# PROTEIN-PROTEIN INTERACTIONS IN ESTROGEN RECEPTOR BIOLOGY : INVOLVEMENT OF A NUCLEAR LOCALIZATION SEQUENCE BINDING PROTEIN AND UBIQUITIN

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**TO**

GOD

and

MY PARENTS

DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
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HYDERABAD

Date: Dec. 05, 1994.

# 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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# CERTIFICATE

This is to certify that Ms. P. Bala Nirmala, 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 "PROTEIN-PROTEIN INTERACTIONS IN ESTROGEN RECEPTOR BIOLOGY: INVOLVEMENT OF A NUCLEAR LOCALIZATION SEQUENCE BINDING PROTEIN AND UBIQUITIN", for submission for the degree of Doctor of Philosophy of this university.

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***Nirmala***

# ABBREVIATIONS

| | |
|---|---|
| AMP | Adenosine 5'monophosphate. |
| AR | Androgen receptor. |
| ATP | Adenosine 5'triphosphate. |
| ATPase | Adenosine triphosphatase. |
| BCIP | 5-Bromo 4-Chloro 3-indolyl phosphate. |
| cAMP | Cyclic 3',5'adenosine monophosphate. |
| cGMP | Cyclic 3',5'guanosine monophosphate. |
| CK | Casein kinase. |
| DNA | Deoxyribonucleic acid. |
| dATPase | Deoxyadenosine triphosphatase. |
| $E_2$ | Estradiol. |
| El | Ubiquitin activating enzyme. |
| E2 | Ubiquitin conjugating enzyme. |
| E3 | Ubiquitin protein ligase. |
| 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. |
| HSP | Heat shock protein. |
| naER | Non activated estrogen receptor. |
| NBT | Nitroblue tetrazolium. |
| NLS(s) | Nuclear localization sequence(s). |
| NLSBP(s) | Nuclear localization sequence 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. |
| ts | Temperature sensitive. |
| TR | Thyroid hormone receptor. |
| WGA | Wheat germ agglutinin. |

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# INTRODUCTION

## 1.0 INTRODUCTION

Steroid hormones exert their biological effects by binding to their receptors. The receptor hormone complex binds to specific DNA binding sites called the `steroid response elements', present upstream of the target gene and modulate its expression. The mechanism of steroid hormone action has been subjected to extensive study and is discussed here briefly, special emphasis being given to the mechanism of the action of estrogens.

## 1.1. MECHANISM OF STEROID HORMONE ACTION.

### *1.1.1 Mechanism of estrogen action.*

The hormone free estrogen receptor (ER) exists as a heteromeric 9S complex (untransformed ER) with heat shock protein (HSP) 90 and other proteins. When the hormone binds to the receptor, the receptor dissociates from the 9S complex and forms a homodimer (5S) and binds to DNA at specific binding sites in the enhancer region of the gene called the estrogen response elements (EREs) ultimately leading to enhanced transcription of specific genes (Beato, 1989). The ERE is a palindromic pair of hexameric half sites AGGTCAnnnTGATCT (Green and Chambon, 1987). What happens to the ER after this step, is not clearly understood. Thampan (1985, '88) observed that estradiol stimulated nuclear ribonucleoprotein transport in the rat uterus. Recent work from our laboratory (Khan *et al*., 1994) gave direct evidence for the presence of ER in the messenger ribonucleoprotein (mRNP) complex of the goat uterus. The ER re-enters the cytoplasm as a constituent of the mRNP complex which carries the mRNA, whose transcription it has promoted.

### *1.1.2. The structure of the estrogen receptor.*

The ER gene has been cloned, sequenced and the predicted amino acid sequence determined. The ER has 595 amino acids and a molecular mass of ~65 kDa. The ER can be divided into six regions (A-F) based on sequence homology between chicken (c) and human (h) ERs.

The "A" region is conserved upto 87% between cER and hER and is required for activating transcription. The region "C" is the most highly conserved region (100%) and contains the DNA binding domain of the ER. The region "E" is the hormone binding domain and is also very well conserved (94%). The B, D and F regions are not well conserved suggesting that their integrity is less important for ER function (Green and Chambon, 1986; Green *et al,* 1986 and Krust *et al,* 1986). The DNA binding domain has a cysteine rich motif. Zinc is tetrahedrally co-ordinated with the cys' and `cys' residues in this region to form two zinc fingers in each ER molecule (Schwabe *et al,* 1990). The zinc fingers determine the target gene specificity by recognizing the ERE (Green *et al,* 1988).

The above mentioned model is the classical ER model. Recent results from our laboratory suggest 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 low affinity and is incapable of binding to the DNA (Anuradha *et al,* 1994). The naER after it binds estradiol dimerizes with the estrogen receptor activation factor (E-RAF), a cytosolic DNA-binding protein with no capacity to bind estradiol (Thampan, 1987; 1989). Thus naER gains access to the DNA by dimerization with E-RAF. Both these proteins have the same molecular mass (~65 kDa) as that of the ER, but, are different proteins as demonstrated by their peptide maps (Zafar and Thampan, 1993). They share common domains as

demonstrated by their immunological cross-reactivity (Anuradha *et ai,* 1994). Recent observations from our laboratory (Karthikeyan, 1994) 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.

### *1.1.3 The cellular localization of the ER.*

The localization of the ER and the other steroid receptors (SRs) in the cell was subjected to scrutiny. A general agreement has been reached regarding the intracellular localization of the estrogen receptors that the ER is primarily localized in the nucleus.Two other models proposed in this context have not withstood the test of time.

**a) Two-step model:** This model proposes that the receptor is cytoplasmic in localization and only upon binding the hormone enters the nucleus. (Gorski *et ai,* 1968; Jensen *et al.*, 1968).

**b) One-step model:** This model proposes that steroid receptors are present exclusively in the nucleus. Enucleation studies and immunolocalization studies have demonstrated exclusive nuclear localization for ER, progesterone receptor (PR) and thyroid hormone receptor (TR) (King and Greene, 1984; McClellan *et ai,* 1984; Welshons *et ai,* 1984 and Perrot-Applanat *et al.*, 1985). Brink *et al.*(1992)demonstrated that the glucocorticoid receptor (GR), too, is exclusively nuclear. Raam *et ai* (1988) questioned the validity of the methods employed by these workers and suggested that the results they had observed were simply artifacts of the fixation procedures employed.

**c) Equilibrium affinity model:** As a mean of the above two models, a third model called the equilibrium affinity model was proposed by Sheridan *et ai*

(1979). This model suggests that the hormone free receptors are in equilibrium partitioned between the nucleus and the cytoplasm according to the free water content of these intracellular compartments.

### *1.1.4 Half-life of the steroid receptors.*

The half-life of the estrogen receptor in the absence of the hormone is ~5 days. In contrast to the rather stable levels, ER levels after estradiol stimulation change significantly and quite rapidly. The ER levels fall within an hour and normal levels are reached within 4 to 6 hours which can be blocked by actinomycin D and cycloheximide (Sarff and Gorski, 1971). By chemical cross-linking and dense amino acid labelling the half-life of ER was studied and was determined to be 4 hours in the presence of estradiol ( Eckert *et al.,* 1984; Monsma *et al,* 1984; Scholl and Lippman, 1984; Miller *et al.,* 1985).

PR is also degraded in a hormone dependent manner. Progesterone administration is followed by a decline in uterine PR content to about 60% in 1-2 hours (Isomaa *et al.,* 1979). Mullick and Katzenellenbogen (1986) have also demonstrated that the hormone bound PR is turned over more rapidly than the unbound PR.

Replenishment of receptors in the cytosol is contributed by protein synthesis or recycling of receptors from the nucleus. By blocking protein synthesis, Horwitz et al. (1983) demonstrated that only 10% of the receptors that are replenished are contributed by recycling. The remaining 90% is due to protein synthesis.

## 1.2 PROTEIN-PROTEIN INTERACTIONS INVOLVING THE ESTROGEN RECEPTORS.

Steroid receptors are associated with several proteins. Described below are the different proteins associated with the steroid receptors and their proposed role in hormone action.

### *1.2.1: Estrogen receptor activation factor (E-RAF).*

E-RAF is a ~65 kDa protein and was first identified by Thampan and Clark (1981). It does not bind estradiol but binds to the DNA. It has DNA-dependent ATPase activity and demonstrates a capacity to introduce structural changes in the DNA. The E-RAF can be resolved by gel filtration into three molecular species I, IIA and IIB. The E-RAF IIA and IIB possess DNA helix destabilizing capacity and E-RAF I has a DNA helix stabilizing function (Thampan, 1987, '89). The E-RAFs share some common immunological domains with both the ER and the naER. E-RAFs I and II are different from the naER and the ER as shown by peptide map analysis.

E-RAF like proteins have been identified recently for other hormone receptors. A thyroid hormone receptor auxiliary protein (TRAP) has been identified which is necessary to enhance the binding of thyroid hormone receptor (TR) to DNA (Darling *et al.*, 1991; O'Donnell *et al.*, 1991). TRAP is also a 65 kDa protein like the E-RAF. Retinoic X receptors (RXRs) have been purified and have been identified to be of three types RXRa, RXRp and RXRy. They dimerize with retinoic acid receptor (RAR) and enhance its DNA binding capacity and also dimerize with TR (Leid *et al.*, 1992). The TRAP protein also heterodimerizes with RAR (Glass *et al.*, 1990).

There probably exists a family of activator proteins akin to the steroid-thyroid receptor superfamily. Members of this family probably share some common domains, as seen by their capacity to heterodimerize with different receptors.

### *1.2.2 Heat shock protein (HSP) 90.*

HSP 90 is a highly conserved, ubiquitous and abundant protein that is associated with all the steroid receptors (Baulieu, 1987; Pratt, 1990; Pratt *et al,* 1992). Such an association has been conserved through out the evolution of eukaryotes from fungi to the highly evolved mammals (Joab *et al.,* 1984; Riehl *et al.*,1985). HSP 90 exists in a stoichiometric ratio of 2:1 (HSP:SR) in the 9S non-transformed receptor complexes. The HSP 90 crosslinks actin filaments (Koyasu *et al,* 1986; Nishida *et al,* 1986) and thus the steroid receptors bind to the actin filaments via HSP 90. (Miyata and Yahara, 1991). The non-transformed ER complex can be reconstituted with HSP 90 and the ER (Inano *et al.,* 1990). 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.* (1990a) 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.

### *1.2.3. p59.*

p59 is associated with all the non-transformed steroid receptor complexes and is associated with HSP 90 (Tai *et al,* 1986; Renoir *et al.,* 1990). In the non-transformed receptor complex, 2 molecules of HSP 90 and one molecule of p59 are associated with one molecule of the steroid receptor (Tai *et al.,* 1993). As

p59 is associated with all the steroid receptors in all the tissues, it must be performing an as yet unidentified but important function.

Several isoforms of the p59 have been identified, and of these, the most notable one is the 56 kDa form (Rexin *et a/.*, 1991). p59 has been cloned and sequenced and the predicted amino acid sequence suggests that the amino acids 41-137 of p59 bears homology to peptidyl prolyl isomerase. It also has an ATP and a calmodulin binding site (Lebeau *et al.*, 1992). p59 was isolated from human lymphocytes and is suggested to be an immunophilin as it binds forskolin (FK 506) and rapamycin. p59 is homologous to other forskolin binding proteins, FKBP 12 and 13, and is proposed to be FKBP 59.

## 1.3 LACUNAE IN OUR KNOWLEDGE OF ESTROGEN ACTION.

Two major lacunae in the known mechanisms of estrogen action have been identified.

a) Though with regard to the intracellular localization of the hormone-free receptor an agreement has not been reached, the hormone-bound steroid receptor is definitely present in the nucleus. Whether the steroid receptor moves to the nucleus before binding to the steroid or after, the problem that still remains, is how it is transported to the nucleus. The mechanism of the ER transport, or any steroid receptor, into the nucleus is not known.

b) The concentration of transcriptional regulators like the steroid receptors has to be regulated in the cell. Steroid receptors have fixed half life in the cell as discussed the section 1.1.4. How these receptors are timed and degraded is not known.

Since practically no information is available about these two mechanisms with regard to the steroid hormone receptors, a survey of the literature on how the other cellular proteins are transported to the nucleus or how they are targetted for degradation was undertaken. As the general cellular pathways are not very different for different proteins, the understanding of the above mechanisms will help to make a head start with similar studies involving the steroid receptors. The mechanism of nuclear transport and of protein degradation is described in sections 1.4 and 1.5 respectively.

## 1.4 NUCLEAR TRANSPORT OF PROTEINS.

The nuclear membrane is the hall mark of eukaryotic cells and a major landmark in evolution. The nuclear membrane functions to separate the genome from the cytoplasm. By importing proteins only at specific times, rapid and discrete changes in intranuclear protein concentration can be achieved, thus allowing them to perform specific functions leading to the development of multicellular organization and organogenesis.

