashion, directly proportional to the amount of $MgCl_2$ or $CaCl_2$ added to the medium (Fig 4.13 A & B). This suggested that the p55 and p12 are Mg^{++} and Ca^{++} dependent ATPases. NaCl and KCl failed to support the ATPase activity of both the proteins (data not shown).

4.14 NUCLEAR TRANSPORT IS INHIBITED BY QUERCETIN:

As seen in the above experiment, both p55 and p12 are Mg^{++}-dependent ATPases. The ATPase activity associated with these two proteins will help in the nuclear transport of the ER from the cytoplasm to the nucleus. It was of interest to see whether quercetin, an inhibitor of Mg^{++}-dependent ATPase could inhibit the nuclear transport by virtue of its function as an inhibitor of the ATPase activity.

The nuclear transport assay was conducted using both intact nuclei and detergent treated nuclei in the presence of varying concentrations of quercetin (0-20 μg/ml). The radioactivity bound to the nuclei was estimated. The nuclear transport activity displayed was inversely proportional to the concentration of quercetin used in the incubation medium (Fig 4.14 A & B). Half-maximal inhibition of activity was observed with quercetin at 8 μg/ml concentration.

4.15. FLUORESCENCE ASSAY OF THE ER TRANSPORT INTO THE NUCLEUS:

The conventional nuclear transport assay that employs ^{3}H-estradiol-labeled ER does not distinguish between binding of the ER to the nuclear membrane and transport of the ER into the nucleus. To confirm the actual transport of ER into the

nucleus, the fluorescence assay for ER transport was performed as descried in the 'methods'.

Goat uterine ER was purified and conjugated with FITC following the procedure of Chard et al (1987). The nuclear transport of ER required an intact nuclear membrane as was observed in the experiments mentioned above using the nuclear transport assay system. To confirm this, the fluorescence assay was performed. Detergent treated nuclei were suspended in the assay buffer. The nuclei were incubated with FITC-ER alone or with 1 μg of p55 at 30^0C for 30 minutes. The transport was examined with the help of a Nikon fluorescence microscope. No transport of ER was seen in the detergent treated nuclei in the absence of the p55 (Fig 4.15A). Following the addition of p55, the FITC-ER was seen to be localized at the nuclear periphery, visualized as rim staining (Fig 4.15 B). Upon addition of 0.3 μg of p12, complete entry of the ER into the nucleus was observed within 2 to 3 minutes of the exposure of the nuclei to the mixture (Fig 4.15D).

To study the ATP dependence of the ER nuclear transport, intact nuclei were incubated with FITC-ER (5 μg) and p55 (0.5 μg) in the assay medium containing no ATP for 30 minutes at 30^0C. In the absence of ATP the FITC-ER was seen bound to the nuclear membrane (rim staining)(Fig 4.15F). Following the addition of ATP into the incubation medium, a rapid entry of FITC-ER into the nucleus could be observed (Fig 4.15G).

Quercetin inhibited the nuclear transport of ER mediated by p55 and p12. To observe this effect in the fluorescence assay, intact nuclei were suspended in the assay buffer in the absence of ATP. The nuclei were incubated with 5 μg of

FITC-ER and 0.5 μg of p55 at 30^{0}C for 30 minutes. These nuclei showed rim staining. In another experiment ATP and quercetin were added simultaneously to final concentrations of 4 mM and 10 μg/ml respectively to the incubation media. The nuclei, at the end of the ten minute's incubation period continued to show only rim staining (4.15H), indicating that quercetin inhibited the transport of the ER into the nuclei.

4.16 p55 DOES NOT ENTER THE NUCLEUS AND IS LEFT BEHIND AT THE NUCLEAR PERIPHERY:

Earlier studies (Nirmala and Thampan, 1995a) and the data presented here regarding p55-p12 interaction gave only an indirect indication regarding the association of p55 with the nucleus. It was not clear whether the p55 gained complete nuclear entry along with the ER or was left behind at the pore complex/nuclear membrane.

There are 2 types of receptors (NLSBPs) that carry nuclear proteins from the cytoplasm to the nucleus. One type, the **docking receptor** binds to the nuclear protein and mediates the transport till the nuclear membrane, transfers the nuclear protein into the nucleus and goes back to the cytoplasm to carryout another round of the transport. The other type is the **shuttling receptor,** which binds to the nuclear protein, transfers the protein to the nuclear membrane and gets co-transported into the nucleus along with the nuclear protein. In the nucleus, it releases the nuclear protein following which it moves back to the cytoplasm and performs another round.

p55 was labeled with FITC following the procedure of Chard et al., (1987). Goat uterine nuclei were incubated with FITC-p55 in the presence of unlabelled ER and 4 mM ATP. The FITC-p55 was seen bound to the nuclear periphery, visualized as rim-staining (Fig 4.16B). (Fig 4.16 A is the phase contrast photograph of Fig. 4.16B). It was clearly indicative of a situation where p55 could not enter the nucleus during the transport process and was retained at the nuclear periphery.

4.17 FLOURESCENCE EVIDENCE FOR THE PRESENCE OF p12 AT THE NUCLEAR MEMBRANE/PORE COMPLEX :

To show the presence of p12 at the nuclear membrane, fluorescence assay was carried out using FITC-p12. P12 was labeled with FITC following the procedure of Chard et al., (1974). Goat uterine nuclei were incubated with p55, ER and FITC-p12 in the presence of 4 mM ATP. Incubation was carried out at 30^0C for 30 mins. There was a clear punctated rim staining observed around the nuclear periphery, showing that p12 is localized at the nuclear membrane/pore complex (Fig 4.16 D & F).

DISCUSSION

A method for the purification of p12 has been developed. The p12 protein purified from the goat uterine nuclear membrane probably recognizes the p55-ER complex, docks them to the NPC by binding to the complex and presents the complex to the NPC transporter and translocates the ER into the nucleus. The nuclear pore complexes retain their structural integrity even after treatment with Triton X-100 and remain attached to the nucleus and perform their usual function (Aaronson and Blobel, 1974). The Triton treatment in the present experiment does

not disturb the actual transporter (NPC). Only nuclei with intact membranes are competent in transporting ER. The nuclei treated with Triton X-100 remained incapable of transporting ER in the presence of p55.

p12 could be extracted from the membrane only through detergent treatment and not with salt wash. If the protein is loosely attached to the membrane through electrostatic interactions or is peripheral in nature it should have appeared in the salt (NaCl) wash. That the p12 was extractable only by Triton X-100 treatment implies that the protein is likely to be either a nuclear membrane integral protein or a detergent extractable nuclear pore complex protein.

One important point that requires special attention here is that the low molecular weight 12 kDa protein is not a histone, because histones are not extractable with Triton X-100 (Aaronson and Blobel, 1974).

Most of the nuclear membrane proteins which participate in nuclear transport are glycoproteins. Nevertheless, the p12 does not appear to be a glycoprotein as it failed to bind either to WGA or Con-A Sepharose columns. Whether the low molecular mass nature of the protein did not favour the glycosylation process is a matter for debate.

The interaction between p55 and p12 was found to be very specific. From among the total mass of cytosolic proteins either free p55 or p55-ER complex alone could interact with p12. Likewise the p55 was found to interact only with p12 from among the total nuclear membrane proteins extracted with the detergent. This shows a very highly specific interaction that exists between these two proteins, required for the selective entry of ER into the nucleus.

Percipalle et al.(1997) observed that importin-β interacts with multiple factors like nucleoporin p62 (equivalent to p12), Ran and NTF2. Similar proteins were not observed among the p55 (equivalent to importin)-Sepharose bound proteins. NTF2 when bound to Sepharose beads, is able to interact specifically with both Ran and xFxFG repeat containing domains of nucleoporin p62 (Clarkson et al., 1996). Paschal and Gerace (1995) identified NTF2 through its interaction with immobilized nucleoporin p62. If one considers the p12 as equivalent to p62, then it should have shown a capacity to interact with other cytoplasmic factor like NTF2. However, other proteins were not observed to be bound to the p12-Sepharose indicating the absence of proteins equivalent to NTF2. Therefore, the concept that 'multiple factors' are involved in the nuclear transport of proteins need not be applicable to all the protein systems. Most of the factors identified in the other systems were recognized based on the import assay using a synthetic NLS-peptide coupled to FITC-labeled BSA. They are not based on the use of any individual protein system. The factors necessary for the import of synthetic peptides need not be equivalent to the factors required for the transport of biochemically active and native proteins. There are no reports regarding individual protein transport systems like the one reported for estrogen receptor, presented in this study.

From this study, what could be concluded is that each nuclear protein will possibly have a very selective and specific pathway for its nuclear transport and also that each nuclear protein will have its own set of cytosolic and nuclear membrane proteins for transporting the protein into the nucleus.

Mechanisms associated with the nuclear transport of ER have been examined through a detailed analysis of the nuclear transport assay. Since this assay does not clearly distinguish between peripheral binding of ER to the nuclear membrane and the actual transport into the nucleus, it was decided to perform a fluorescence assay for nuclear transport of ER using FITC labeled ER, p55 and p12. The nuclear binding or the nuclear transport observed in the transport assay reflects truly the ER that is transported into the nucleus. The transport assay did not detect any rim staining i.e., peripheral binding. In the case when clear rim staining was observed in the fluorescence assay, the transport assay did not detect any binding activity. This indicated that the peripheral binding was probably not strong enough to withstand the stringent washing conditions of the nuclear transport assay.

There is a clear interdependence of these proteins for the nuclear transport. In the absence of one factor (p55 or p12) the other will not support the nuclear transport. This shows clearly that both the proteins are equally important for the nuclear transport function.

Transport of ER into the nucleus was very rapid and was completed within 10-15 minutes of incubation. The transport was ATP and magnesium dependent. Requirement for 4 mM ATP and 5 mM $MgCl_2$ and the absence of transport in the presence of quercetin along with 4 mM ATP all suggested the possibility that ATP was hydrolyzed and the energy released was utilized in the translocation of ER into the nucleus. This was also confirmed by the observation that quercetin, an ATPase inhibitor, inhibited translocation of ER into the nucleus. Requirement of

ATP and $MgCl_2$ for the nuclear transport of other proteins have also been demonstrated (Newmeyer et al., 1986; Richardson et al., 1988).

Both p55 and p12 are ATPase and there is an enhancement in the total ATPase activity when the two proteins are put together. Both the proteins are Mg^{++}-dependent ATPases. The inhibition of transport by quercetin could be due to the inhibition of the ATPase activity associated with the two proteins.

Since the first step in the nuclear transport, the p55-mediated peripheral binding of the ER did not require ATP, the ATPase activity of the p55 and p12 comes into play only after binding of the p55-ER complex to the nuclear membrane. The p55 stimulates the ATPase activity associated with the p12. The energy released at this step is probably utilized in the actual transport of the ER-p55 complex into the nucleus.

Based on the results of the work presented in this chapter a model for the transport of ER to the nuclei has been proposed (Fig 4.17). From the punctated appearance of the detergent-treated nuclei with FITC-labeled p12 it may be assumed that the p12 is localized possibly at the nuclear pore complex. The ER-p55 complex gets docked at the p12 of the pore complex. The p55-ER interaction weakens, facilitating the solitary entry of the ER into the nucleus. The p55 is left behind, for its re-entry into the cytosol and binding to a fresh receptor molecule.

Figure 4.1 Purification of p12 following gel filtration on Sephadex G-100.

Goat uterine nuclei were isolated as described in the 'methods'. The nuclei were washed with 0.05% Triton X-100. The Triton X-100 wash fraction was dialyzed overnight against TEMN buffer. The dialysate was chromatographed over a column of DE-52 equilibrated with TEMN buffer. The flow through fraction as well as one column wash from the DE-52 column was concentrated and about 300 µl of the concentrated material was chromatographed on a pre-calibrated column of Sephadex G-100 (75X1.5 cm). The column was developed with TEM buffer containing 0.3 M NaCl. Two milliliter fractions were collected and subjected to the nuclear transport assay which employed detergent treated nuclei. The active fractions were collected, dialyzed and concentrated through ultrafiltration. Three hundred microliters of the material was re-chromatographed on the same Sephadex G-100 column. Two milliliter fractions were collected and assayed for the activity. The figure 4.1 displays the activity profile (●) as well as the 280 nm absorbance profile (0) of the fractions recovered from the second Sephadex G-100 column.

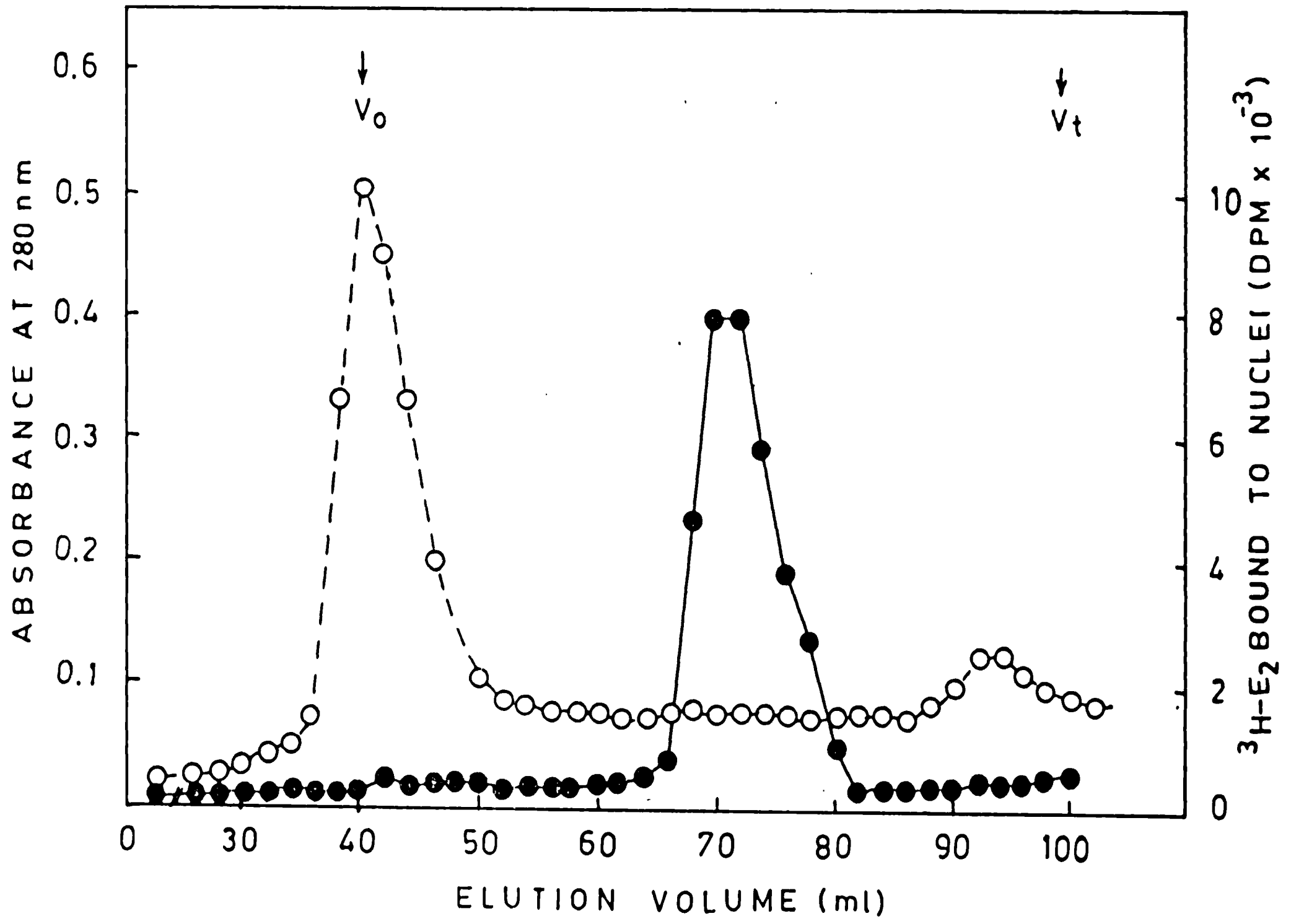

0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 280 nm
V_o
V_t
10
8
6
4
2
0
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
30
40
50
60
70
80
90
100
ELUTION VOLUME (ml)

Figure 4.2 SDS-PAGE analysis of the Sephadex G-100 fractions.

The peak fractions from the first Sephadex G-100 column (A) and the second Sephadex G-100 column (B) displaying activity were pooled individually and subjected to SDS-PAGE on a12% acrylamide gel. Both the fractions were run side by side for comparision. The gel was stained with silver nitrate.

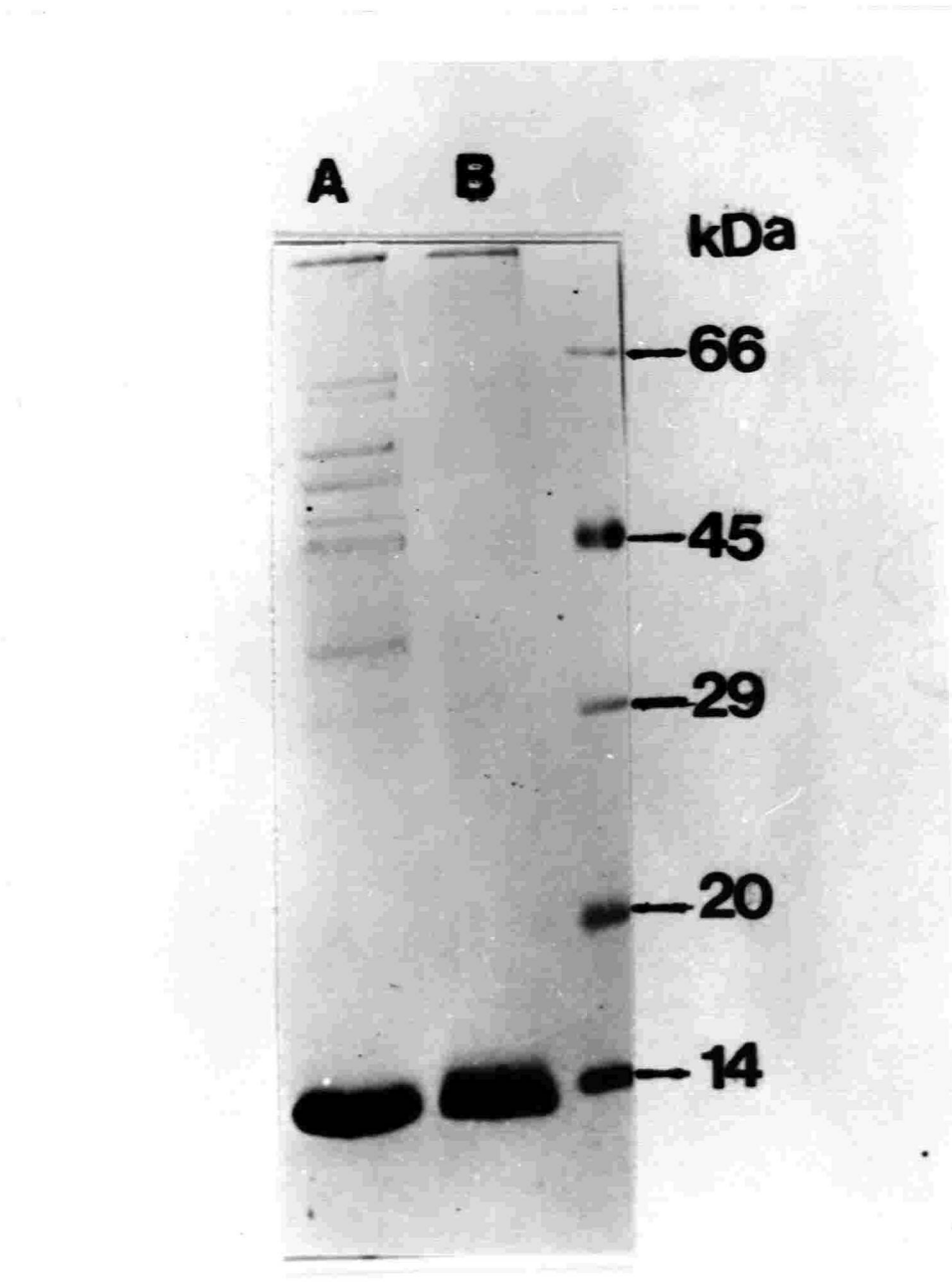

A
B
kDa
66
45
29
20
14

Figure 4.3. Determination of the Stokes radius of p12.

The purified p12 was chromatographed over a precalibrated column of Sephadex G-100 (75 X 1.5 cm). The column was developed with TEM buffer containing 0.3 M NaCl and 2 ml fractions were collected. The fractions were subjected to the nuclear transport assay. The column was pre-calibrated with bovine serum albumin (BSA), ovalbumin (OV) and cytochrome C (CYT). The inset is a standard graph of kd (distribution coefficient) vs R_S (Stokes radius).