### *1.4.1 The structure of the nuclear envelope.*

The nuclear membrane consists of two concentric lipid bilayers, the outer and the inner nuclear membranes, separated by a perinuclear cisternal space. The inner nuclear membrane is lined with the nuclear lamina, a layer that is composed of A and B type lamins, a specialized type of intermediate filament protein (Fisher *et al.*, 1986; Gerace and Burke, 1988). Pores traverse the nuclear envelope at sites where the inner and outer membranes are fused, serving as channels for molecular exchange between the nucleus and the cytoplasm. The nuclear pore complexes also appear to function as rivets that hold the outer and inner layers of membrane together.

*1.4.2 The nuclear pore complex.*

Molecular trafficking between the nucleus and the cytoplasm occurs through the nuclear pore complex (NPC). NPCs are roughly cylindrical in shape (~120 nm in diameter and 70 nm high) and are embedded in the nuclear envelope. The number and density of NPCs per nucleus varies among cell types, and their arrangement in the envelope is not random (Franke, 1974; Maul, 1977; Franke *et al.*, 1981). Pore number and density appear to be influenced by the state of cellular metabolism (Carmo-Fonseca, 1982; Carmo-Fonseca and David-Ferreira, 1981). The NPC allows passive diffusion of ions and small molecules through an aqueous channel 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 nuclear pore complex: The structure of the NPC has been extensively investigated by electron microscopy, and there is slowly emerging a consensus on its basic architectural frame-work (Akey, 1989; '90; Akey and Radermacher, 1993). The nuclear pore is a large and complex structure of 124 million daltons (~30 times the size of an eukaryotic ribosome) (Reichelt *et al.*, 1990). The NPC consists of four separate structural elements:

(a) the scaffold, which includes the majority of the pore as seen in the electron microscopic studies.

(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).

**Figure 1.1 A structural model of the nuclear pore complex.**

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

**Figure 1.2 Selected projection maps** of **the nuclear pore complex.**

**A** comparison of selected projection maps of the nuclear pore complex transporter in different transport related configurations with corresponding simulated projections of the double iris. (a and b) The docked form; (c and d) the **"in** transit" form; (e and f) the open form. The annulus of eight strong peripheral densities in the open and the "in transit" form is generated by the juxtaposition of four different subunits of the double iris near the hinge region (Figure reproduced from Akey, 1990).

Figure 1.3 A hypothetical nuclear import sequence.

A hypothetical nuclear import sequence with a karyophilic substrate (K) is presented here.

a) Substrate binds peripherally to the transporter,

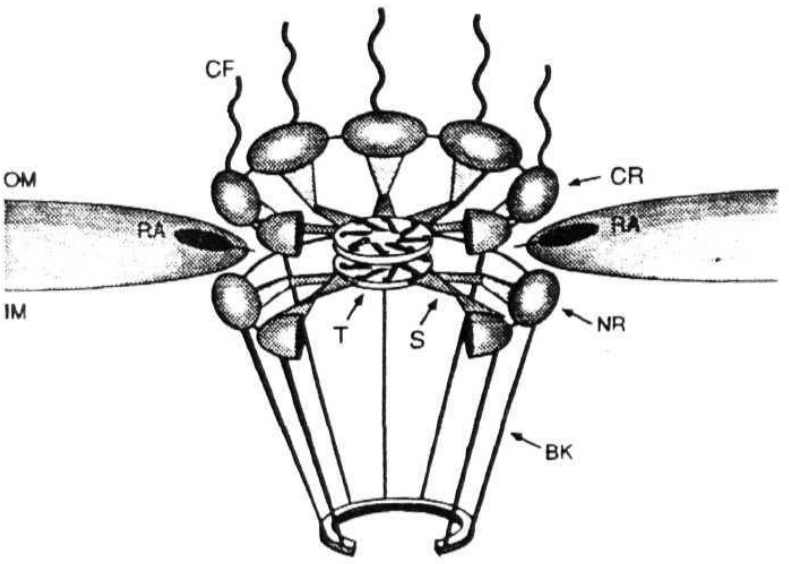
b) Substrate docks over the central transport pore (P),

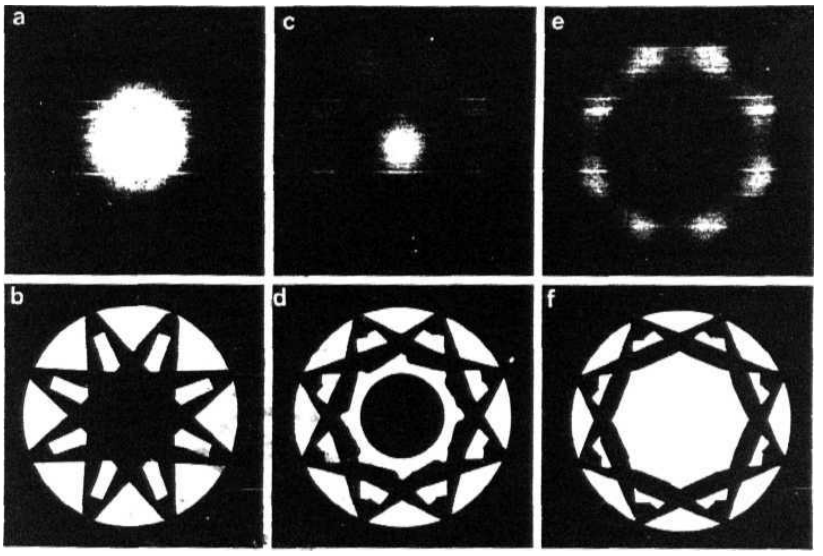
c) Top iris opens to admit substrate,

d) Bottom iris opens as substrate moves further into the transporter creating a symmetrical "in transit" form,

e) Top iris closes behind the substrate as it moves further into the expanded portion and

f) Substrate dissociates from the inner surface of the nuclear pore complex transporter (Figure reproduced from Akey, 1990).


CF
OM
RA
IM
CR
RA
T
S
NR
BK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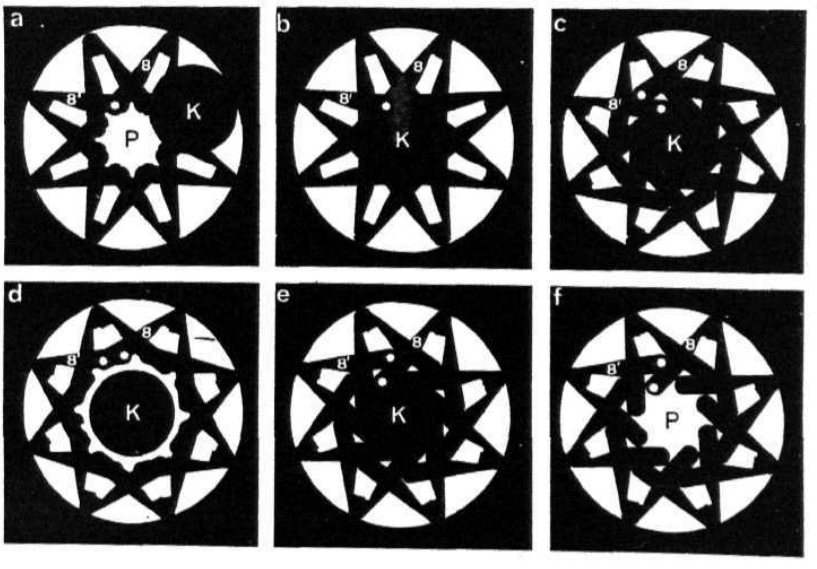
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(a) The scaffold appears as a stack of three closely apposed rings: the cytoplasmic ring (CR), the nucleoplasmic ring (NR), and a central ring of thick spokes (S). Each ring has an eight-fold symmetry. The spokes are connected at their inner edge and support a central hub or transporter (T) of ~36-38 nm. Interspersed between the spokes are large 9 nm aqueous channels, which presumably allow the observed passive diffusion of small proteins and metabolites (Milligan, 1986). The scaffold of the pore is thought to (i) maintain the fusion of the two nuclear membranes that creates the 90 nm opening in the nuclear envelope, (ii) provide the 9 nm diffusion channels and (iii) support the smaller central transporter that regulates actual import and export.

(b) The central hub or the transporter: The 36-38 nm central hub, which was termed the transporter (T), is a proteinaceous ring. The transporter consists of two irises of eight arms each. The two irises are predicted to stack atop one another and to open sequentially, each like the diaphragm of a camera, to let the protein or RNA through (Akey and Goldfarb, 1989; Akey, 1990; 1991; 1992).

The transporter can be resolved by electron microscopic analysis into four distinct forms (Fig 1.2): a) Closed form (Fig 1.3 a), b) Docked form (Fig 1.2 a and b), c) "In transit" form (Fig 1.2 c and d) and d) open form (Fig 1.2 e and f). By assuming that the four forms seen represent sequential intermediates in nuclear imports, the authors have placed these forms as a model for transport in the following order (Fig 1.2 and 1.3). Nuclear protein (K) first binds at the periphery of the transporter ring (Fig 1.3 a); it then moves to the central channel where it docks and induces the channel to open (Fig 1.3 b). In response to the signal for import, the first iris opens, lets a protein pass into the pore (P) (Fig 1.3c)), the second iris would then open, further passage would ensue (Fig 1.3d),

the first iris closes (Fig 1.3e), followed by the second, and translocation is completed (Fig 1.3f). 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.

(c & d) Recent electron microscopic studies indicate that the nuclear pore is also in contact with important accessory structures. On the cytoplasmic face of the pore, thick fibres (3 nm diameter) that extend into the cytoplasm have been observed (CF) (Ris, 1991). These filaments are a staging area for nuclear proteins to bind prior to transport through the NPC. On the nucleoplasmic side of the pore a large basket (BK) like structure was seen in the scanning electron microscopic studies of Ris (1990) and appears to consist of eight filaments extending from the nucleoplasmic ring of the pore (Jarnik *et al*, 1991). The function of this basket is the subject of speculation at this point but its nucleoplasmic location may indicate RNA export.

1.4.2-2 The Chemical composition of the NPC: In contrast to the relatively large amount of structural studies, less is known about the chemical composition of the NPC. Only a small number of NPC proteins have been identified and described to date, probably accounting for less than 10% of the NPC mass. The first NPC polypeptide examined in detail has been gp 210, a transmembrane glycoprotein, and is proposed to anchor the NPC and probably plays a role in membrane folding during nuclear pore formation (Greber *et al*, 1990; Jarnik and Aebi, 1991;Gerace, 1992; Hinshaw *et al*, 1992).

Nucleoporins, the NPC proteins, have been identified to be glycoproteins containing n-acetyl glucosamine residues and bind the lectin, wheat germ agglutinin. ~8-12 nucleoporins of molecular mass 210, 180, 145, 100, 62, 58, 54, and 45 kDa have been identified (Davis and Blobel, 1986; '87; Davis and Fink, 1990; Snow *et al*, 1987). These proteins are present in roughly 1-10 copies per NPC (Holt *et al*, 1987). These proteins appear to be involved in nucleocytoplasmic transport as monoclonal antibodies against these proteins as well as the wheat germ agglutinin (WGA) inhibit transport (Finlay *et al*, 1987, Dabauvalle *et al*, 1988).

A NTPase activity was found to be associated with the isolated nuclei. A 46 kDa NTPase was identified and was proposed to play an important role in RNA efflux (Clawson *et al*, 1984; '85; '88). A second ATPase, of 188 kDa, was also identified in nuclear envelope-pore complex lamina of different vertebrates. This protein accounts for most of the ATPase activity of the nucleus and appears to be a form of myosin heavy chain localized to the nuclear periphery. This protein has been speculated to play an important role in pore function (Akey, 1990; Berrois *et al*, 1983a; '83b; Berrois and Fischer, 1986). Myosin, has been identified to be a component of the NPC (Berrois et al., 1991). Actin is present in the nucleus, in the nuclear membrane - lamina fraction (Clark and Merriam, 1977; Comings and Harris, 1975; Jockush *et al*, 1974; Le Stourgeon *et al*., 1975; Sanger, 1975; Fukui, 1978; Le Stourgen, 1978). Actin along with myosin, a and β tropomyosin and tubulin constituted 38% of the non-histone proteins of the rat liver nucleus (Douvas *et al*, 1975).

*1.4.3* ***Nuclear*** *localization signals/sequences.*

Since the initial proposal that nuclear proteins must contain in their primary structure a signal that enables them to accumulate selectively in the nucleus (De Robertis *et al.*, 1978), regions important for nuclear localization have been defined for several proteins and are referred to as the **nuclear localization signals/sequences (NLSs).** Two approaches have **been adopted to** identify NLSs. The first, the subtractive approach, in which a putative NLS **is** deleted or mutated to demonstrate that it is necessary for the nuclear localization of a given protein. The second one, the additive approach, involves **the** transposition of the sequence under study onto a non-nuclear protein **to** demonstrate that it is sufficient for nuclear localization. In many cases both **the** approaches have been used in concert to identify the NLSs. In spite of a lot of data regarding the NLSs, no consensus NLS sequence has emerged except **that** it is a sequence rich in the basic amino acids lysine and/or arginine.