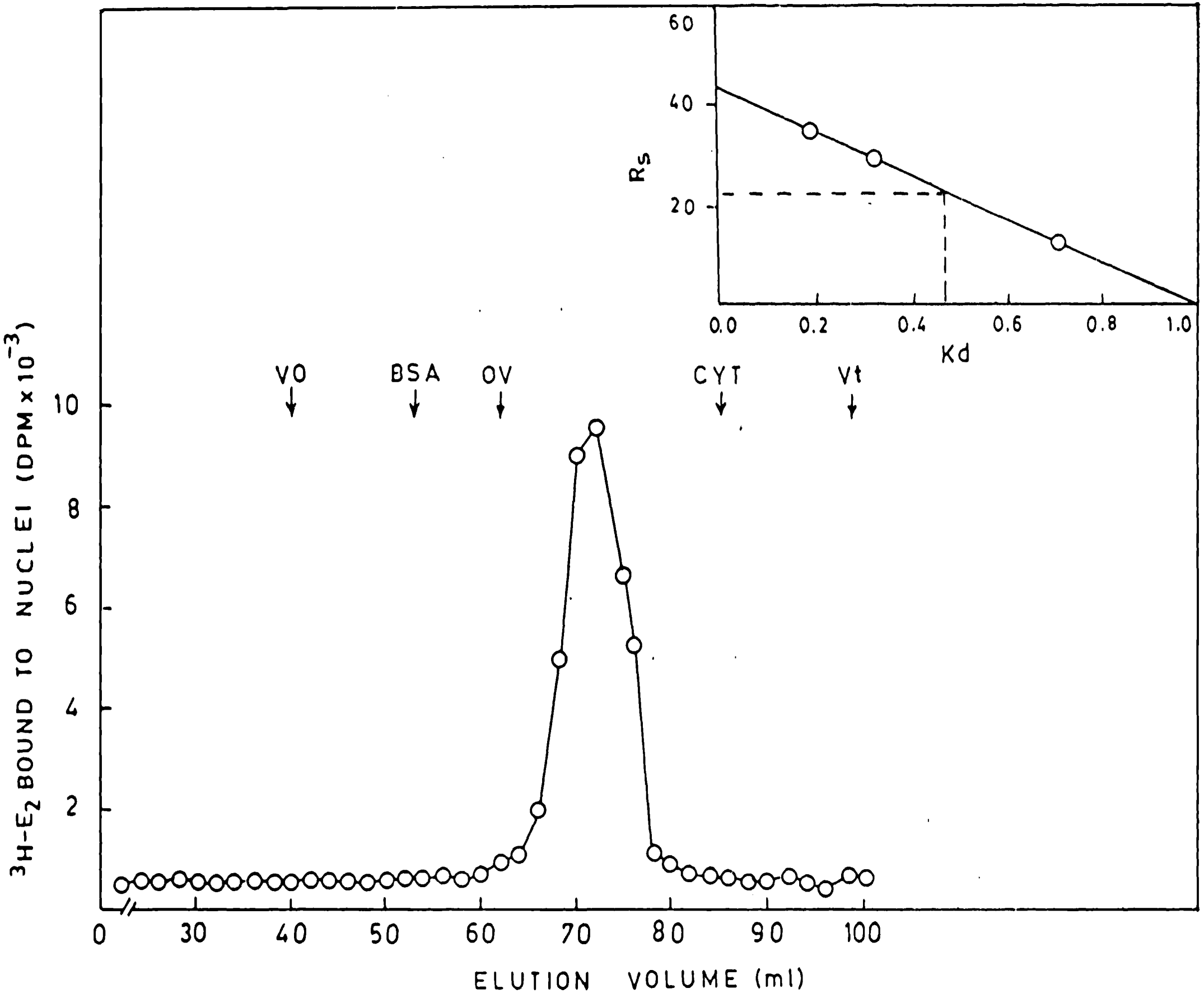
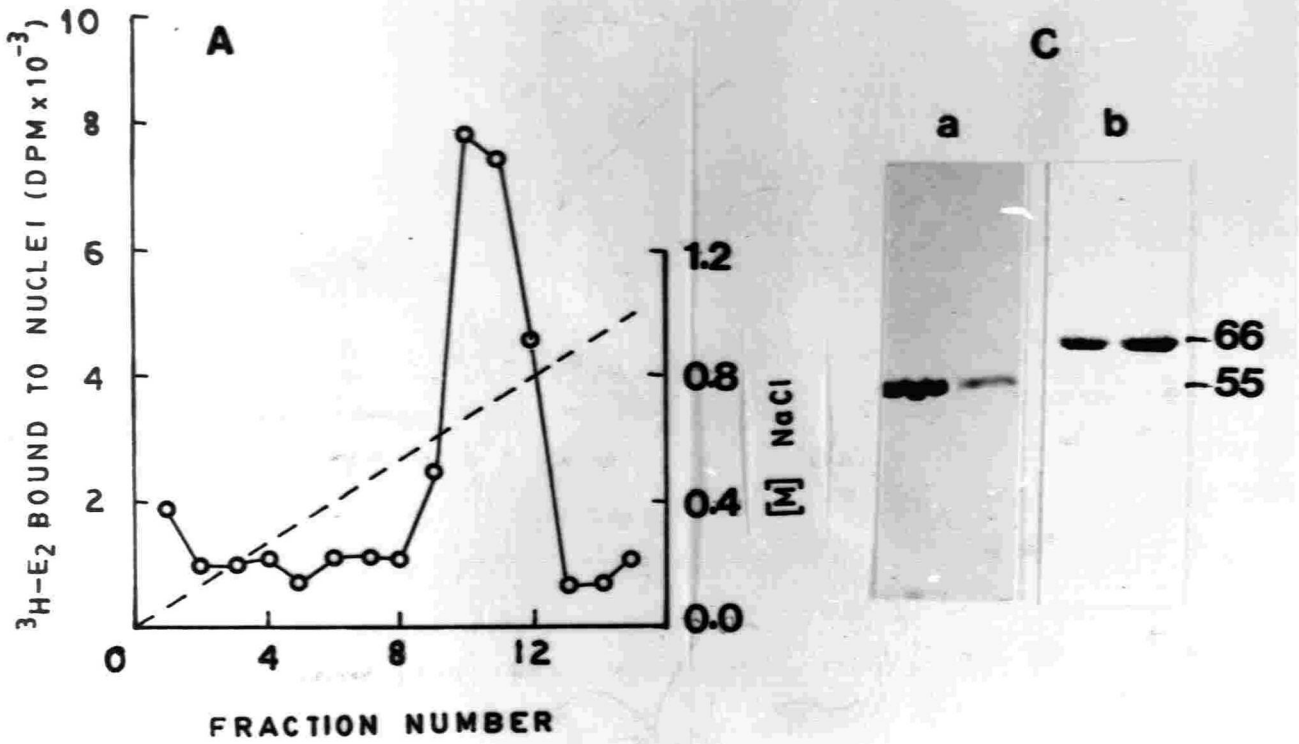

60
40
20
Rs
0.0
0.2
0.4
0.6
0.8
1.0
Kd
VO
BSA
OV
CYT
Vt
10
8
6
4
2
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
30
40
50
60
70
80
90
100
ELUTION VOLUME (ml)

Figure 4.4 Specific binding of p55 to p12-Sepharose.

(A) Goat uterine cytosol was chromatographed on a column of p12-Sepharose. The column was washed with TEMN buffer and eluted with the buffer containing 10 mM ATP. The column was washed with the TEMN buffer and elution was achieved using a 50 mM to 1M NaCl gradient in the same buffer. The eluate was collected in 2 ml fractions and were subjected to the nuclear transport assay.

(B) The ATP eluate and the salt eluted fractions were subjected to SDS-PAGE on a 10% gel. The gel was stained with silver nitrate. The numbers on the lanes correspond to the fraction numbers shown in (A).

(C) (presented along with 'A') The 1M NaCl eluate and the ATP eluate were subjected to SDS-PAGE on 10% gels. The proteins were transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated overnight one with anti p55 antibody (a) and the other with anti ER antibody (b) both having been used at 1:50 dilution. The blots were washed in order to remove the unbound antibody and were re-incubated with HRPO-coupled anti-rabbit IgG (1:500). The blots were stained with 4-chloro-1-naphthol and H_2O_2.


A
^{3}H-E$_2$ BOUND TO NUCLEI (DPM x 10^{-3})
10
8
6
4
2
0
4
8
12
FRACTION NUMBER
1.2
0.8
0.4
0.0
[M] NaCl
C
a
b
66
55

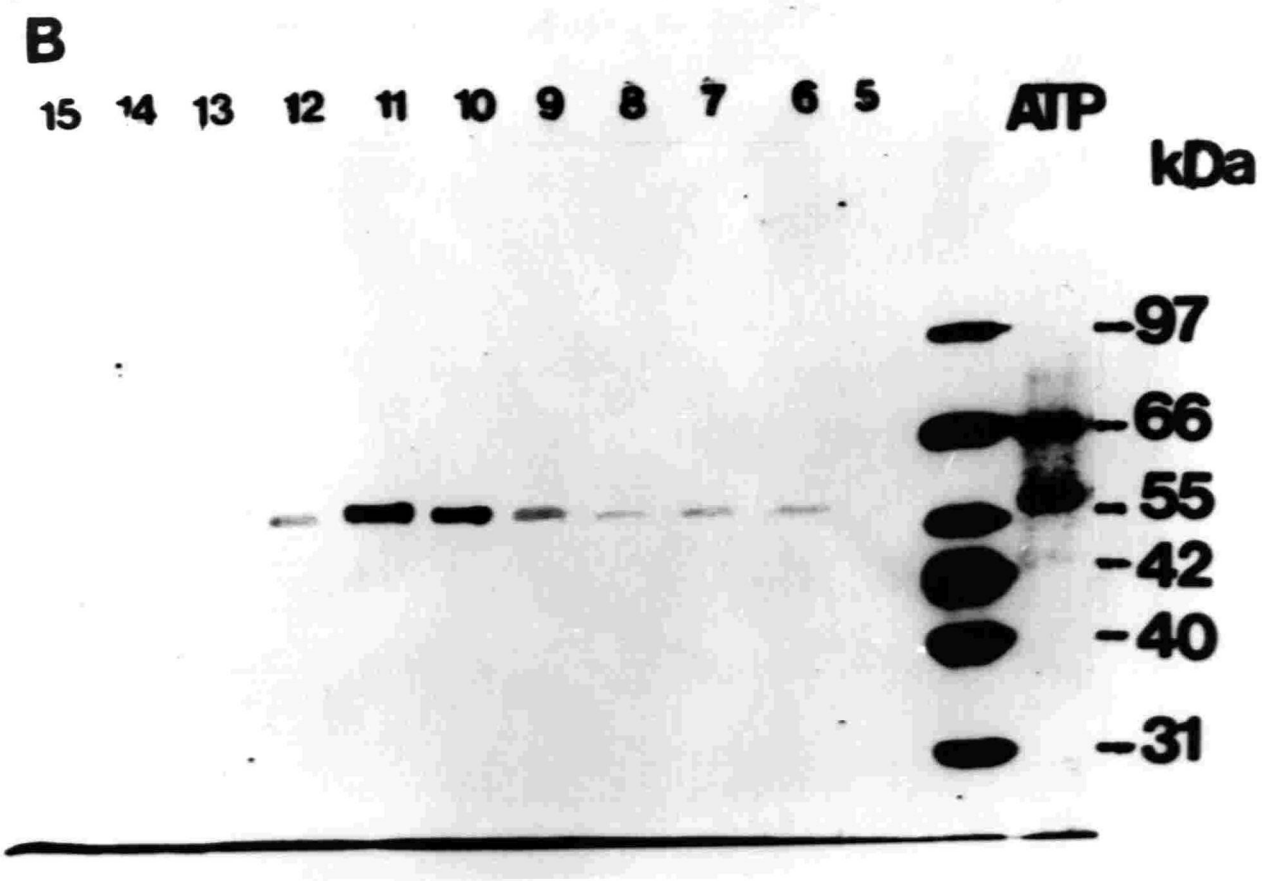

B
15 14 13 12 11 10 9 8 7 6 5
ATP
kDa
97
66
55
42
40
31

Figure 4.5 (A) SDS-PAGE analysis of p55.

p55 was purified as described in the 'methods'. The purified protein was subjected to SDS-PAGE on a 10% gel. The gel was stained with silver nitrate.