The NLSs for several proteins, such as Polyoma T, N1/N2, lamin A, c-myc, c-rel, adenovirus E1 A, p53, histones, etc. have been identified (Dang and Lee, 1988; '89), but only a few of them, which have been studied extensively, **are** described here.

In the Simian virus 40 (S V40) large T antigen NLS, a sequence of eight **amino** acids pro.lys.lys(128).lys.arg.lys.val.gly is necessary for its nuclear localization. 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). 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).

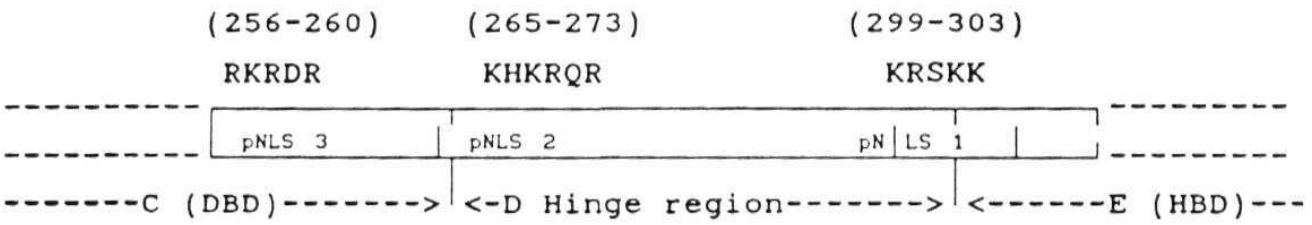
This phosphorylation site was identified to be a casein kinase (CK) II site and a survey of other nuclear proteins shows that all nuclear proteins have CK II sites in the vicinity of the NLS at a distance of 10-30 amino acids. Phosphorylation might modulate the affinity of NLS for its receptor (Rihs *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 NLS may be a general element of nuclear transport regulation.

The nucleoplasmin NLS is a bipartite sequence and both the basic clusters are interdependent. The two domains can still bring about nuclear localization of a fusion protein when the intervening spacer is increased but does not when the spacer is decreased (Burglin and DeRobertis, 1987). A nucleoplasmin like motif is seen in several proteins such as p53, N1/N2, No38 and the steroid receptors (Robbins *et al,* 1991).

### *1.4.3-1 Steroid hormone receptor NLSs.*

The NLSs of the members of the steroid/thyroid hormone receptor superfamily are located in the hinge region (region D) between the DNA and the hormone binding regions. A 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.,* 1990b). The above results have been obtained from studies involving nuclear targetting of β-galactosidase with NLSs of the GR and ER. Ylikomi *et al.* (1992) preferred to analyze SR NLSs in their natural amino acid


(256-260)
RKRDR
(265-273)
KHKRQR
(299-303)
KRSKK
pNLS 3
pNLS 2
pN
LS 1
C (DBD)
D Hinge region
E (HBD)

sequence context, as the possible co-operation between NLSs and the effect of the other domains of the protein on the activity can be assessed only in their natural environment.

Thus, Ylikomi *et al.* (1992) identified three **proto-NLSs (p-NLSs)** for ER. None of the three NLSs is an efficient NLS on its own and has to co-operate with the others to become efficient in nuclear targetting, which is why they **are termed** as **the proto-NLSs'.**

The three pNLSs are constitutive in the sense that they promote transport of ER even in the absence of 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 co-operate with the constitutive hER 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 constitutive and one hormone-inducible p-NLSs are also seen in the PR. The constitutive nuclear targetting sequence of PR and GR are equally potent, but the activity of the constitutive GR NLS is inhibited by the ligand-free hormone binding domain (Ylikomi *et al.*, 1992).

Despite the identification of the steroid receptor NLSs a good eight years ago, no headway has been made with regard to how these signals are recognized.

### *1.4.4 Nuclear localization sequence binding proteins (NLSBPs).*

The nuclear accumulation of NLS-BSA showed saturation kinetics and this accumulation could be reduced markedly by free NLSs (Goldfarb *et al.*, **1986).** The saturation kinetics and the effect of the free peptide on **nuclear transport are** indicative of a limiting component of the uptake apparatus. This **component may be a** receptor for the signal peptide. A family of **proteins bearing** homologous NLSs may share a common receptor (Goldfarb *et al.,* 1986). **Thus a** search for **"receptors"** that bind NLSs had started. The first attempt was made by Yoneda *et al.* (1988) using a novel approach. They assumed that since the SV40 large T antigen NLS consisted of positively charged amino acids lys.lys.lys.arg.lys, probably the receptor that interacts with the signal sequence, has an electrostatically complementary negatively charged sequence containing either asp.asp.asp.glu.asp (DDDED) or glu.glu.glu.asp.glu. (EEEDE). The anti-DDDED antibody blocked transport of nuclear proteins into the nucleus. The anti-EEEDE antibody had only 1/10 or 1/15 the inhibitory effect of the anti-DDDED. The anti-DDDED cross-reacted with two proteins of molecular weight 69 and 59 kDa in rat liver nuclei.

Putative import receptors that can bind SV40 large T antigen NLS peptides have been identified by *in vitro* cross linking reactions and by ligand blotting. Adequate proof of a functional role for the NLSBP in nuclear transport requires demonstration of stimulation of transport by the purified protein **and/or inhibition** of transport by **an** antibody raised against the protein. In the case of **many** of **the** import receptors identified so far, neither of these results have been obtained.

**The import receptors were termed as the nuclear localization sequence binding proteins (NLSBPs)** by Silver *et al.* (1989). The NLSBPs retain their NLS

binding ability despite subjecting them to heating, SDS treatment and electroblotting suggesting that the binding activity is probably present in a small region of the protein. Imamoto-Sonobe *et al.* (1990) identified in the rat liver a NLSBP of molecular mass 69 kDa (p69) which binds to anti-DDDED and nucleoplasmin. p69 also binds to the SV40 large T antigen NLS.

**A table of the NLSBPs identified and in some cases purified is given below:**

| Species | Mol. Mass (kDa) | Cellular location | Properties | References |
|---|---|---|---|---|
| Rat | 70, 60 | N+C, N+C | Stimulates import | Adam *et al.*, 1989. |
| Cow | 56, 54 | C | -Do- | Adam & Gerace '91. |
| Rat | 140, 55<br>100 | N, N<br>C- | - | Yamasaki *et al.*,'89. |
| Rat | 140 | Nucleolus | - | Meier & Blobel, '90. |
| Rat | 76, 67 | N | - | Benditt *et al.*, |
| | 59, 58 | N | - | '89. |
| Rat | 69 | N | Binds anti-DDDED antibody | Imamoto-Sonobe *et al.*, '90 |
| Human | 38 | Nucleolus | - | Goldfarb, '88 |
| Human | 66 | N | - | Li & Thomas,'89 |
| Yeast | 140, 95, 59 | N | - | Silver *et al.*'89 |
| Yeast | 70 | N+C | - | Stochaj *et al.*'91 |
| Yeast | 67 | Nucleolus | - | Lee & Melese '89 |

N- nucleus; C - cytoplasm.

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-ethylmaleimide (NEM), a sulphydryl alkylating reagent. Stochaj *et al* (1991) purified a 70 kDa NLSBP (NBP 70) from the yeast *Saccharomyces cerevisiae.* Antibodies raised against NBP70 cross reacted with the purified 70 kDa protein (NBP 70) from *lea mays* (endosperm), HeLa cells and *Drosophila.* Thus NBP 70 seems to be conserved throughout evolution, from yeast to man. The NBP 70 was present in the nucleus as well as the cytoplasm (Stochaj and Silver, 1992).

Nopp 140, a nucleolar protein was purified, cloned and sequenced (Meier and Blobel, 1990;'92). The Nopp 140 sequence consists of 7 stretches of glutamate and aspartate residues [the sequence suggested by Yoneda *et al.* (1988) to be electrostatically complementary to the NLS].

While my work was in progress, LaCasse et al. (1993) identified two NLSBPs for GRs and TRs by cross linking cytosolic proteins with GR/TR NLS peptides. The molecular mass of these proteins was 74 and 58 kDa, of these, the 58 kDa protein binds the NLS peptide with a higher affinity. These proteins have been identified in the rat liver nuclei and nuclear envelopes as well. They are yet to be purified and a functional assay needs to be designed to confirm these proteins as the NLSBPs.

### *1.4.5. Mechanism of Nuclear Transport*

It is becoming increasingly evident that nucleocytoplasmic partitioning of macromolecules plays an important role in the control of nuclear activities. A

major role in signal transduction is played by the subcellular organization of the signal transducers.

NLS-containing proteins enter the nucleus more rapidly than can be accounted for by diffusion through the NPC. The concentration of nuclear proteins in the nucleus is higher than in the cytoplasm. Since the transport is against the concentration gradient and requires ATP hydrolysis, facilitated transport is ruled out and the mechanism of transport of proteins into the nucleus is, therefore, most probably by active transport.

Molecules with a diameter <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; '84).

Nuclear transport can be experimentally separated into two distinct steps: (1) perinuclear accumulation and (2) translocation. The first step, the perinuclear accumulation is due to specific recognition of the transport signal on proteins by the NLSBPs and this complex is transported to the nuclear envelope. These proteins are associated with the fibrillar components of the NPC. The rate of accumulation is very rapid and is ATP independent. This step is not inhibited by wheat germ agglutinin (WGA). The second step, translocation through the pore is a slower process than binding and is ATP and temperature dependent. This step can be inhibited by WGA (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). WGA binds to the nucleoporins and blocks the nuclear pore. An intact nuclear membrane is required for nuclear transport (Zimmer *et al.*, 1988).

Nuclei treated with Triton X-100, which removes the nuclear membranes, lose their ability to transport proteins (Imamoto-Sonobe *et al.*, 1988).

Based on the electron microscopic analysis of the structure of the NPC, Akey and Goldfarb (1989) proposed that the mechanism of protein import through the NPC is a multi-step process.

(i) Binding of the proteins to the cytoplasmic ring, followed by,

(ii) docking of the protein to the centre of the nuclear pore, at the transporter, which probably results in a gating event which facilitates transport through the pore.

(iii) Actual translocation through the pore and release of the protein at the nucleoplasmic side.

Mechanism of translocation through the transporter of the NPC was proposed by Akey (1990) in the double iris model again based on electron microscopic studies of the NPC. (described in detail in 1.4.2-1). 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 opens at the nucleoplasmic side to let the protein move out into the nucleoplasm. WGA may prevent the dilation of the first iris by cross linking the subunits of the iris. The motive force of transport is probably provided by a myosin $Mg^{++}$ATPase and actin-myosin like control of the pore diameter (Schindler and Jiang, 1986).

Moore and Blobel (1992) have shown that two cytosolic factors A and B are required for nuclear transport. Factor A recognizes NLSs and transports proteins to the nuclear periphery suggesting that the factor A could be the NLSBP. Factor B is ATP dependent and helps in transport of the proteins into the nucleus. Factor

B is comprised of two interacting components B1 and B2. B1 was purified to homogeneity and identified as a ~25 kDa GTP binding protein RAN/TC4 (Ras related nuclear protein). GTP hydrolysis by RAN seems to be an important step in the nuclear transport (Moore and Blobel, 1993).

Newmeyer and Forbes (1990) have identified two cytosolic factors NIF1 and NIF2 important for nuclear transport of proteins. NIF1 co-purifies with the NLSBP activity and may be the same as factor A identified by Moore and Blobel (1992).

There is no 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 Kennady, 1990).

## 1.5 UBIQUITINATION OF PROTEINS.

In eukaryotic cells, proteins are degraded through lysosomal and non-lysosomal pathways, significant lysosomal degradation of intracellular protein is observed primarily under conditions of nutritional deprivation. Non-lysosomal, ATP-dependent mechanisms are probably responsible for most of the highly selective turnover of intracellular proteins under normal metabolic conditions. The rate limiting step in the degradation of proteins is energy dependent (Goldberg and John, 1976).