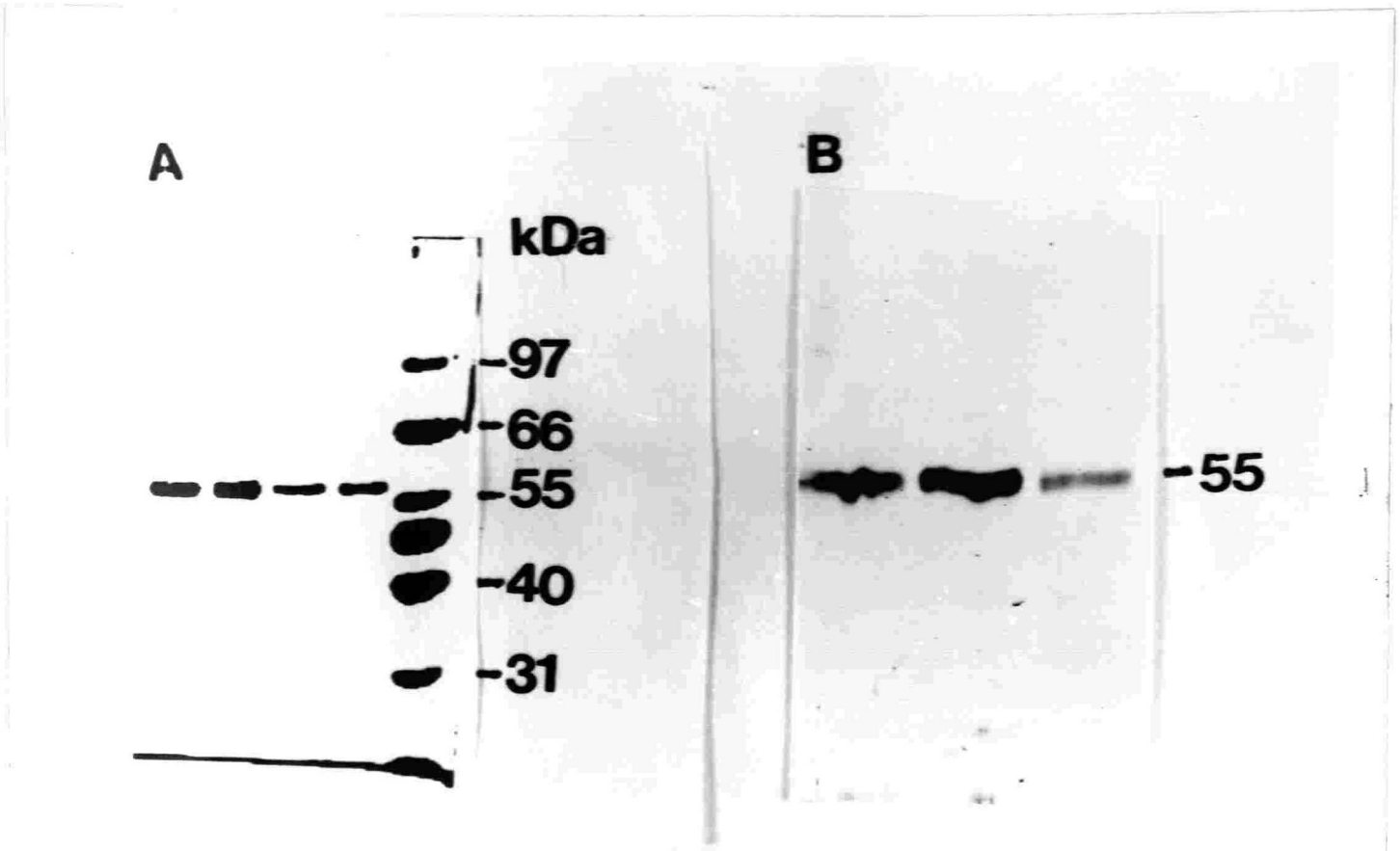
B) Western blot analysis of p55.

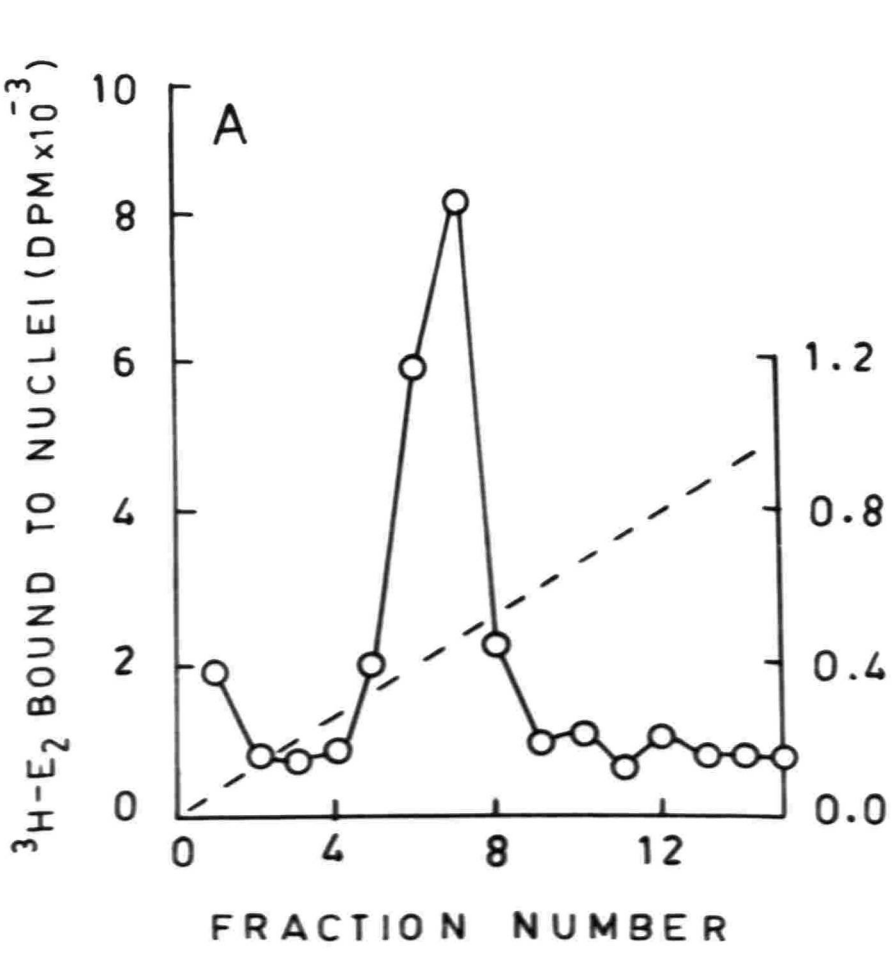
Isolated p55 was subjected to SDS-PAGE on a 10% gel and the proteins were transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated overnight with p55 antibody (1:50). The membranes were re-exposed to HRPO-coupled anti-rabbit IgG (1:500). The blots were stained with 4-chloro-1-naphthol and H_2O_2.

Figure 4.6. Specific interaction of p12 with p55-Sepharose.

(A) Goat uterine nuclei were isolated as described in methods. The nuclei were washed with 0.05% Triton X-100. The Triton X-100 wash fraction was dialyzed against TEMN buffer. The dialysate was chromatographed over a p55-Sepharose column. The column was washed with TEMN buffer and eluted with a linear NaCl gradient (50 mM to 1M NaCl in TEM). 2 ml fractions were collected and subjected to the nuclear transport assay.

(B). The fractions showing maximum activity were subjected to SDS-PAGE on a 15% acrylamide gel. The gel was stained with silver nitrate. The numbers 6 and 7 on the lanes correspond to the fraction numbers shown in (A). The middle lane represents the molecular weight markers.


A
kDa
97
66
55
40
31
B
55


A
10
8
6
4
2
0
^{3}H-E$_2$ BOUND TO NUCLEI (DPM x10^{-3})
1.2
0.8
0.4
0.0
[M] NaCl
0
4
8
12
FRACTION NUMBER

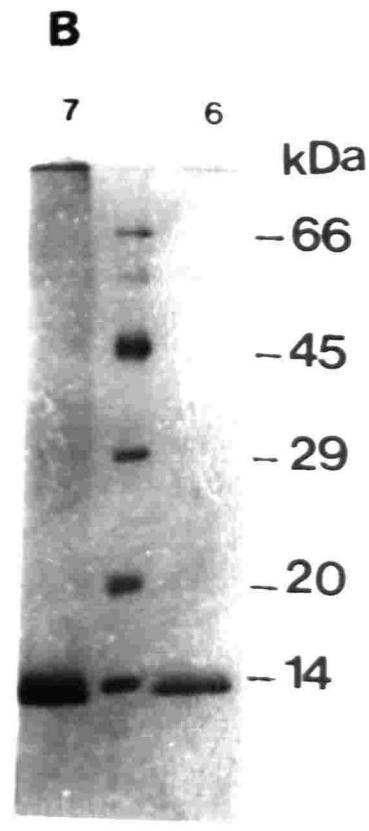

B
7
6
kDa
66
45
29
20
14

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}H-E_2-ER complexes and intact nuclei at 30^{0}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 (O). The ^{3}H-E_2-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}H-estradiol bound to nuclei.

(B) 0 to 0.5 μg of p12 was incubated with ^{3}H-E_2-ER complexes, 0.5 μg of p55 and detergent treated nuclei (without membrane) at 30^{0}C for 30 min (●) and the assay was performed as described previoulsy. Control set was incubated without p55 in the assay medium (O).

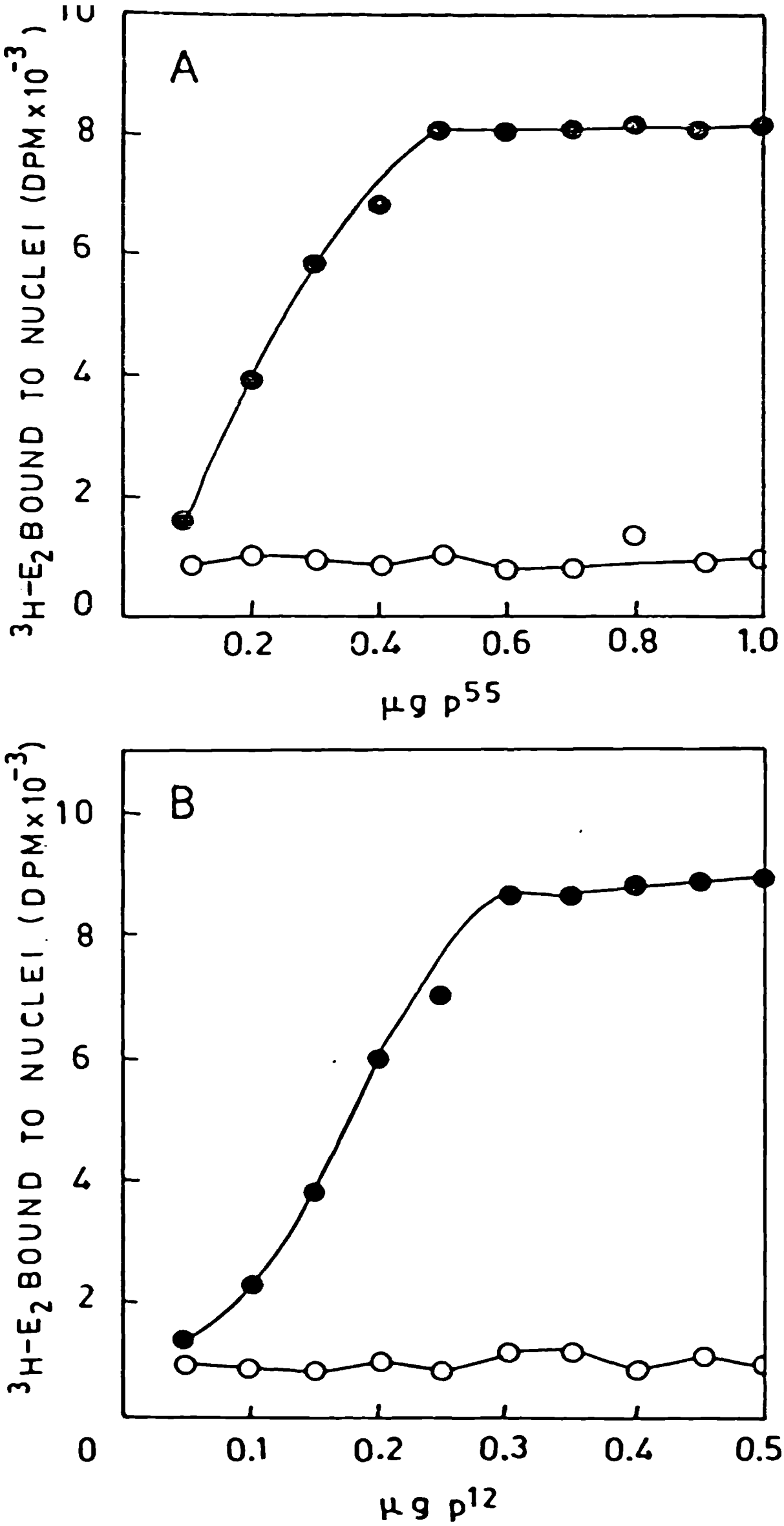

A
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
2
4
6
8
0.2
0.4
0.6
0.8
1.0
µg p^{55}
B
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.5
µg p^{12}

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

(A) Detergent treated nuclei (without membranes) were incubated with ^{3}H-E_2-ER complexes, 0.5 μg p55 and 0.3 μg of p12 at 30^{0}C for different time intervals (●). The control incubation was performed without adding p12 to the incubation medium (O). 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}H-estradiol bound to nuclei.