A large number of cellular proteins are in a dynamic state of turnover; protein breakdown is responsible for essential cellular functions such as the modulation of the levels of key enzymes and regulatory proteins and removal of abnormal

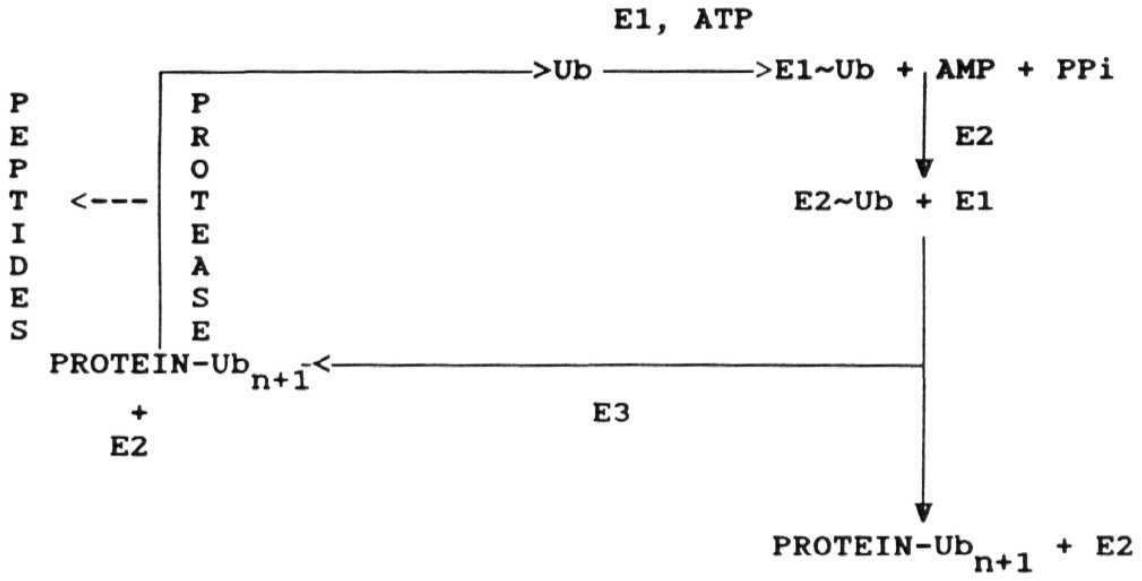
proteins that arise by biosynthetic errors or post-synthetic damages. The process is highly selective and half lives of proteins vary from several minutes to many days (Goldberg and John, 1976).

A novel mechanism for selective protein degradation was proposed by Hershko and Tomkins (1971) which consists of several essential components, most notably ubiquitin (Hershko and Ciechanover, 1982). The selective degradation of many proteins in eukaryotic cells is carried out by a ubiquitin mediated pathway.

### 7.5.7 *Structure of ubiquitin.*

Ubiquitin is an abundant polypeptide of 76 amino acids, has a molecular mass of ~8.5 kDa and is one of the most highly conserved proteins (Goldstein *et al.*, 1976). The tertiary structure determined by X-ray crystallography and 2 D NMR shows that human ubiquitin is highly compact and tightly bonded. The ubiquitin moieties of the ubiquitin-protein conjugates are believed to be recycled when the target protein is degraded by the ubiquitin-dependent protease. The rigid structure may serve to reduce its susceptibility to degradation (Vijay-Kumar *et al.*, 1987; Weber *et al.*, 1987).

Ubiquitin has two functional sites. A C-terminal site through which ubiquitin is ligated to the e-amino group of the lysine residues in the acceptor proteins. A second site is the lys-48 of ubiquitin. This residue can serve as an acceptor site for ubiquitination.




Ub: **ubiquitin.**

### *1.5.2* ***Ubiquitination.***

Ciechanover *et al.* (1980) demonstrated that several molecules of ubiquitin were conjugated to the proteins to be degraded in an ATP-dependent manner by isopeptide linkages to the e-amino lysine of the protein substrate. Since ubiquitin protein conjugates are degraded rapidly with the release of free and reusable ubiquitin, a model was proposed according to which the conjugation of ubiquitin with protein /is an obligatory event in protein breakdown (Hershko *et al.*, 1980). The enzymatic pathways for ubiquitin conjugation and degradation of the proteins conjugated to ubiquitin are described below.

#### **1.5.2-1 Conjugation** of **ubiquitin.**

The conjugation of ubiquitin to protein substrates is a multistep process. In **an** initial ATP requiring step, ubiquitin is activated by an ubiquitin activating enzyme (E1). Ubiquitin is then transferred to a specific cysteine of one of the several ubiquitin conjugating (E2) enzymes. Finally these E2 enzymes donate ubiquitin to protein substrates in the presence or absence of E3 proteins (ubiquitin ligases). This results in branched protein conjugates in which the C-terminus of ubiquitin is linked by an isopeptide bond to specific internal lysine residues of target proteins.

Ubiquitin may be conjugated onto the first ubiquitin, or onto another lysine of the protein resulting in multiubiquitinated proteins (Finley and Varshavsky, 1985). The individual reactions and the enzymes involved in the above described pathway are detailed below.

*a) Ubiquitin activating enzyme (El):* The first reaction in the ubiquitin conjugation system is the activation of ubiquitin to a high-energy

intermediate. This reaction is catalyzed by E1, the ubiquitin activating enzyme. The reaction takes place in two steps, during which ubiquitin first forms an adenylate intermediate before being transferred to a thiol site with the release of AMP (Ciechanover *et al.*, 1982).

$$E1 + Ub + ATP \xrightarrow{Mg^{++}} E1_{AMP\sim Ub} + PPi \longrightarrow E1^{-S\sim Ub} + AMP$$

El from animals and plants is immunologically cross-reactive, indicating that this enzyme, like ubiquitin, has been conserved through evolution (Hatfield and Vierstra, 1989). Ubiquitin conjugate formation is essential for cell viability. A mutant mouse cell line ts 85 has a block at the El function. This mutation is a lethal mutation and no ubiquitin conjugation was observed in these mutants (Ciechanover *et al.*, 1984; Finley *et al*., 1984). In yeast too, gene UBA1 coding for El when deleted resulted in cell death (McGrath *et al*., 1991).

*b) Ubiquitin conjugating enzymes UBCs(E2):* Hershko *et al.* (1983) first identified E2s and termed them as carrier proteins. Five E2 proteins of molecular mass 14, 17, 20, 24 and 32 kDa were identified in reticulocytes. Ubiquitin transfer from El to E2 at submicromolar concentrations is very rapid (in less than a minute). E2s are heterogeneous in character based on their dependence on E3 to transfer ubiquitin. $E2_{14}$ transfers ubiquitin to proteins in an E3 dependent manner and is the most active enzyme among the E2s. The other E2s conjugate E2 to proteins in an E3 independent manner (Pickart and Rose, 1985). They catalyze monoubiquitination rapidly and support polyubiquitination at slow rates (Haas and Bright, 1988).

Most of the UBC mutations result in a pleiotropic phenotype. Pleiotropic phenotype demonstrates slow growth rate, severe defects in induced mutagenesis, extreme sensitivity to DNA damaging agents, increased rate of spontaneous mutations, etc (Finley and Chau, 1991).

***c) Ubiquitin protein*** *ligases (E3):* E3 enzyme catalyzes the formation of an isopeptide bond between the C-terminus of ubiquitin and the $\varepsilon$-$NH_2$ lysine of the protein substrates (Hershko *et al.*, 1983, 1986).

Based on the N-end rule (1.5.3) three types of protein substrates have been identified ( Mayer *et al.,* 1989; Reiss *et al.,* 1988).

Type I protein substrates have basic amino acids at the N-terminus, Arg, Lys or His.

Type II protein substrates have bulky, hydrophobic N-termini, Phe, Tyr, Trp or Leu.

Type III protein substrates have N-termini that are neither basic nor hydrophobic. The signal for this class is not known.

Two species of E3, termed E3$\alpha$ and E3$\beta$, have been isolated from reticulocyte extracts (Reiss and Hershko, 1990). E3$\alpha$ conjugates ubiquitin to both type I and type II substrates. Heller and Hershko (1990) have characterized E3$\beta$ that can conjugate ubiquitin efficiently to type III substrate.

Very little is known about E3 enzymes other than E3a. Since the cellular protein substrates of E3a are very limited, it may be expected that other species of E3 exist that may recognize other specific features in different cellular proteins. It may seem reasonable to assume that many different ligases exist,

which are highly specific for target proteins. Along these lines is the identification, recently, of a specific protein ligase which catalyzes the ubiquitination of oncogene p53, which is a heterodimer of human papilloma virus HPV E6 oncoprotein and a E6-associated protein (Scheffner *et al,* 1993).

**1.5.2-2. Degradation of the ubiquitinated proteins.**

Very little is known about the degradation of ubiquitin-protein conjugates. Hershko *et al.* (1984) have observed that ubiquitin conjugated proteins were degraded in an ATP dependent manner. Hough *et al.* (1986) partially characterized a large ATP dependent protease complex from rabbit reticulocytes that degrades proteins ligated to ubiquitin. This protease complex had a sedimentation coefficient of 26 S and molecular mass between 1000 to 1500 kDa.

Ganoth *et al.* (1988) resolved three separate factors required for degradation of protein-ubiquitin conjugates. These were termed CF-1, CF-2 and CF-3 (CF for Conjugate degradation Factors). The apparent molecular masses were 600, 250 and 650 kDa respectively. The CF-3 component has been identified as the 20 S protease complex (Eytan *et al.,* 1989; Driscoll and Goldberg, 1990). The 20 S protease has also been termed as multicatalytic proteinase complex, alkaline protease, macropain, prosome and proteasome. It is a cylindrical particle organized as a stack of four rings of subunits (Goldberg, 1992).

CF-1, CF-2 and the 20 S proteasome form the 26 S protease complex in an ATP dependent manner. The functions of CF-1 and CF-2 is unknown, they may provide the recognition elements of the complex. The subunits of the 20 S complex are strongly conserved in evolution from yeast to mammals. A mutation

in a subunit of the 20 S complex results in accumulation of ubiquitin-protein conjugates, while deletion of the genes for the subunits is lethal (Fujiwara *et al.*, 1990; Heinemeyer *et al.*, 1991).

*1.5.3 How are substrates recognized by the ubiquitin pathway?*

A necessary step in the degradation of proteins by the ubiquitin system is the recognition by the ubiquitin-protein ligases. Various structural features on the proteins have been studied in conjunction with their susceptibility for degradation. The major recognition signal might reside in the N-terminus of the protein as shown by Bachmair and colleagues (1986). They examined the half-lives of β-galactosidase after substituting its amino terminal amino acid Met with 19 other amino acids. The results show that molecules with either Met, Ser, Ala, Thr, Val, Gly, Pro, Cys have relatively long half-lives of ~20 hours or more; while those with Ile, Glu, Tyr, Gln, His, Arg, Lys, Fhe, Trp, Leu, Asn or Asp at the N-terminus have extremely short half lives of 2-30 minutes. Thus the 20 amino acids can be divided into `stabilizing' and `destabilizing' residues. The authors proposed the `N-end rule' according to which the *in vivo* half life of a protein is a function of its N-terminal residue.

While the N-terminus of a protein is certainly an important structural determinant, other findings suggest that it is not the only recognition signal and it may not be the predominant one. Although, proteins have stabilizing residues at their N-termini, they are efficiently degraded by the ubiquitin system. They may have recognition sites down stream from the N-terminus called the `body site'. In fact, body' type proteins seem to be the most abundant among the proteins which are turned over rapidly (Ciechanover and Schwartz, 1989) suggesting a limited role for the `N-end rule'.

Signals that are distinct from the N-terminal residue have been under study. Rogers *et al.* (1986) have noticed that extremely short lived proteins contain stretches **of** amino acids enriched in Proline (P), Glutamic acid (E), Serine (S) and the Threonine (T). These PEST sequences were flanked by positively charged amino acids and probably are the signals for degradation. However, whether the PEST sequences are recognized by the ubiquitin system is not yet known. Ornithine decarboxylase has two PEST sequences and is degraded by **the** 26 S proteasome but is not ubiquitinated (Bercovich *et al.,* 1989). On the **other** hand, phytochrome and p53, both contain PEST sequences and their degradation is known to be mediated through the ubiquitin pathway. A second well characterized example of a proteolytic signal of the ubiquitin pathway is the degradation signal of the cyclins. They have a conserved sequence motif of nine amino acids characterized by RAALGNISN which is required for degradation of cyclin (Glotzer *et al*., 1991).

### *1.5.4 Examples **of** proteins known to be degraded by **the ubiquitin pathway:***

Studies on phytochrome turnover provided the first evidence for a natural endogenous substrates of the ubiquitin pathway. The newly synthesized phytochrome (P670) absorbs light at 670 nm and shifts to a new spectral state, P730 and this photoconversion increases the rate of degradation as much as 100-**fold.** Light stimulates ubiquitin-phytochrome conjugation which increases until 90 minutes followed by rapid degradation of phytochrome (Shanklin *et al*., 1987).

MATa2 repressor of yeast molecules is rapidly degraded through out the cell cycle. MATα2-ubiquitin conjugation has been demonstrated which is followed by proteolysis of MATa2. It has a half life of two minutes (Hochstrasser *et al,* 1991).

The cyclins are proteins that are associated with protein kinases of the $p34^{cdc2}$ family to form important regulators of the cell cycle. Cyclins accumulate during G1 and S phases and are rapidly degraded at metaphase. Glotzer *et al.* (1991) demonstrated conjugation of ubiquitin to cyclin followed by degradation at metaphase.