(B) Control nuclei (with membranes) were incubated with ^{3}H-E_2-ER complexes, 0.5 μg of p55 either in presence or absence of additional p12 at 30^{0}C for different time intervals. The ^{3}H-E_2-ER transported into the nuclei were measured as described before. The activity was expressed as ^{3}H-estradiol bound to the nuclei. (●) without added p12; (O) with exogenously added p12.

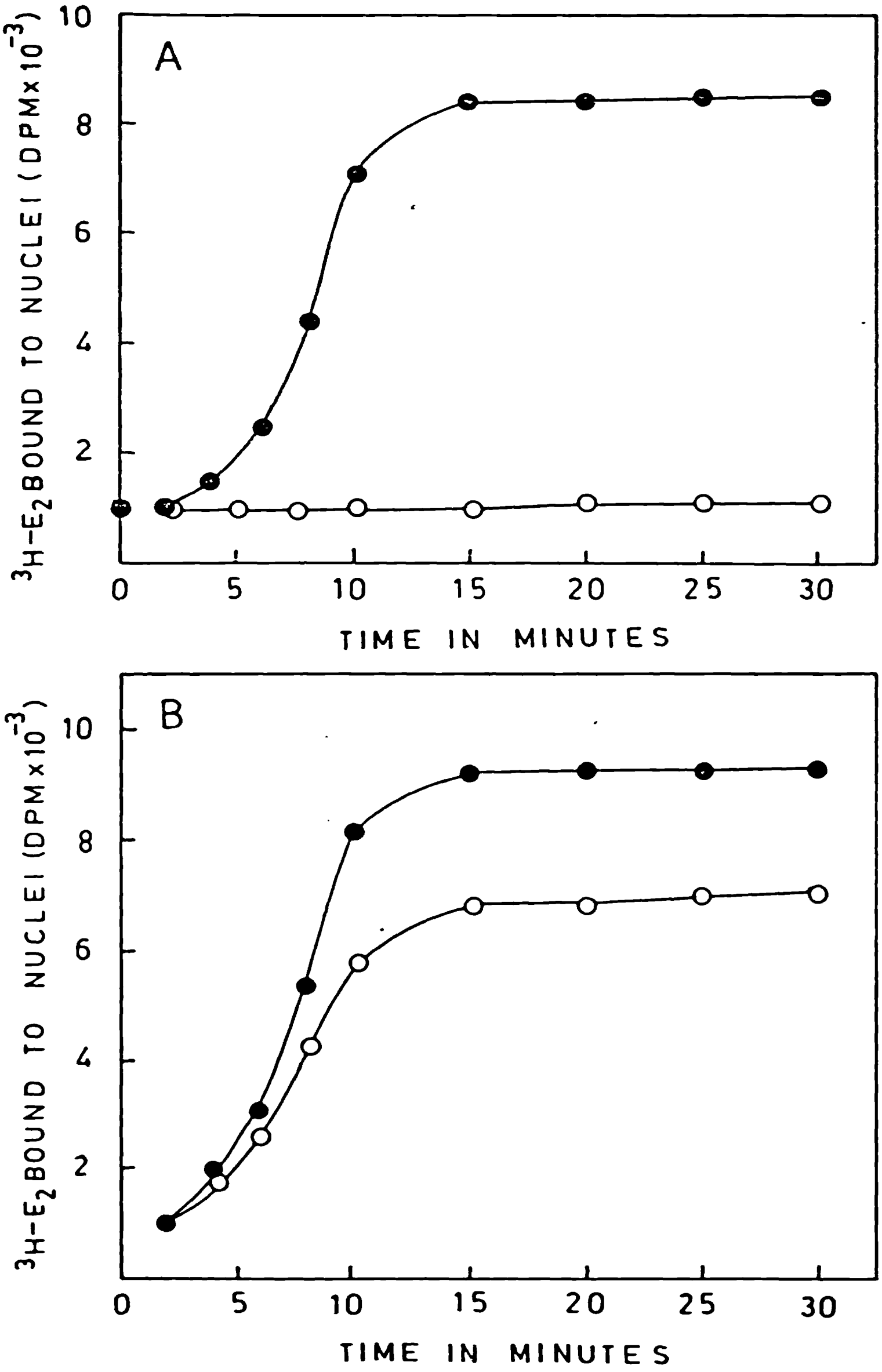

A
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
10
8
6
4
2
0
5
10
15
20
25
30
TIME IN MINUTES
B
NUCLEI (DPM x 10^{-3})
^3H-E_2 BOUND TO
10
8
6
4
2
0
5
10
15
20
25
30
TIME IN MINUTES

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

(A) Detergent treated nuclei were incubated with ^{3}H-E_2-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 $30^{0}C$ for 30 minutes. The ^{3}H-E_2-ER transported into the nuclei were measured and the activity was expressed as ^{3}H-estradiol bound to the nuclei.

(B) Control nuclei (not detergent-treated) were incubated with ^{3}H-E_2-ER complexes and 0.5 μg of p55 in the presence of 0 to 8 mM ATP. The assay was performed as described above.

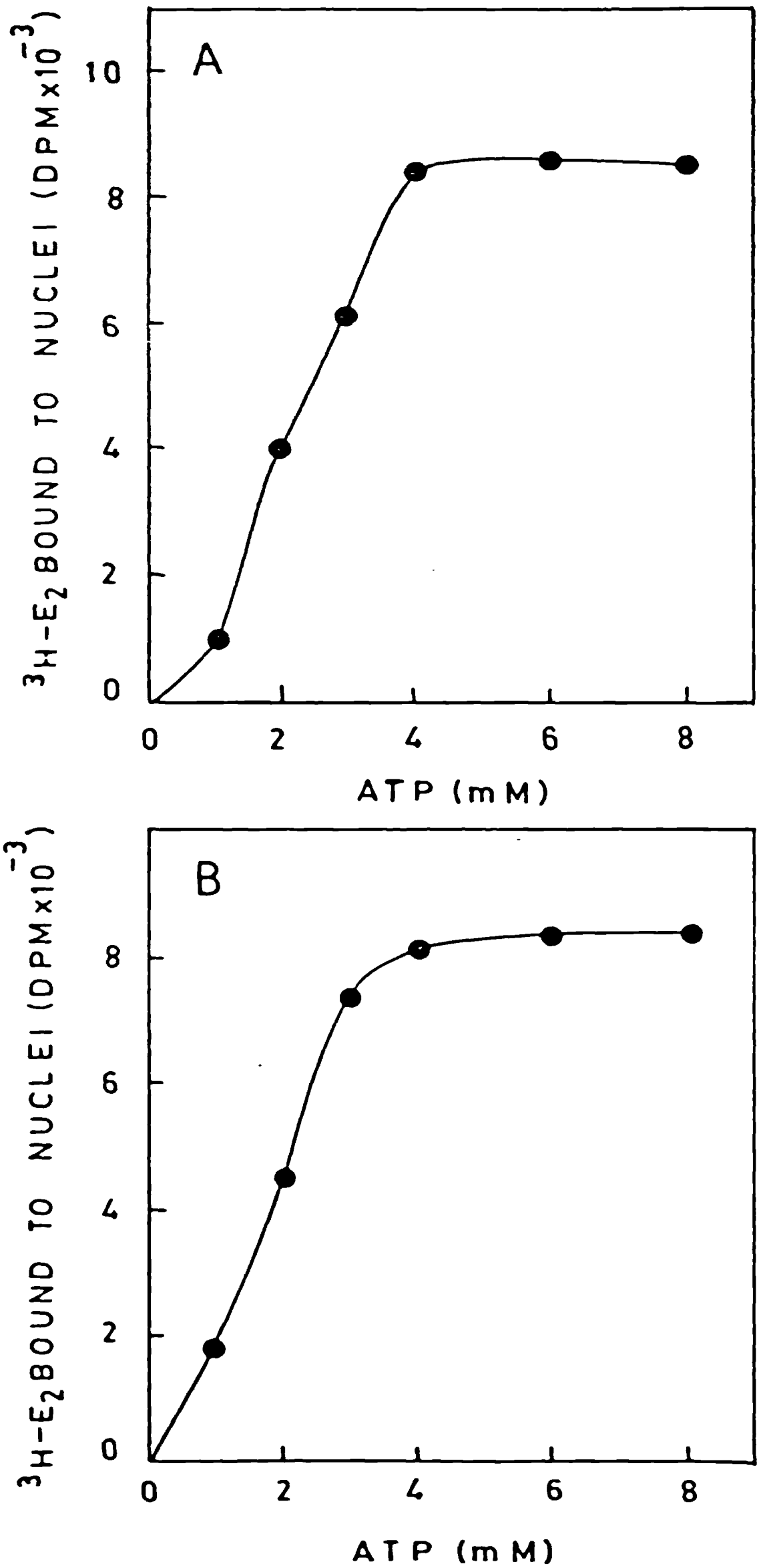

A
B
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
2
4
6
8
10
ATP (mM)

Figure 4.10. Mg^{++}-dependence of the transport of ER into the nucleus.

(A) Detergent treated nuclei were incubated with ^{3}H-E_2-ER complexes, 0.5 μg of p55 and 0.3 μg of p12 in the presence of 0 to 8 mM $MgCl_2$. The incubation was carried out at $30^{0}C$ for 30 minutes. The ^{3}H-E_2-ER transported into the nuclei were measured and the activity was expressed as ^{3}H-estradiol bound to nuclei.

(B) Control nuclei (not detergent-treated) were incubated with ^{3}H-E_2-ER complexes and 0.5 μg of p55 in the presence of 0 to 8 mM $MgCl_2$. The assay was performed as described above.