Several oncogenic viruses encode proteins that inactivate p53, for example, papilloma virus E6 protein. Scheffner *et al* (1993) demonstrated that E6 and an E6 associated protein forms a part of the complex that functions as a p53 specific ligase and conjugates ubiquitin to p53. Ciechanover *et al.* (1991) observed that proteolysis of Myc, Fos, E1A and p53 was inhibited by using antibodies against E1.

# SCOPE OF THE THESIS

Having accepted the theory that the mammalian estrogen receptors are primarily localized within the nuclei of target cells it was imperative that the details regarding the transport of the ER from the site of its synthesis in the cytosol to the nucleus be made known. From the plethora of molecular events I opted to identify the protein(s) that are directly involved in the transport process. Consequently a 55 kDa protein, apparently of cytosolic origin and a group of 14-11 kDa proteins, presumably associated with the nuclear pore complex/nuclear membrane have been identified and isolated. With the studies on these two classes of proteins what has been achieved is the knowledge that the receptor entry into the nucleus is a 2-stage process, the first stage, which is mediated by the 55 kDa nuclear localization sequence binding protein (NLSBP) and ATP-independent, and the second which is ATP-dependent and is mediated by the 14 kDa protein(s) of the nuclear membrane.

"Estrogen receptor degradation" is a topic that has received scanty information inflow. Ubiquitination is considered to be one of the prime events involved in the process of protein degradation. It was of interest to study whether estrogen receptor gets ubiquitinated and if so whether this process is hormone-dependent. The results, presented in this thesis, are in the affirmative, suggesting that the degradation of the estrogen receptor takes place by the ubiquitin pathway of protein degradation.

Thus two practically unattended areas in the field of estrogen action have been examined and it is anticipated that the studies will lead to additional insights into the vast realm of the biology of estrogen action.

# 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. SV40 large T antigen NLS peptide (pro-lys-lys-lys-arg-lys-val-glu-asp-pro-tyr-cys), Poly-L-lysine hydrobromide, Poly-L-aspartic acid, anti-actin, anti-tubulin and anti-ubiquitin antibodies, 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), 5-Bromo 4-chloro 3-indolyl phosphate (BCIP) Fluorescein isothiocyanate (FITC) isomer I, Estradiol 173, testosterone, dexamethasone, progesterone, Triton X-100, Nonidet P-40, Quercetin, CM-Sephadex, DEAE-Sepharose, Protein A-Sepharose, Adenosine 5'-monophosphate, 4-chloro-1-naphthol 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. HRPO (Horse radish peroxidase) and alkaline phosphatase coupled goat anti-rabbit IgG was purchased from Genei, Bangalore. Phenylmethylsulfonyl fluoride and silver nitrate were purchased from E-Merck, Germany. All the other buffers and 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) deionizer. 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 pH 7.6
1 mM EDTA,
12 mM Monothioglycerol,
0.2 mM PMSF

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

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

TCKM buffer was made as a 5X buffer and stored at 4°C. It was diluted to X **and** the following buffers were made for the isolation of nuclei.
***Buffer A:*** TCKM (X) + 250 mM sucrose.
***Buffer B:*** TCKM (X) + 250 mM sucrose + 0.05% Triton X 100.
***Buffer** C:* TCKM (X) + 340 mM sucrose.

**4. NLSBP assay buffer:** 50 mM Tris-HCl pH 7.6,
3 mM $CaCl_2$ ,
5 mM $MgCl_2$ ,
25 mM KC1,
250 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) PMG buffer:** 10 mM sodium phosphate pH 6.5, 10 mM $MgCl_2$, 0.1 mM GTP.

**9) Alsever's reagent:** 2.05 g glucose, 420 mg sodium chloride, 800 mg sodium citrate, 55 mg citric acid, in 100 ml DDW.

**10) Towbin buffer:** 25 mM Tris-HCl pH 8.3, 192 mM Glycine, 20%methanol.

11) **TNN buffer:** 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1%NP-40.

12) **TMg buffer:** 10 mM Tris-Hcl pH 7.6, 6 mM $MgCl_2$.

13) **Scintillation Cocktail:** 5 gPPO, 500 mg POPOP in Toluene:Triton X-100 (67:33 V/V).

## 2.3 PREPARATION OF GOAT UTERINE CYTOSOL.

Goat uteri were obtained from a local slaughter house, transported on ice to the laboratory and kept frozen at -75°C until further use. Goat uterine cytosol was prepared following the procedure of van der Hoeven (1981). Goat uteri, stored frozen at -75°C, were thawed on ice and minced finely. The uteri were homogenized in the cold using a pre-chilled Waring blendor. The tissue was homogenized in TEMN buffer at high speed for 30 seconds and the blendor was left to cool for sufficient time. The homogenization was continued for another 30 seconds and the procedure was repeated five times. The homogenate was filtered through a nylon mesh. The filtrate was centrifuged at 10,000 X g **for** 3

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 precipitate with 5% PEG were pelleted at 10,000 X g for 30 minutes. The post-mitochondrial supernatant (cytosol) was filtered again through glass wool.

## 2.4 ISOLATION OF GOAT UTERINE NUCLEI.

Goat uterine nuclei were isolated following the procedure of 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 (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 fine nylon mesh and through a nylon membrane of 80-120 microns. The filtrate was then centrifiiged 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 in 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 solubilized the nuclear membranes.

## 2.5 NLSBP ASSAY FOR ER TRANSPORT INTO THE NUCLEUS.

Goat uterine nuclei were isolated as described above. The nuclei to be used in the NLSBP assay were isolated with their membranes kept intact. The nuclei were suspended in 2X NLSBP 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 the 20 nM $^{3}$H-estradiol (50 µl). The unbound hormone was removed following adsorption of free hormone to dextran coated charcoal (100 µl) (1% charcoal and 0.1 % dextran in TEMN buffer). 200 µl of $^{3}$H-$E_2$-ER complexes (containing ~2 µg) were incubated with goat uterine nuclei (250 µl) in the presence of 50 µl aliquot of the fractions containing the NLSBP for 30 minutes at 30$^{o}$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 5,000Xg for 2 minutes. The nuclei were washed once again with 2 ml assay buffer and finally the radioactivity 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 in a Beckman LS 1701. The efficiency of the counting was monitored and the radioactivity in counts per minute (CPM) was converted into disintegrations per minute (DPM) to give a more accurate value.

## 2.6 FLUORESCENCE ASSAY OF THE NLSBP-MEDIATED ER TRANSPORT INTO THE NUCLEUS.

ER was labelled with FITC as described in the section 2.14. Goat uterine nuclei (N+M) were isolated as described above and were suspended in the NLSBP assay buffer. The nuclei were incubated with FITC-ER (5 μg) in the presence or absence of the NLSBP (0.5 μg) at 30°C. Aliquots of the incubation mixture was transferred to a glass slide, covered with a glass coverslip and sealed with vacuum grease. The transport of the ER into the nucleus was observed in a Leitz 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 1 M sodium bicarbonate pH 10.9. The volume of Sepharose 4B was measured and two volumes of 1 M sodium bicarbonate pH 10.9 was added. The slurry was stirred slowly on ice to keep the temperature at 4°C. All the following operations were carried out in a fume hood. The rate of stirring was increased and 0.05 volumes of cyanogen bromide solution (2 mg CNBr/ml acetonitrile) was added. The slurry was stirred vigorously for 10 minutes at 4°C. The slurry was poured into a sintered glass funnel and washed with 10 volumes each of 0.1 M sodium bicarbonate, pH 9.5; double distilled water and 0.4 M sodium bicarbonate, pH 9.5. After the last wash, the slurry was filtered to a moist cake. The activated Sepharose 4B was transferred to a bottle containing one volume of the protein solution. The protein to be coupled to Sepharose 4B was dissolved in the required concentration in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M sodium chloride. Coupling was done at 4°C for 20 hours

with gentle stirring on a rotary shaker. The protein solution was removed, Sepharose was washed with 1 M glycine and stirred with 1 M glycine for an additional four hours to mask the unreacted groups. The protein-Sepharose was washed with 20 volumes each of 0.1 M sodium acetate, pH 4.0 and 0.1 M sodium bicarbonate, pH 8.3; both the buffers contained 0.5 M sodium chloride. The matrix was subsequently washed with the TEMN buffer and stored at 4°C in TEMN buffer containing 0.02% sodium azide.

## 2.8 PREPARATION OF ss-DNA-SEPHAROSE.

DNA was isolated from goat liver following the procedure of Marmur (1961). DNA (1 g) 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 HC1 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 (i) 1000 ml of 50 mM sodium bicarbonate for 10 mins; (ii) 500 ml of 1 mM EDTA pH 7.0 for 10 mins; (iii) twice with 500 ml of double distilled water for 5 mins and (iv) five times each with 250 ml of acetone (at room temperature) for 10 mins each followed by

filtration under vacuum. The final residue was lyophilized in order to remove any traces of acetone. Twenty five grams of skeletal muscle acetone powder was obtained and stored at -70°C until further use.

Ten grams of acetone powder was extracted with 250 ml TMC buffer containing 0.2 mM ATP for 30 mins and 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 3 M potassium chloride, 1 M magnesium chloride and ATP powder were added to the final concentrations of 50 mM, 2 mM and 1mM respectively. The supernatant was stirred with a glass rod and left undisturbed for 2 hours. The KC1 concentration of the supernatant was increased to 0.6 M and stirred gently for an additional one and a half hours to polymerize actin. 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 KC1 and washed with the same buffer. Elution was carried out with 300 mM KC1 in imidazole buffer. The eluate was dialyzed overnight against 2 1 TMC buffer containing 50 mM KC1, 2 mM $MgCl_2$ and 1 mM ATP to effect actin polymerization. The dialysate was centrifuged in order to pellet actin at 80,000 X g for 3 hours. The pellet was resuspended in TMC buffer with 0.2 mM ATP and dialyzed against the same buffer for two days. The dialysate was centrifuged at 80,000 X g for 3 hours. The supernatant which had pure actin was lyophilized. Actin was dissolved in 0.2 M sodium bicarbonate pH 8.3 containing 0.5 M NaCl (~3mg/ml)

and coupled to equal volume of CNBr activated Sepharose 4B as described above.

## 2.10 PREPARATION OF ESTROGEN RECEPTOR-SEPHAROSE.

The activated ER was purified from goat uterine cytosol following the procedure of Zafar and Thampan (1993). All the chromatographic procedures were performed by 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 0.3 M NaCl in TEM buffer. The NaCl concentration in the phosphocellulose eluate was diluted to 0.1 M with TEM buffer and mixed with ss-DNA-Sepharose equilibrated with TEMN buffer. DNA Sepharose was washed with TEMN buffer and eluted with 10 mM ATP in TEMN buffer. 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 isolations was pooled. 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 10 ml of CNBr activated Sepharose 4B as described above.

## 2.11 PREPARATION OF NLS-BSA-SEPHAROSE.

The commercially available SV40 T antigen NLS peptide was coupled to bovine serum albumin (BSA)-Sepharose following the procedure described in the Pierce catalogue with some modifications. BSA (5 mg) was first coupled to CNBr activated Sepharose 4B as described above. The column was washed extensively with 100 mM sodium phosphate buffer pH 6.5. Excess buffer was

drained off and 2 ml of fresh phosphate buffer was added following which 1 ml of Maleimidobenzoic acid N-hydroxysuccinimide ester(MBS) in DMSO (Dimethylsulphoxide) was added. The final concentration of MBS in the incubation mixture was 5 mg/ml. The incubation proceeded at room temperature for one hour. The MBS was washed off with the phosphate buffer. The activated BSA-Sepharose was incubated with 1 mg SV40 T antigen NLS peptide at room temperature for three hours with continuous and gentle shaking. The column was washed with the phosphate buffer and the unreacted sites were blocked with 1M cysteine in the same buffer for an additional hour. The column was washed with the phosphate buffer to remove cysteine and stored at 4°C in the same buffer until use.

## 2.12 PREPARATION OF TUBULIN-SEPHAROSE.

Tubulin was prepared following the procedure of Weisenberg *et al.* (1968). Goat brain was minced well and washed with three volumes of PM buffer containing 0.24 M sucrose. The mince was homogenized in one volume of PM buffer containing 0.24 M sucrose using a Waring blendor. The homogenate was centrifuged in the cold at 16,000 X g for 30 minutes. To the supernatant ammonium sulphate was added to a final 32% saturation and stirred for 30 minutes, allowing maximal precipitation. The supernatant was collected by centnfugation at 10,000 X g for 20 mins. To the supernatant ammonium sulphate was added to 43% saturation. The proteins precipitated at this concentration were pelleted out at 10,000 X g for 20 mins. The pellet was resuspended in PMG buffer (PM buffer containing 0.1 mM GTP) and mixed with DEAE Sephadex pre-equilibrated with the same buffer. The DEAE Sephadex (30 ml) was washed with five volumes of 0.5 M KC1 and the bound proteins were eluted with 0.8 M KC1 in PMG buffer. To the eluate, ammonium sulphate was

added (24.8 g/100 ml) and stirred for 30 minutes. The solution was centrifuged at 35,000 X g for 20 mins. The pellet was dissolved in PMG buffer and was dialyzed extensively against the same buffer in order to remove ammonium sulphate. The dialysate was clarified following centrifugation at 10,000 X g for 30 mins. The supernatant contained pure tubulin which was stored at -70°C until further use. Tubulin (3 mg/ml) was coupled to CNBr-activated Sepharose 4B as described above.