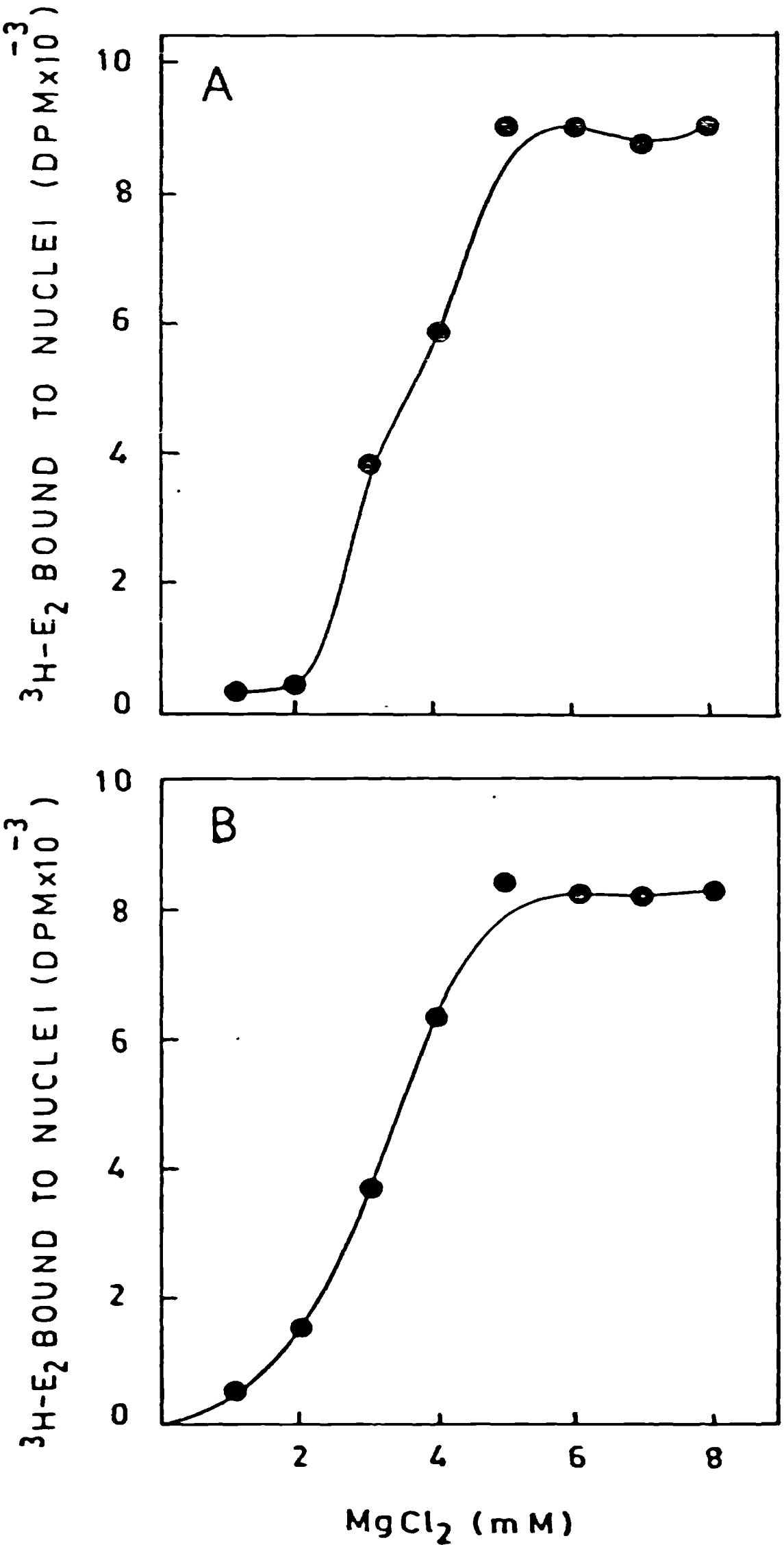

A
B
^3H-E_2 BOUND TO NUCLEI (DPMx10^{-3})
0
2
4
6
8
10
2
4
6
8
$MgCl_2$ (mM)

Figure 4.11. ATPase activity of p55 and p12.

Both p55 and p12 (0 to 2.0 μg) were incubated independently with 6 mM ATP at 30^0C 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 μmoles Pi released /hr.

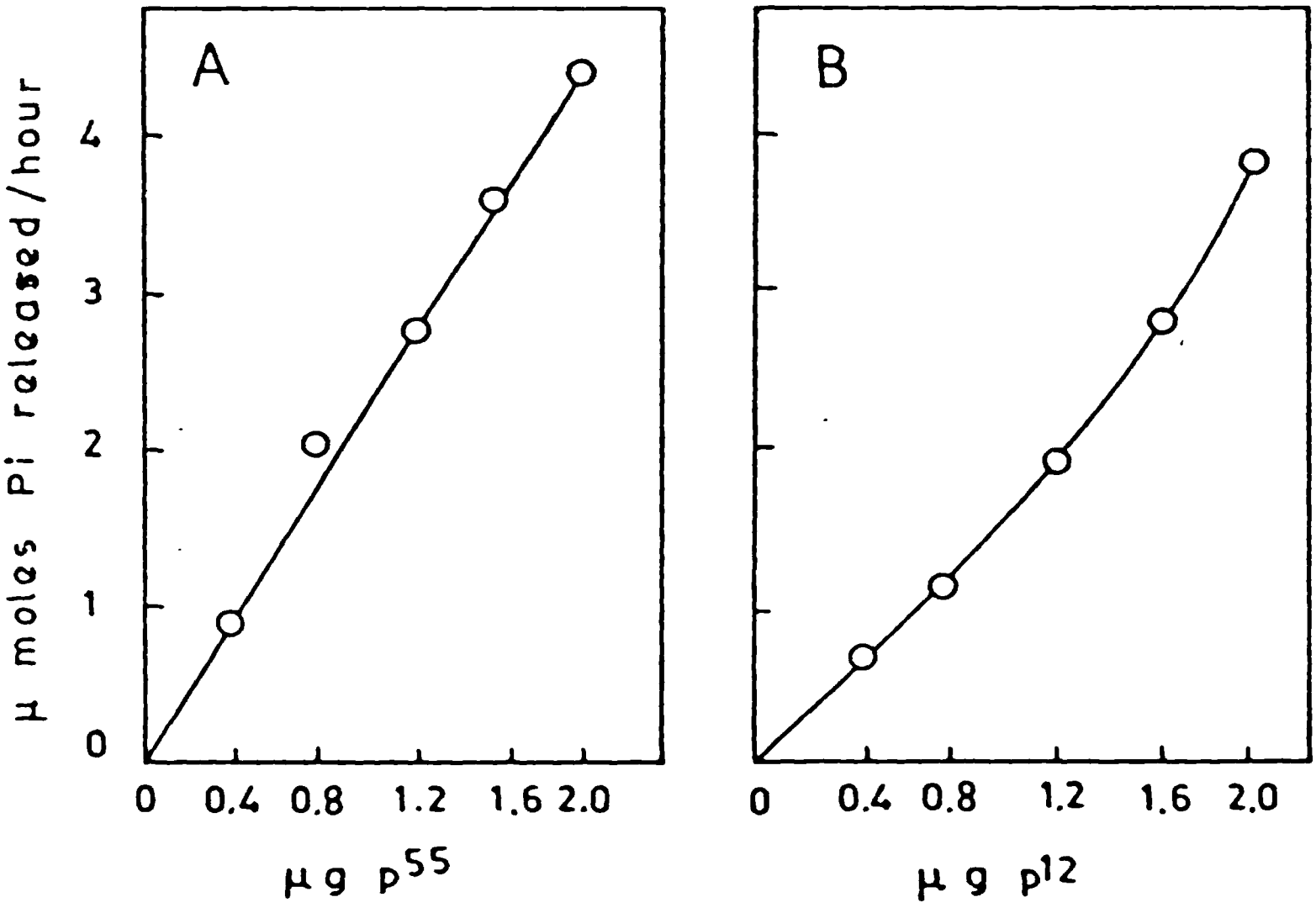

A
B
μ moles Pi released/hour
0
1
2
3
4
0
0.4
0.8
1.2
1.6
2.0
μ g p^{55}
μ g p^{12}

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 30^0C for 30 min. In another set only p12 was incubated in the same protein concentration range, with 6 mM ATP at 30^0C 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 μmoles/Hour.

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

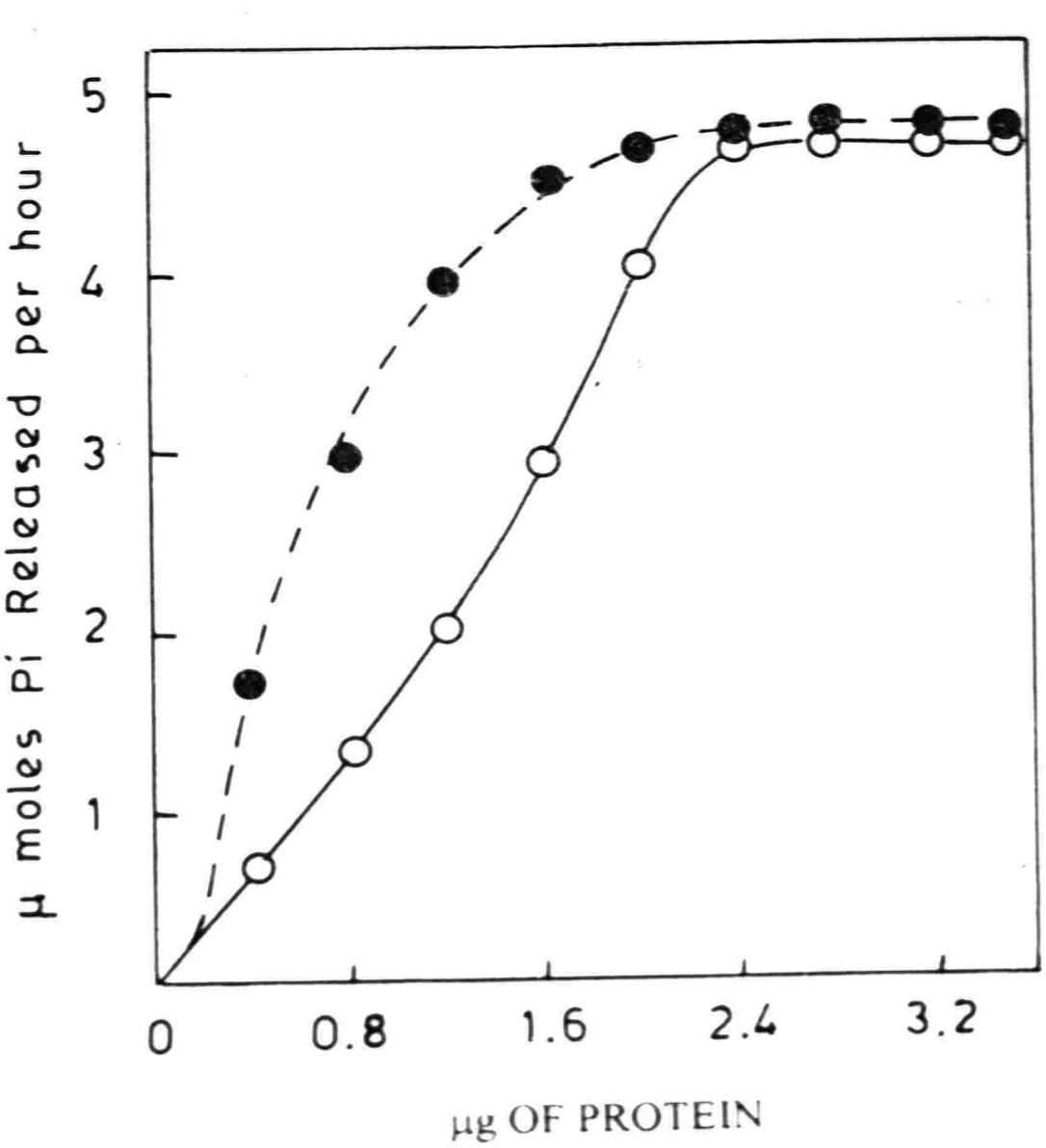

μ moles Pi Released per hour
5
4
3
2
1
0
0.8
1.6
2.4
3.2
μg OF PROTEIN

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

p55 (2 μg) and p12 (2 μg) were incubated independently in the presence of varying molar concentrations of (A) $MgCl_2$ or (B) $CaCl_2$ at 30^0C for 30 minutes. The incubation was terminated by the addition of TCA. The Pi released into the TCA soluble supernatant was estimated as described in the methods. The Pi released is expressed here as μmoles Pi released/ hour.

(●) p55; (O) p12.