## 2.13 PURIFICATION OF UBIQUITIN.

Ubiquitin was purified from rat red blood cells (RBCs) following the procedure of Parakh and Kannan (1992). Rats were killed by decapitation and the blood was collected into Alsever's reagent. The RBCs were pelleted by centrifugation at 1000 X g for 5 mins. RBCs were washed thrice with Alsever's reagent and were lysed with equal volume of double distilled water. The lysate was stirred and its pH adjusted to 4.5 by the addition of glacial acetic acid. The stirring was continued for an additional 30 mins. The lysate was centrifuged at 5000 X g for 30 mins. The supernatant was dialyzed against 250 ml of 25 mM ammonium acetate pH 4.5 in a dialysis bag with a 12 kDa cut off limit. Ubiquitin (~8 kDa) moved out of the dialysis bag into the buffer. The dialysis buffer was chromatographed over a CM-Sephadex column (30 ml) equilibrated with 25 mM ammonium acetate pH 4.5. The column was washed with the same buffer and eluted with 250 mM ammonium acetate pH 7.2. The fractions collected were examined for their absorbance at 280 nm. The peak fractions were pooled and dialyzed against TEM buffer in a dialysis bag (with a 3.5 kDa cut off limit). The dialysate containing pure ubiquitin was lyophilized and stored at -70°C.

## 2.14 FITC LABELLING OF THE ESTROGEN RECEPTOR.

Goat uterine ER was purified following the procedure described by Zafar and Thampan (1993). The ER was labelled with FITC following the procedure of Chard (1987). One mg of ER was dissolved in 250 µl of 0.15 M $Na_2PO_4.2H_2O$ pH 9.5. 100 µl of the FITC solution (1mg/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 incubated overnight at 4°C. The unbound FITC was removed following chromatography on a column of Sephadex G-25.

## 2.15 IMMUNIZATION PROTOCOL.

The following immunization protocol was followed. Protein 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 day 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. If the serum tested positive, the rabbits were given another booster injection and bled from the ear vein a week laterTheserum waisolated, aliquoted and stored at -200C.

Antibodies were raised against goat uterine estrogen receptor, rat erythrocyte ubiquitin and the synthetic poly-amino acid poly-L-aspartic acid.

## 2.16 ISOLATION OF IgG FROM THE ANTISERUM.

The IgG was purified following chromatography 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 (500 µl) were collected into tubes containing 50 µl of 1 M Tris-HCl pH 8.0. The absorbance of the fractions at 280 nm was monitored. IgG eluted as a single peak. The fractions containing the IgG were pooled and dialyzed extensively against 10 mM Tris-HCl pH 8.0 in order to remove the glycine.

## 2.17 PREPARATION OF PROTEINS FOR SDS-PAGE.

To the protein solution 100% TCA was added to give a final concentration of 30%. The solution was vortexed thoroughly and left on ice for a minimum of two hours (or overnight). The samples were transferred to clean Eppendorf tubes and were centrifuged at 12,000 X g for 20 mins. 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 20 µl of SDS sample buffer. The samples were denatured following heating in a boiling water bath for 3 mins.

## 2.18 SDS-PAGE.

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

**Solutions used:**

*0 Acrylamide solution:* 30 g acrylamide and 800 mg methylene bis-fccrylamide 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°C.

*it) 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, 4 ml 10% SDS was added and the pH of the solution was adjusted to pH 8.8 with 12 N HC1. The volume was then made upto 100 ml. The lower Tris was filtered through Whatman No. 1 paper and stored in glass bottles at 4°C.

***iii)*** *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, 4 ml 10% SDS was added following which the pH of the mixture was adjusted to 6.8 with 12 N HC1. The final volume was then made upto 100 ml. The upper Tris was filtered and stored at 4°C as above.

*iv) Reservoir buffer (8X):* 24 g Tris and 115.2 g glycine were dissolved in double distilled water and the volume was made upto a litre. The buffer was stored at 4°C and diluted just before use.

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

*vi) 10% SDS:* 10 g SDS was dissolved in double distilled water and the volume was made upto 100 ml and stored at room temperature.

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

***viii) 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 was made upto 10 ml **with** double distilled water. 10 mg bromophenol blue was added to the sample buffer. The sample buffer was stored at -20°C 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 were easy to handle and were of the optimal thickness for silver staining. 5 to 15% gels were prepared as given in the table below. (the volumes of the solutions are in ml).

| Lower gel: | 5% | 7.5% | 10% | 12% | 15% |
|---|---|---|---|---|---|
| Acryl amide | 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 | 130 μl |
| TEMED | 10 μl |

Standard gels were run at a constant current of 30 mA and the samples in mini gels at a constant voltage of 60 V through the stacking gels and at 100 V through the separating gels. When the tracking dye reached the end of the gels, the run was terminated and the gels were transferred to the gel boxes containing the fixer (section 2.19). 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.

## 2.19 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.

i) Fixer: 50% methanol, 12% glacial acetic acid and 100 µl 37% formaldehyde/200 ml.

ii) 50% ethyl alcohol.

**iii)** Thiosulphate solution: 40 mg sodium thiosulphate was dissolved in 200 ml double distilled water.

iv) Silver nitrate solution: 400 mg silver nitrate was dissolved in 200 ml double distilled water. 150 µl of 37% formaldehyde was added just before use.

v) Developer: 12 gm of anhydrous sodium carbonate and 100 µl of 37% formaldehyde was dissolved in 200 ml double distilled water.

vi) **12%** glacial acetic acid.

The gels were transferred to a staining tray containing 200 ml of the fixer and left on a shaker for one hour or overnight. The gels were washed with 50% ethanol for 30 minutes. The 50% ethanol was removed and the gels were treated with the thiosulphate solution for exactly one minute. The gels were washed thrice with distilled water for about twenty seconds each in order to

remove thiosulphate. The gels were then treated 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.

## 2.20 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 SDS-PAGE in mini 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 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 - white panel. Care was taken to remove any air bubbles trapped between the gel and the nitrocellulose membrane and the other layers as well. 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 70 V (0.25 A 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 re-incubated with anti-rabbit IgG-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 NP 40) 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 HRPO-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 AP-IgG.

## 2.21 GEL FILTRATION ANALYSIS.

A Sephadex G-100 column (66 X 1.8 cm) was used to perform gel filtration analysis in order to determine the Stokes radius (Rs) of the purified protein following the procedure of Siegel and Monty (1966). The Sephadex G-100 column was calibrated with the gel filtration markers and proteins of known Stoke's radii (blue dextran, bovine serum albumin (BSA), ovalbumin (OV), cytochrome C (CYT-C) and glycine). The column was equilibrated with TEM buffer containing 0.3 M NaCl. The marker proteins were dissolved (~5 mg/ml) in the equilibration buffer and about 500 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 Rs of the marker proteins was made.

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

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 from the standard graph.

## 2.22 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:**

**i)** 20 μg/ml potassium phosphate solution (for the standard) in 0.05 N hydrochloric acid.

ii) 0.05 N hydrochloric acid.

iii) Acid molybdate: 1.25% ammonium molybdate in 2.5 N sulphuric acid.

iv) Fiske-Subbarow reagent: 7.312 gm sodium metabisulphite, 400 mg 1-amino-2-naphthol-4-sulphonic 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 gm of this ANSA 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 HC1 to different concentrations (1 μg to 10 μg/500 μl). To the phosphate, 1 ml acid molybdate was added and mixed, to be 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 read at 660 nm against a blank containing 500 µl 0.05 N HC1 similarly treated. The standard was determined as an absorbance of 0.76 for 10 µg phosphate.

**2.23 ATPASE** ASSAY:

The pure NLSBP was incubated with 6 mM ATP in TMg buffer at 30°C for different time intervals. 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 cold at 10,000Xg for 20 minutes. The Pi present in the supernatants was estimated as described above. The concentration of the Pi released was calculated from the standard graph and the value was converted to its molar concentration (20 µg Pi/ml is 0.65 mmol/l). The ATPase activity was expressed as µmoles Pi released/hour. The ATPase assay was performed as above in the presence of goat uterine nuclei in order to estimate the nuclear ATPase activity. The ATPase activity of the nuclei was expressed as µmoles Pi released/mg DNA.

**2.24 ESTIMATION** OF DNA:

DNA was estimated following the method described by Burton (1956). The diphenylamine (DPA) reagent was prepared by dissolving 1.5 g diphenylamine in 100 ml glacial acetic acid, 1.5 ml of conc. sulphuric acid and 0.1 ml of aqueous acetaldehyde (0.16 %). DNA was dissolved in 0.5 N perchloric acid and 1ml of this solution was mixed with 2 ml of the DPA reagent and boiled for twenty minutes. The tubes were then transferred to an ice bath and the color developed was measured at 600 nm against a blank of 1 ml 0.5

N perchloric acid, treated as above. The standard value obtained was 0.01 at 600 nm for 20 jig/ml calf thymus DNA.

## 2.25 ESTIMATION OF PROTEINS.

Proteins were estimated by the procedure of Lowry et al. (1951). Alkaline copper sulphate solution was prepared by mixing 2% sodium-potassium tartarate, 1% copper sulphate and 2% sodium carbonate in 0.1 N sodium hydroxide in the ratio 1:1:98. One ml of protein solution was mixed with 5 ml of alkaline copper sulphate solution, mixed well and incubated at room temperature for 15 minutes. After 15 mins, 0.5 ml of Folin's reagent was added, vortexed and the mixture was left for an additional 30 mins at room temperature. The color developed was measured spectrophotometrically at 670 nm. A standard graph was prepared with bovine serum albumin (BSA) in the range of 20-200 μg. The standard was taken as 0.24 absorbance at 670 nm for 100 μg BSA.

## 2.26. MAINTENANCE AND SURGERY OF ANIMALS:

3 month old female rats from an inbred colony of Wistar strain were used in the experimental studies. The animals were maintained under natural dark and light cycles (12 hours of light and 12 hours of darkness). Food (standard pellet food supplied by Hindustan Lever Ltd. India) and water were available *ad libitum* to the animals. Ovariectomy and subcutaneous implantations of hormone-bees-wax (estradiol:Bees wax 1:1000) pellets were done in rats under light ether anaesthesia.

# TRANSPORT OF THE ER INTO THE NUCLEUS IS MEDIATED BY A 55 kDa CYTOSOLIC PROTEIN.

# 3.0 TRANSPORT OF THE ESTROGEN RECEPTOR INTO THE NUCLEUS IS MEDIATED BY A 55 kDa CYTOSOLIC PROTEIN.

The mechanisms associated with the estrogen receptor transport into the target cell nucleus are not known. It is believed that the process is mediated by a protein that recognizes nuclear localization sequences (NLSs) in the ER. Receptors deficient in the NLS were demonstrated to be cytoplasmic in localization even in the presence of the hormone (Picard *et al.*, 1990b; Ylikomi et al., 1992). It is already known that the NLS containing karyophilic proteins are transported to the nucleus by a protein, the NLSBP, which recognizes the NLS and transports the protein into the nucleus (section 1.4.4). The protein(s) that transport ER into the nucleus require to be identified. In order to identify and purify the NLSBP for the ER, it was important to develop and standardize a suitable functional assay.

## 3.1 THE NLSBP ASSAY.

The NLSBP assay mentioned in this chapter is described in section 2.5. To develop a NLSBP assay with optimal activity, it was important to identify a simple and rapid procedure to isolate intact nuclei (with maximum yield and activity). Thus the procedure developed by Thampan (1985) was followed. This procedure was quite simple and the yield of intact nuclei was between 70-80% as detennined by phase contrast microscopy (data not shown). The method is detailed in `materials and methods' (section 2.4).

The nuclei to be used in the NLSBP assay were isolated with their membranes kept intact (i.e., the isolation procedure should not involve any detergent). Only such nuclei were transport competent. We observed that ER does not get transported into the nucleus by itself (data shown elsewhere). Transport of ER into the nucleus was seen only in the presence of the cytosolic proteins. This assay was repeated several times and consistently repeatable results were obtained.

Using this assay a protocol to purify the NLSBP was developed. A multipronged approach was undertaken. The usual conventional chromatographic procedures were avoided and purification was planned to be undertaken solely by affinity chromatography procedures. Not withstanding the fact that purification of the NLSBP was not achieved on some of these affinity matrices, they are described here in detail, as they gave a certain insight to the structure and the function of the NLSBP. The affinity chromatography procedures are described below along with the reason why they were used.