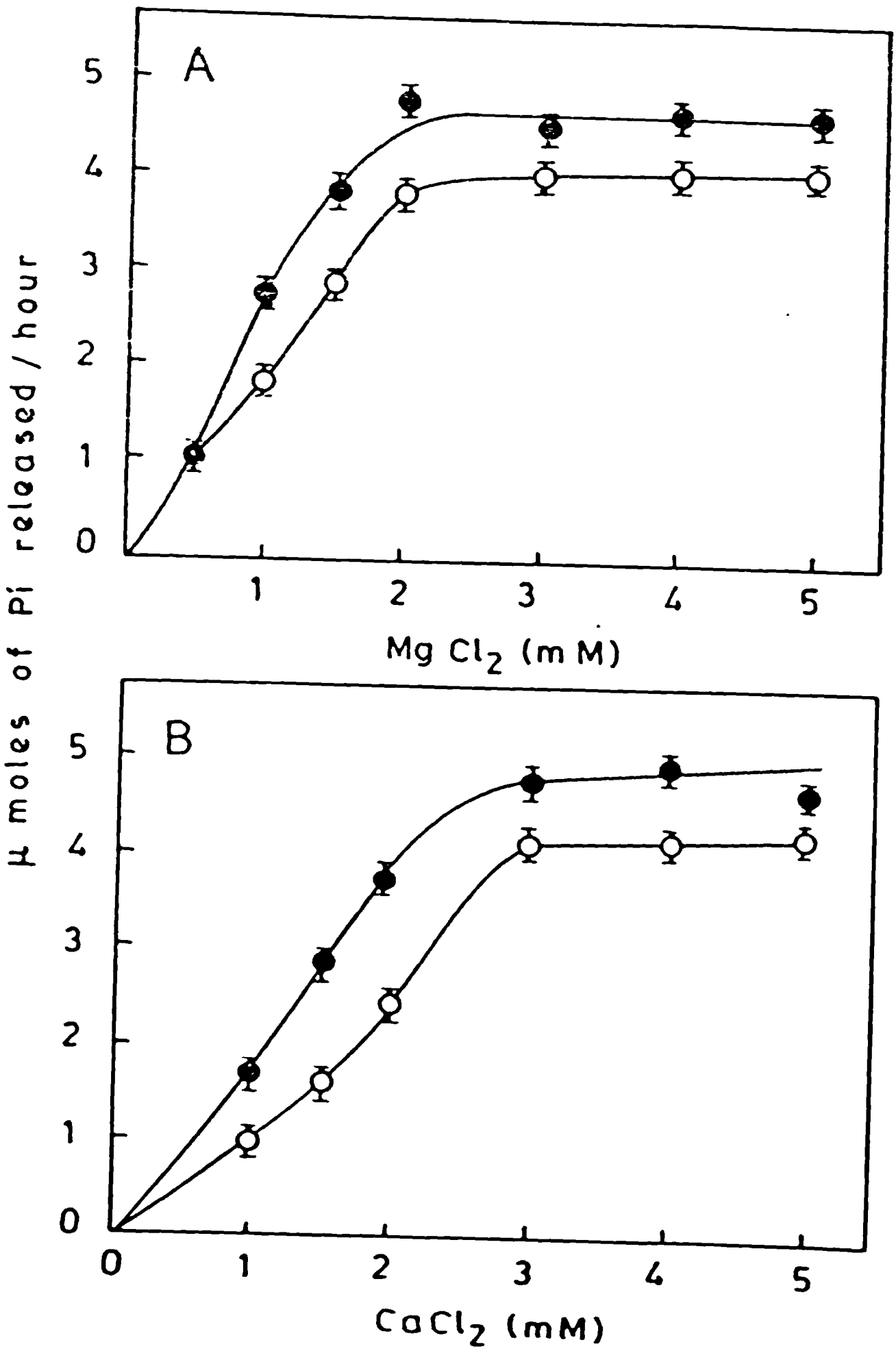

A
5
4
3
2
1
0
1
2
3
4
5
Mg Cl2 (m M)
B
μ moles of Pi released / hour
0
CaCl2 (mM)

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 ^{3}H-E_2-ER complexes in the presence of varying concentrations (0 to 20 μg/ml) of quercetin at $30^{0}C$ for 30 minutes. The assay was performed as described in the methods. The ^{3}H-E_2-ER transported into the nucleus was measured. The activity was expressed as ^{3}H-estradiol bound to nuclei.

(B) Control nuclei (not detergent treated) were incubated with 0.5 μg p55, ^{3}H-E_2-ER complexes and 0 to 20 μg/ml quercetin. The incubation was carried out at $30^{0}C$ for 30 min. The ^{3}H-E_2-ER transported into the nucleus was measured.

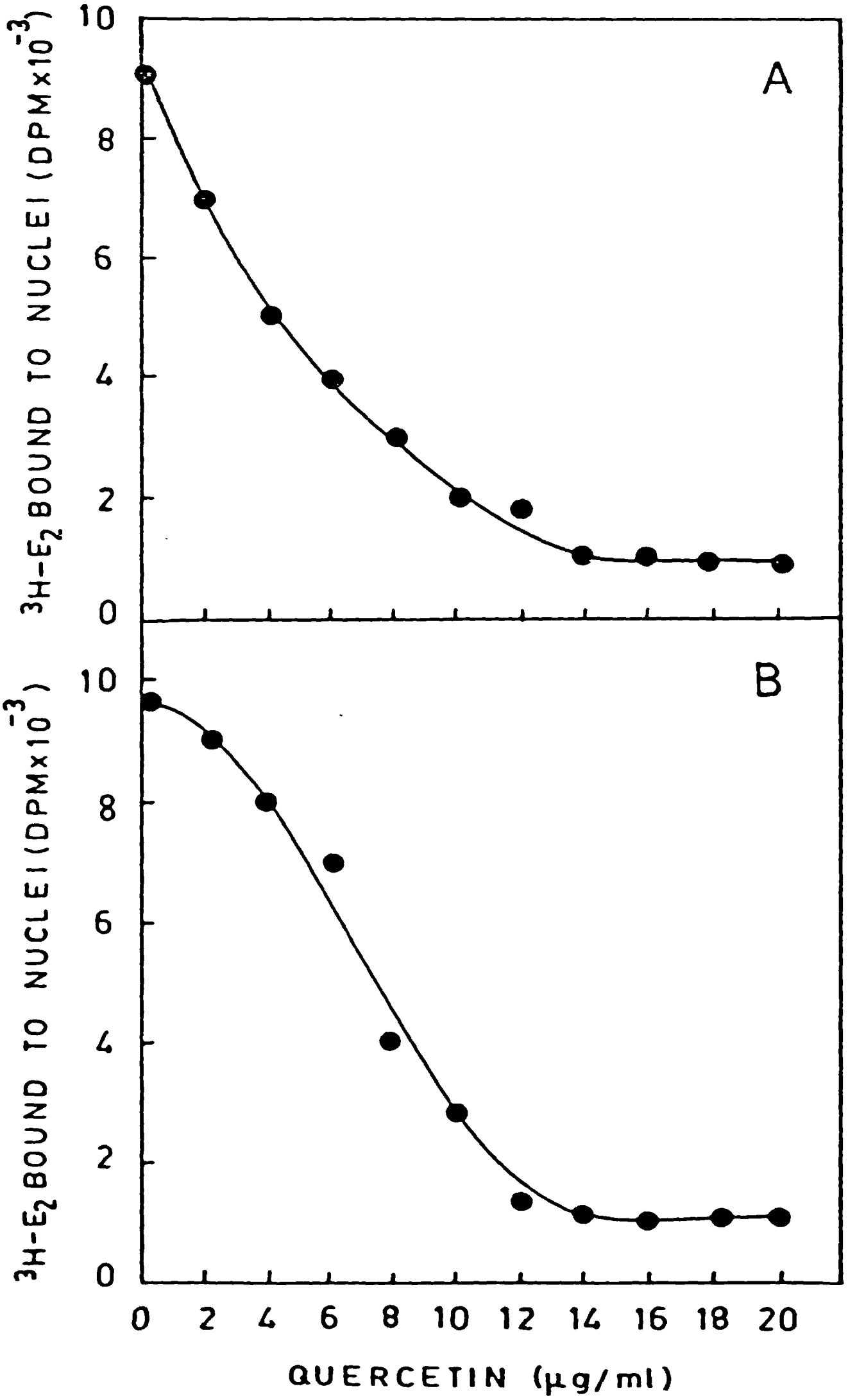

A
B
^3H-E_2 BOUND TO NUCLEI (DPM x 10^{-3})
0
2
4
6
8
10
0 2 4 6 8 10 12 14 16 18 20
QUERCETIN (µg/ml)

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 μg) at 30^0C

(A) Detergent treated nuclei incubated in the absence of p55 (100X).
(B) Detergent treated nuclei incubated with 0.5 μg of p55, in the absence of p12 (100X).
(C)Phase contrast micrograph of B (100X).
(D)Nucleus incubated with 0.5μg p55 and 0.5 μg of p12 (100X).
(F)Goat uterine nuclei (with membranes) were suspended in the assay buffer without ATP and were incubated with FITC-ER (5μg) and p55 (0.5μg) at 30^0C 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 μg/ml) were added simultaneously to the medium and visualized after 30 minutes (100X).

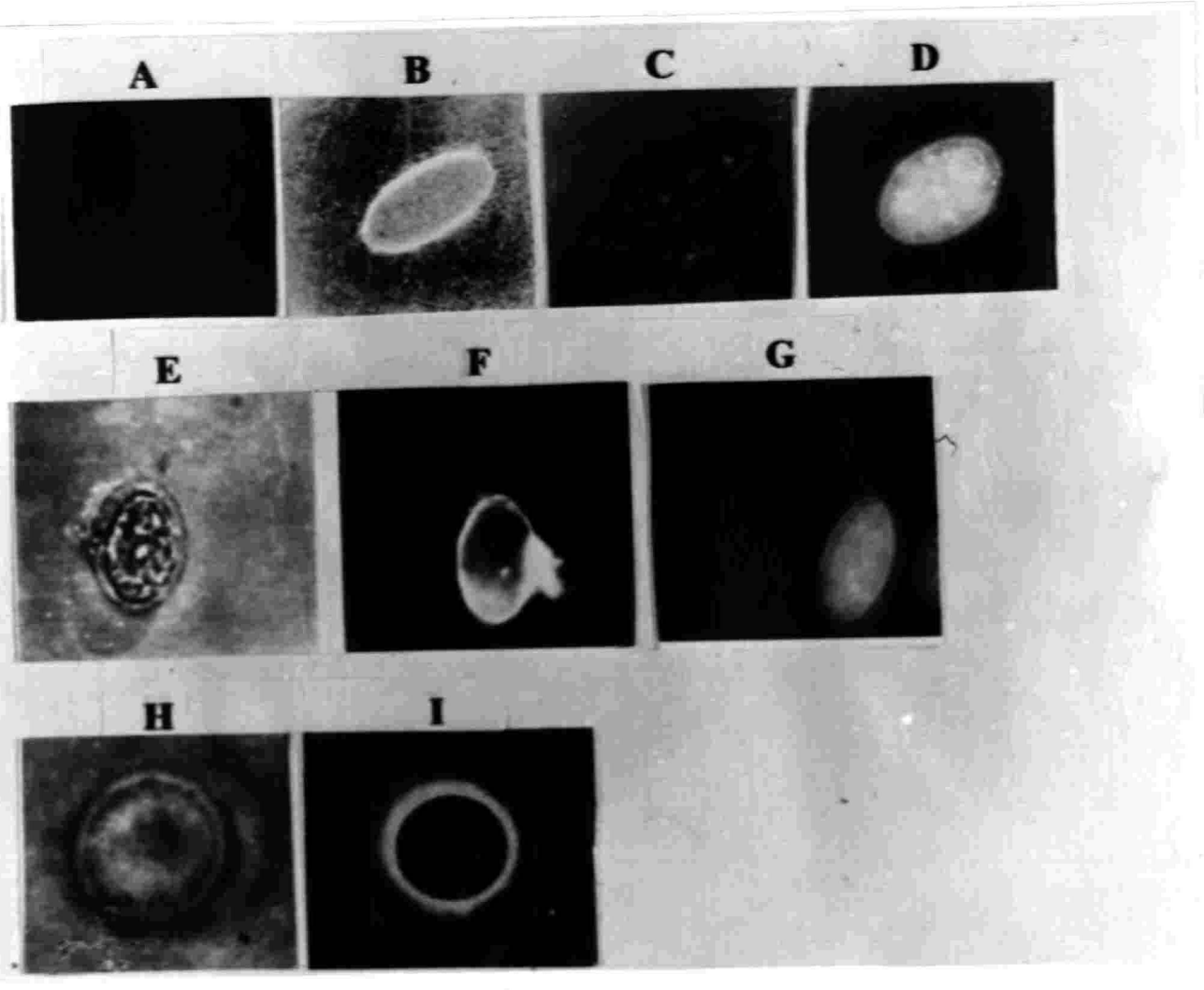

A
B
C
D
E
F
G
H
I

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 30^0C 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 (5μg), unlabelled p55 (1 μg) and about 0.5 μg of FITC-p12. The incubation was carried at 30^0Cfor 30 minutes.