## 3.2 CHROMATOGRAPHY OF GOAT UTERINE CYTOSOL ON ESTROGEN RECEPTOR-SEPHAROSE.

The NLSBP in order to transport ER into the nucleus ought to recognize the NLS on the ER. Thus the first obvious choice for purification was estrogen receptor-Sepharose. It was felt that this matrix should prove useful in isolating the NLSBP by chromatography on estrogen receptor-Sepharose column. 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 the `methods' (section 2.10).

Goat uterine cytosol was prepared as described in the methods. The cytosol was chromatographed on an estrogen receptor-Sepharose column, pre-equilibrated with TEMN buffer. The flowthrough fraction was recycled onto the column in order to optimize the binding of proteins to the estrogen receptor-Sepharose. The column was washed extensively with TEMN buffer and a linear lysine gradient (0 to 1.0 M) was applied, so that, the proteins that bind ER in a lysine dependent manner could be eluted first. The column was washed with TEMN to remove lysine and a linear NaCl gradient (50 mM to 1 M) was applied, The fractions collected (1.5 ml/fraction) were subjected to the NLSBP assay. The NLSBP activity eluted from the estrogen receptor-Sepharose as a single peak of activity with ~0.7 M lysine (Fig 3.1A). This suggested a very high affinity of NLSBP for binding to the ER. All the NLSBP activity was eluted at this step and no additional NLSBP activity was observed in the NaCl eluted fractions (data not shown). The fractions demonstrating the NLSBP activity were precipitated with trichloroacetic acid (TCA) and were subjected to SDS-PAGE. Upon silver staining three major protein bands were visualized, of molecular mass 55, 48 and 27 kDa. (Fig 3.1B). The estrogen receptor-Sepharose columns had a short half life. A column, on an average, could be used for about 4 isolation procedures. It was not viable to use estrogen receptor-Sepharose columns extensively to purify NLSBP. Thus a search for an alternate method of purification was undertaken.

## 3.3 CHROMATOGRAPHY ON POLY-L-LYSINE SEPHAROSE.

The putative NLS of the SV40 T antigen is a stretch of basic amino acids, predominantly comprised of lysine residues. A mutation in one of the lysines in the NLS results in the loss of nuclear localizing capacity or activity (Kalderon *et al*, 1984). This apparently indicated that the NLSBP recognizes the lysine residues in the NLS. The ER proto-NLSs are also largely comprised of basic residues RKRDR-KHKRQR-KRSKK (13 out of the 16 residues are basic) (Ylikomi *et al*., 1992). The best affinity column to be used would have been the ER-NLS peptide coupled to Sepharose (via a spacer). The cost of getting a sufficient amount of this peptide synthesized was very high which prompted us to search for other avenues. There are a number of lysines in the ER proto-NLSs and a large number of NLSs identified so far are predominantly lysine rich. The NLSBP activity was found to elute from the estrogen receptor-Sepharose with ~0.7 M lysine, suggesting that the interaction of the NLSBP with ER was via lysines. For the above reasons, estrogen receptor-Sepharose was substituted with poly-L-lysine-Sepharose in order to attempt purification of the NLSBP.

Goat uterine cytosol was chromatographed over a column of poly-L-lysine-Sepharose (15 ml). The flowthrough from this column was recycled on the column for maximal binding. Poly-L-lysine-Sepharose was washed extensively with TEMN and eluted with a linear lysine gradient (0-1 M lysine in TEMN). The column was washed again with TEMN buffer to remove lysine and eluted with a linear NaCl gradient (50 mM to 1 M NaCl). The eluate was collected as 2 ml fractions. All the fractions were subjected to the NLSBP assay. The NLSBP activity eluted from the poly-L-lysine column as a single peak of activity with 0.7-0.8 M lysine (Fig 3.2A). This resembled very closely the elution pattern from

the estrogen receptor-Sepharose column. The peak activity fractions were subjected to SDS-PAGE. The fractions had three major proteins, of molecular mass 55, 48 and 27 kDa (Fig 3.2B). There was no NLSBP activity seen in the fractions eluted with NaCl (data not shown).

Since the estrogen receptor-Sepharose elution profile and the poly-lysine elution profiles were essentially the same, the poly-L-lysine Sepharose column was used thereafter to attempt further purification of the NLSBP.

It was possible that poly-lysine binding of the NLSBP activity or the elution of the activity from estrogen receptor-Sepharose with lysine, could be purely based on charge. Poly-lysine could therefore function as an anion exchanger. To determine this, either cytosol or the poly-lysine eluate was chromatographed over a DE-52 column, a well known anion exchanger. The column was eluted with an increasing salt gradient (50 mM to 1 M NaCl). The fractions collected were subjected to NLSBP assay. It was observed that the NLSBP bound very weakly to DE 52 and eluted with ~100-150 mM NaCl (data not shown) suggesting that the binding of the NLSBP to the poly-lysine column was due to lysines and not due to the charge on the column.

## 3.4 CHROMATOGRAPHY ON ANTI-POLY-L-ASPARTATE IgG-SEPHAROSE.

Yoneda *et al*. (1988) had proposed that since the SV40 large T antigen NLS is KKKRK, the sequence on the NLSBP that recognizes this sequence would probably be an electrostatically complementary DDDED or EEEDE. They found that antibodies against DDDED or EEEDE inhibited nuclear transport and recognized two cytosolic proteins of molecular mass 69 and 58 kDa respectively.

Encouraged by these results, we decided to explore the possibility of using antibodies raised against poly-L-aspartate to further purify the NLSBP. It was assumed that poly-L-aspartate would elicit a similar kind of antibody response as a stretch of 5 to 6 aspartates coupled to a carrier protein. In any case the idea was to raise antibodies against a peptide of aspartates. Antibodies were raised against poly-L-aspartate in rabbits as described in the "methods". The IgG fraction was isolated from the antiserum by chromatography on protein A-Sepharose and was coupled to Sepharose 4B.

Goat uterine cytosol was chromatographed on poly-L-lysine-Sepharose. The column was washed with TEMN, followed by 0.5 M lysine in TEMN and eluted with 1 M lysine. The 1 M lysine eluate was dialyzed overnight against TEMN buffer to remove lysine. The dialysate was re-chromatographed over anti-poly-L-aspartate Sepharose (5 ml). The column was washed extensively with TEMN buffer. The anti-poly-L-aspartate IgG bound proteins were eluted consecutively with 1M NaCl ($N_1$ to $N_{10}$); 30 mM aspartate in TEM buffer pH 3.5 ($A_1$ to $A_{10}$); and 50 mM glycine-HCl pH 2.5 ($G_1$ to $G_{12}$). In between each elution and after the final elution the column was washed extensively with TEM buffer. The eluates were collected as 1.5 ml fractions. The pH of the aspartate and glycine eluates was adjusted to neutrality with 1 M Tris. All the fractions were subjected to NLSBP assay. NLSBP activity was found to elute from the anti-poly-L-aspartate IgG-Sepharose column only with 30 mM aspartate at pH 3.5 (Fig 3.3A). Two protein bands of molecular mass 55 and 48 kDa were seen here as well (Fig 3.3B)

In another experiment, cytosol was chromatographed directly over anti—poly-L-aspartate-Sepharose, and eluted as described above. The elution method and the elution profiles were the same as above. The yield of the 55 kDa and the 48 kDa proteins in this experiment was higher than that mentioned above (Fig 3.3B).

The poly-L-lysine, ER, or anti-poly-L-aspartate fractions showing the maximum NLSBP activity were subjected to western blot analysis. The blots were incubated with anti-poly-L-aspartate IgG followed by HRPO-coupled anti-rabbit IgG. The blots were stained with 4-chloro 1-naphthol and $H_2O_2$. The poly-aspartate antibody recognized only the 55 kDa protein (Fig 3.4). The 48 kDa protein may be very tightly associated with the 55 kDa protein as it was seen to bind to the anti-poly-aspartate IgG-Sepharose but does not crossreact with this antibody. The 55 kDa protein obtained here may be the same as the 58 kDa protein identified by Yoneda *et al.* (1988).

Tubulin and actin coprecipitated with the estrogen receptor when goat uterine cytosol was immunoprecipitated with ER antibody (Zafar and Thampan, 1994). Tubulin and actin were also demonstrated to be associated with the glucocorticoid receptor via HSP 90 (Sanchez *et al.*, 1988; Miyata and Yahara, 1991). The association of the ER with the contractile apparatus of the cell suggests that the transport of the ER from the cytoplasm to the nucleus is probably aided by the microfilaments or the microtubules. It was decided to test whether the NLSBP possessed the capacity to bind actin and/or tubulin and explore the possibility of purifying the NLSBP using the actin-Sepharose or the tubulin-Sepharose columns.

## 3.5 CHROMATOGRAPHY ON TUBULIN-SEPHAROSE.

**It** was important to obtain a highly pure preparation of tubulin to prepare an affinity column. Tubulin was purified from goat brain following the procedure of Weisenberg *et al*. (1968). The purity of the material obtained was determined by SDS-PAGE followed by silver staining. A single band of tubulin, of molecular mass 55 kDa was obtained (Fig 3.5A). To confirm that the protein obtained was tubulin it was further subjected to western blot analysis. The commercially available anti-tubulin antibody crossreacted with the tubulin preparation (Fig 3.5B). Tubulin (~30 mg) was coupled to 10 ml CNBr activated Sepharose 4B.

The 1 M lysine eluate from the poly-L-lysine column was dialyzed against TEMN buffer in order to remove lysine. The dialysate was rechromatographed over the tubulin-Sepharose column. The column was washed extensively with TEMN buffer and was eluted with a linear salt gradient (50 mM to 1 M NaCl). The fractions collected were subjected to NLSBP assay. The NLSBP activity eluted from the tubulin-Sepharose column with ~0.6 M NaCl (Fig 3.6A). The fractions with the maximum NLSBP activity were subjected to SDS-PAGE. The fractions showed a major 55 kDa band, but faint bands of 48 and 27 kDa were also seen(Fig 3.6B). The above result demonstrates that the NLSBP binds strongly to tubulin

## 3.6 CHROMATOGRAPHY ON ACTIN-SEPHAROSE.

**It** was imperative to obtain a highly pure preparation of actin to prepare an affinity column. Actin was purified from the goat skeletal muscle following the procedure of Pardee and Spudich (1982) in repeated polymerization and depolymerization cycles. The polymerization of actin in the first step was

enhanced several fold in the presence of 1 mM ATP resulting in a higher yield of actin. After several polymerization and depolymerization cycles actin was further purified by chromatography on the DE-52 column. The final actin preparation was subjected to SDS-PAGE and the gels were stained with silver nitrate. 100% pure actin was obtained (Fig 3.7A) as determined by scanning densitometry. The pure protein was subjected to western blot analysis. The commercially available actin antibody cross-reacted with the protein confirming it as actin (Fig 3.7B). Actin (30 mg) was coupled to 10 ml CNBr activated Sepharose 4B.

NLSBP activity eluted from the Poly-L-lysine-Sepharose column (1 M lysine eluate) was dialyzed against TEMN buffer and the dialysate was chromatographed on a column of actin-Sepharose. The column was washed with 10 mM ATP in TEMN buffer. The column was washed with TEMN buffer and elution was achieved using an increasing salt gradient (50 mM to 1M NaCl). The eluate was collected as 1.5 ml fractions and was subjected to NLSBP assay. A single peak of NLSBP activity was eluted with 0.5 to 0.6 M NaCl (Fig 3.8A). The fractions with the NLSBP activity were subjected to SDS-PAGE. Upon silver staining, a single band of molecular weight 55 kDa was obtained (3.8B).

In another experiment cytosol was chromatographed directly over actin-Sepharose column. The column was washed and eluted as described above. The fractions were subjected to NLSBP assay. The NLSBP activity was eluted with ~0.6 M NaCl (Fig 3.9A). When these fractions were subjected to SDS-PAGE a single protein band of molecular mass 55 kDa was observed (Fig. 3.9B).

The yield of the protein was higher than in the previous experiment. Thus, a procedure for purifying the NLSBP in a single step was identified.

Almost all of the 48 kDa protein got eluted in the 10 mM ATP wash. This ATP eluted fraction did not show any NLSBP activity (data not shown). This demonstrated that the 48 kDa protein was not an NLSBP protein and that it dissociated from the 55 kDa protein in the presence of 10 mM ATP. The 55 kDa protein might have an ATP binding site which is confirmed by the demonstration of an ATPase activity in the next chapter 4.9. This binding site probably overlaps the binding site for the 48 kDa and the binding affinity of the 55 kDa protein for ATP is probably higher than that for the 48 kDa protein. Alternatively, the binding of ATP to the 55 kDa protein might change its conformation to a form that does not bind to the 48 kDa protein.

Actin-Sepharose columns had a limited half-life. The column gave the maximum yield during its first use. It was possible to use the column twice again but the yield of the protein thus obtained decreased several fold. Actin-Sepharose does not lose any binding activity upon storage but lost activity only after use. Therefore actin-Sepharose was prepared in large quantities and stored at $4^{0}C$; 10 ml columns were used for two isolations and discarded.