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

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

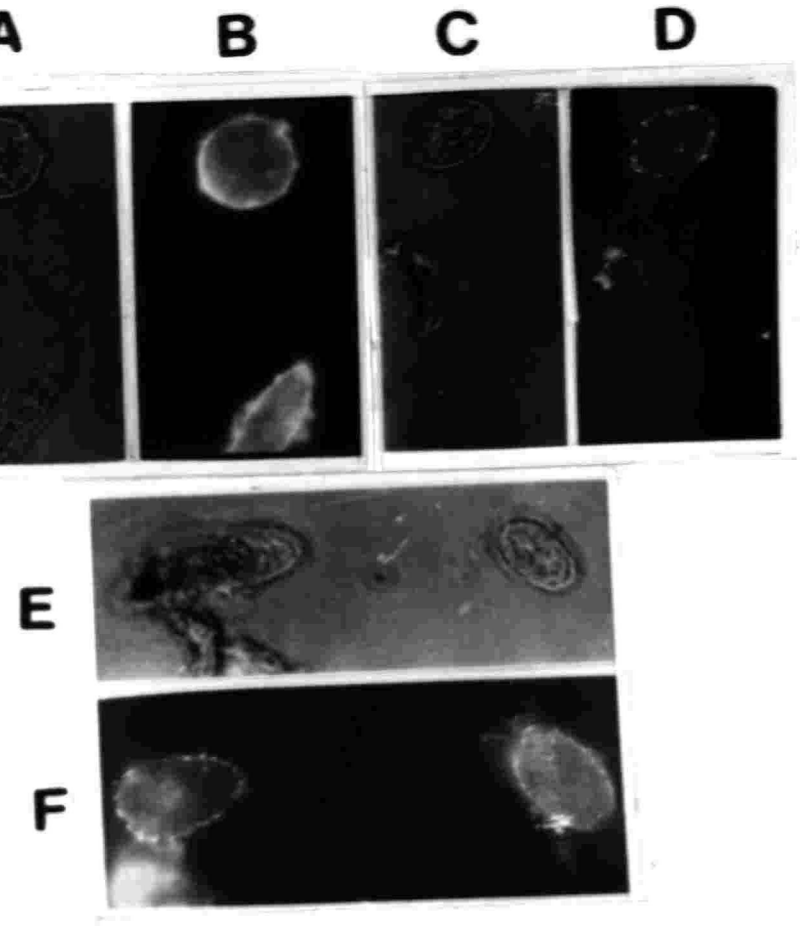

A
B
C
D
E
F

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.

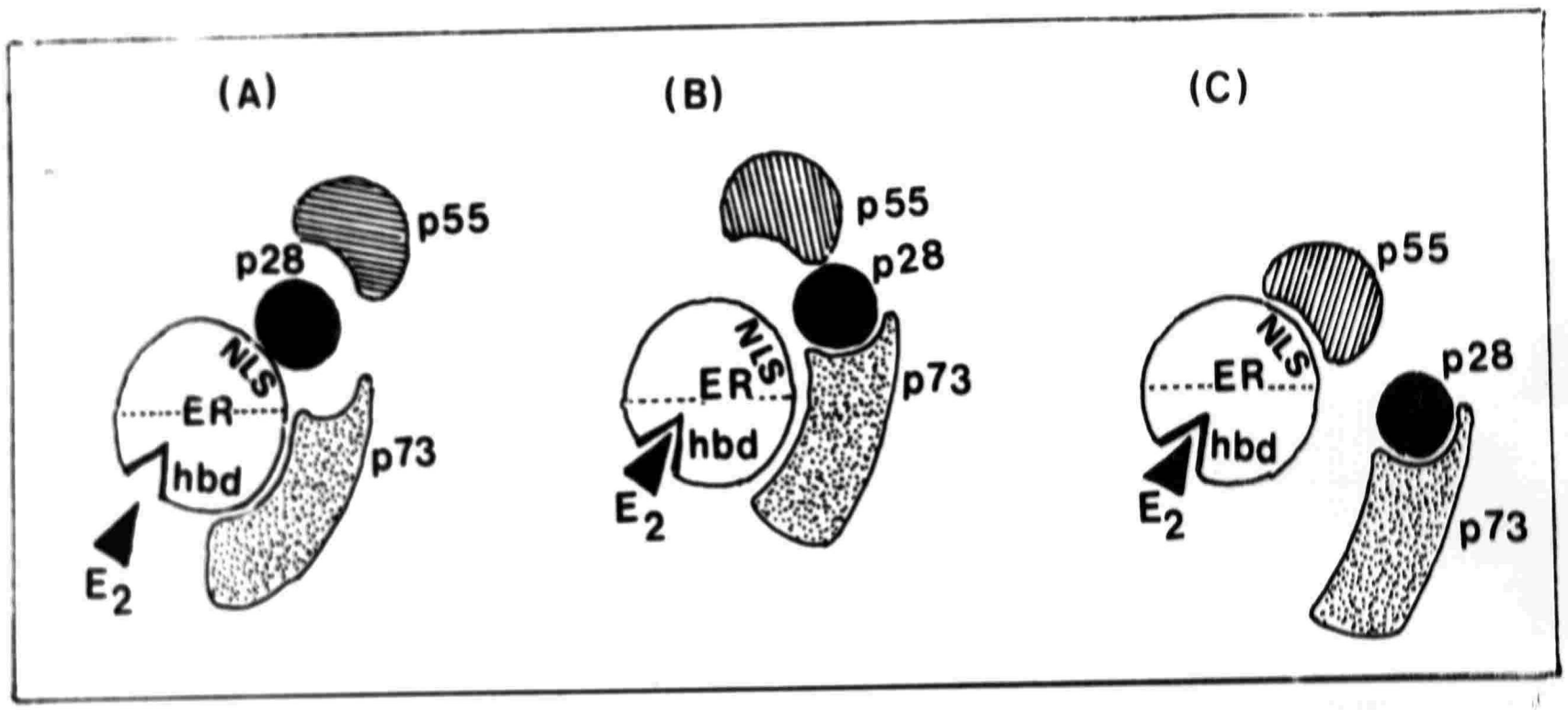
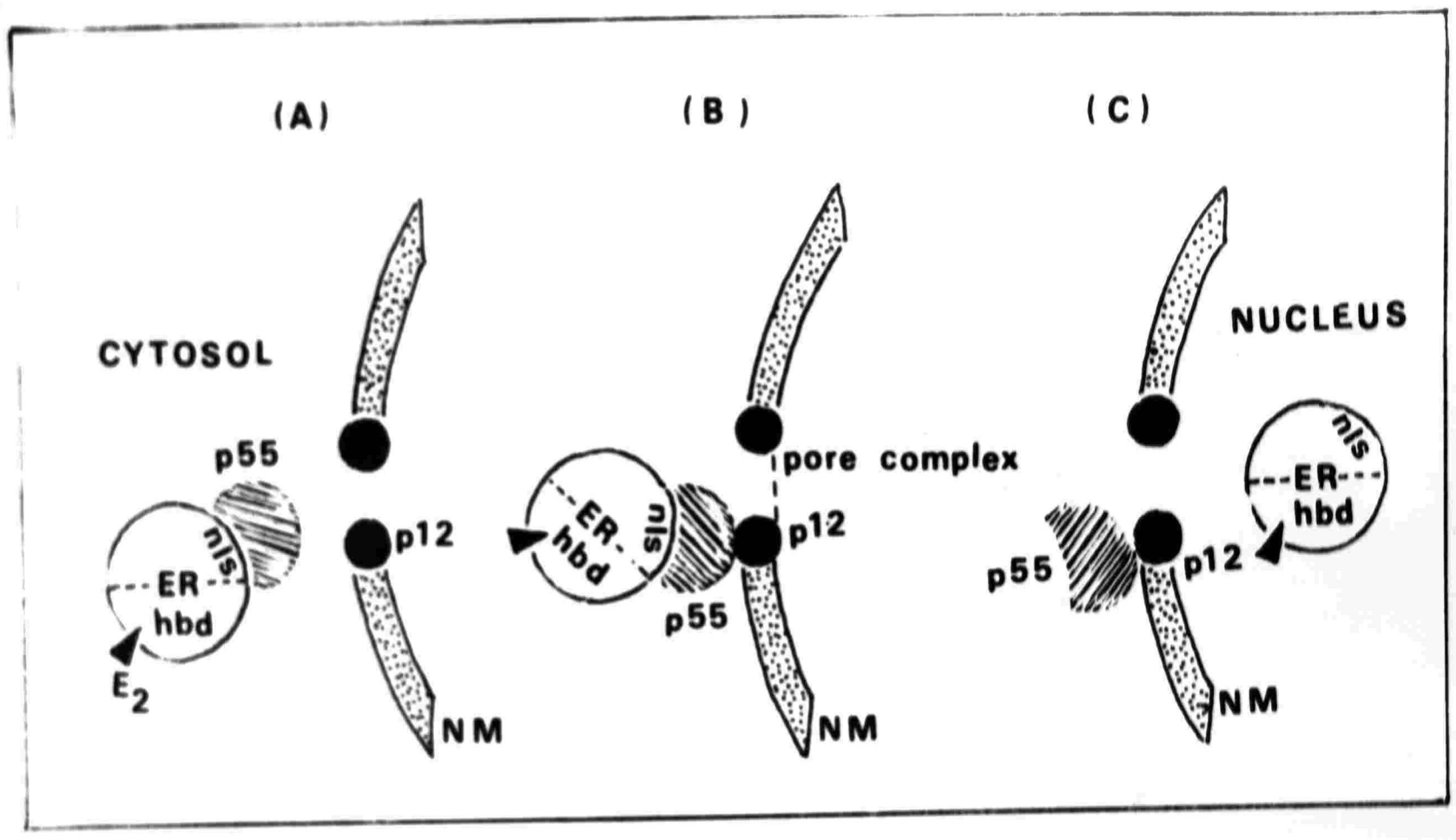

(A)
(B)
(C)
p55
p28
NLS
ER
hbd
p73
E2

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.


(A)
(B)
(C)
CYTOSOL
NUCLEUS
p55
pore complex
nls
ER
hbd
p12
NM
E_2

CONCLUSIONS

CONCLUSIONS

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}-dependentg 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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