NLSBP was purified by the chromatography of cytosol on actin-Sepharose as described above. NLSBP purified thus was used for further characterization of the protein.

## 3.7 CALCULATION OF THE STOKES RADIUS.

The Stokes radius of the NLSBP was calculated by performing gel filtration analysis as described in the "methods" (section 2.21). Pure NLSBP was concentrated to a small volume and about 300 µl was chromatographed on a column of Sephadex G-100 (66X1.8 cm). The column was developed with TEM buffer containing 0.2 M NaCl. Sodium chloride was added to the buffer to prevent non-specific adsorption of proteins to the Sephadex beads and self-aggregation of the protein. The fractions (2.5 ml) collected were subjected to the NLSBP assay. NLSBP eluted as a single peak of activity with 55-60 ml of the buffer (Fig 3.10).

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 NLSBP was calculated as below.

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

where Ve - volume with which NLSBP was eluted.

$$\text{The NLSBP } Kd = \frac{57-28.5}{82.5-28.5} = 0.527$$

The corresponding Stokes radius for the Kd of 0.527 is 25 $A^0$ . Thus the Stokes radius of the NLSBP was determined to be 25 A °.

## 3.8 THE NLSBP BINDS TO THE ER-SEPHAROSE IN A LYSINE DEPENDENT MANNER.

In the first experiment described in this section (3.1), NLSBP activity was found to elute from estrogen receptor-Sepharose column with ~0.7 M

lysine. Though the major proteins eluted were the 55 kDa (NLSBP) and the 48 kDa protein; it was not clear if the 55 kDa protein directly bound to estrogen receptor-Sepharose or its binding to ER was mediated by another protein, probably the 48 kDa. Therefore in order to determine if the pure NLSBP binds to estrogen receptor-Sepharose, NLSBP isolated from actin-Sepharose was dialyzed and chromatographed over a column of estrogen receptor-Sepharose (10 ml). The column was washed extensively with TEMN buffer and eluted with an increasing lysine gradient (0 to 1 M) followed by an increasing NaCl gradient (50 mM to 1 M NaCl). The eluate collected in 1.5 ml fractions were, subjected to the NLSBP assay. The fractions containing NLSBP activity were subjected to SDS-PAGE. The 55 kDa protein was found to elute with ~0.7 M lysine (Fig 3.11A and B). There was no NLSBP activity seen in the NaCl eluted fractions. This experiment confirmed that the NLSBP binds to the ER. The binding is probably lysine dependent as it elutes with a high concentration of lysine.

### 3.9 **THE NLSBP** ALSO **BINDS** TO THE SV40 T **ANTIGEN** NLS.

The NLSBP identified here might be similar to the one identified by Adam and co-workers (1989, '91). The molecular weight of the protein they had identified was ~55 kDa. They identified this protein from rat liver cytosol and bovine erythrocytes. This protein binds to a heterologous NLS, the SV40 large T antigen NLS. Thus it was necessary to check whether the purified NLSBP could bind to the SV40 large T antigen NLS. Commercially available SV40 NLS peptide was cross linked to BSA-Sepharose using a bifunctional crosslinker, MBS, as described in the methods' (section 2.11).

The pure NLSBP that eluted from the actin-Sepharose column was dialyzed against Tris buffer (50 mM Tris-HCl pH 7.6). The dialysate was

chromatographed over a column of SV40 NLS-BSA-Sepharose (3 ml). The column was washed extensively with Tris buffer and NLSBP was eluted from the column with a linear lysine gradient (0 to 1 M) in Tris buffer. One ml fractions were collected and were subjected to SDS-PAGE. The NLSBP was eluted from the NLS peptide column with ~0.5 M lysine (Fig 3.12). The binding of NLSBP to SV40 large T antigen NLS peptide, a heterologous peptide, suggested that it may be involved in the transport of other nuclear proteins with NLSs resembling the SV40 NLS.

## 3.10 THE NLSBP IS NOT TUBULIN.

Previous studies from our laboratory demonstrated that the ER was associated with both tubulin and actin (Zafar and Thampan, 1994). The molecular weight of tubulin and the NLSBP appeared to be the same (55 kDa). It was therefore necessary to confirm whether tubulin and the protein that we purified were the same proteins. In order to examine this possibility, western blot analysis of NLSBP and tubulin were performed in duplicates. One of the blots was exposed to commercially available tubulin antibody and the other to poly-aspartate antibody. The blots were re-exposed to HRPO-coupled anti-rabbit IgG and stained with 4-chloro 1-naphthol. The tubulin antibody did not crossreact with the NLSBP (Fig 3.13A) and the poly-L-aspartate antibody did not crossreact with tubulin (Fig 3.13B) confirming that the NLSBP was not tubulin.

## 3.11 THE ANTI-POLY-ASPARTATE IgG CROSSREACTS WITH THE NLSBP AND INHIBITS TRANSPORT OF THE ER INTO THE NUCLEUS.

In an earlier experiment, it was demonstrated that the 55 kDa protein with NLSBP activity isolated by chromatography on poly-aspartate IgG-Sepharose (section 3.3) showed crossreactivity to the poly-aspartate antibody. But, it was considered important to demonstrate the crossreactivity of the pure NLSBP with the poly-aspartate antibody.

Increasing concentrations of pure NLSBP (0.4 to 2 μg) were subjected to SDS-PAGE. The proteins were transferred from the gel to nitrocellulose membranes. The membranes were exposed overnight to poly-L-aspartate antibody. The blots were washed and re-exposed to HRPO-coupled anti-rabbit IgG and stained subsequently with 4-chloro 1-naphthol and $H_2O_2$ . The pure NLSBP was seen to crossreact with the poly-L-aspartate antibody (3.14).

The anti-poly-L-aspartate IgG recognizes the NLSBP. Theoretically, addition of this IgG to the NLSBP assay medium should inhibit the transport of ER into the nucleus. To test this assumption the following experiment was carried out. The NLSBP assay mixture including the $^3$H-$E_2$-ER complexes, pure NLSBP and nuclei was incubated with anti-poly-L-aspartate IgG (1:10) in water bath maintained at 30°C for 30 minutes. A control set without the antibody was also incubated.

There was no transport of the ER into the nucleus in the presence of the poly-L-aspartate antibody. The antibody inhibition could not be overcome by the addition either of excess ER or of nuclei but only by the addition of excess

NLSBP (Fig 3.15). This ruled out the possibility that poly-L-aspartate antibody inhibits transport by binding to the ER or by blocking the nuclear pore by binding to the nuclear envelope. The antibody bound to the NLSBP and made it unavailable for the transport of ER into the nucleus. This also suggested that the site on the NLSBP which recognized the ER was probably a stretch of aspartates, as suggested earlier by Yoneda *et al*. (1988).

## 3.12 DISCUSSION.

A single step procedure for the purification of the NLSBP has been identified. The 55 kDa protein purified from the goat uterine cytosol binds to the ER and transports it to the nucleus. This was demonstrated by the NLSBP assay. Because the NLSBP assay does not distinguish between binding of the ER to the nuclear membrane and transport into the nucleus, we decided to confirm transport by
performing the fluorescence assay for ER transport (described in the following chapter). NLSBP mediated transport of ER reflects the true transport of ER into the nucleus. More details of the mechanism of transport of ER are discussed in the section 4.13.

The NLSBP-mediated transport of ER into the nucleus was not influenced by the presence of estradiol in the assay medium. ER nuclear transport was seen in the absence of estradiol, when NLSBP assay was performed using C-labelled ER (data not shown) or FITC-labelled ER (data shown in next section). The incubation of ER with H-estradiol in the NLSBP assay was only to label the ER.

Though there is no direct evidence to demonstrate that the NLSBP binds to the NLSs of the ER, there are several indirect evidences to suggest so.

1. The NLSBP binds to ER with high affinity and can be eluted only with a high concentration of lysine, suggesting that it probably binds to the ER at a lysine rich region.

2. In the primary structure of the ER the region rich in lysine is the NLS of the ER (Picard *et al,* 1990b; Ylikomi *et a!.,* 1992).

3.ER deficient in the NLS region cannot be transported into the nucleus (Ylikomi et al., 1992), nuclear transport ought to be only by recognition of the NLSs by the NLSBP. We propose that the 55 kDa protein we have purified is the estrogen receptor nuclear localization sequence binding protein (NLSBP). The NLSBP recognizes the NLS on the ER, binds to it and transports it to the nucleus.

The site on the NLSBP which recognizes the ER is probably aspartate rich, as the NLSBP crossreacts with the anti-poly-L-aspartate IgG. The addition of the poly-L-aspartate antibody to the NLSBP assay inhibits nuclear transport of the ER and this inhibition can be overcome by the addition of excess NLSBP.

The fact that the NLSBP also binds to the SV40 large T antigen NLS suggests a broad specificity for the NLSBP. The NLSBP may also transport the other steroid receptors, but this also has to be studied in detail using the same assay system but with other purified steroid receptors.

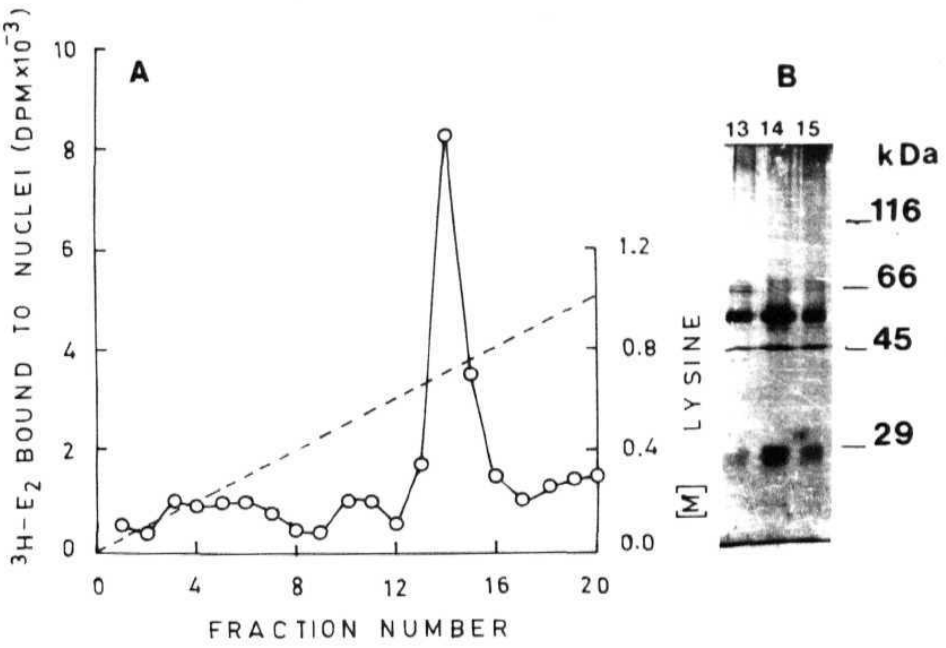
The NLSBP was seen to bind to both actin and tubulin with high affinity. The interaction of both the ER and the NLSBP with the cytoskeletal elements of the cell suggests the possible involvement of the cytoskeletal proteins in the transport of ER into the nucleus.

A 59 kDa protein was observed to be present as a component of the non-transformed estrogen, androgen, progestogen and glucocorticoid receptors (Rexin *et al*;., 1991; Tai et al., 1986; 1993). p59 is associated with the HSP 90 and has an ATP binding site (LeBeau et al., 1992). It has a short stretch of DDED residues a site that is electrostatically complementary to the NLS. The function of this protein has not been identified. Keeping the above observations in mind and the fact that it is associated with all the steroid receptors, we propose that the NLSBP we purified is the p59 protein identified by the other research groups.

Figure 3.1. Chromatography of goat uterine cytosol on estrogen receptor-Sepharose.
(A) Goat uterine cytosol was chromatographed on a estrogen receptor-Sepharose column (10 ml). The column was washed with TEMN buffer and eluted with a linear lysine gradient (0 to 1 M). Fractions (1.5 ml) collected were subjected to the NLSBP assay. The $^{3}H$-$E_2$-ER complexes transported to the nucleus was measured and were converted to DPM (disintegrations per minute). The lysine concentration in the fractions was also estimated (broken line).

The fractions that demonstrated NLSBP activity were precipitated with TCA and subjected to SDS-PAGE in 10% gels. The gels were stained with silver nitrate as described in the "methods". The lane numbers depicted are the corresponding fraction numbers from figure 1 A.

Figure 3.2. Chromatography of cytosol on poly-lysine-Sepharose.
(A) Goat uterine cytosol was chromatographed on poly-lysine-Sepharose equilibrated with TEMN buffer. The column was washed with the same buffer and eluted with a linear lysine gradient (0 to 1 M lysine in TEMN). The eluate was collected as 2.0 ml fractions and were subjected to NLSBP assay.
(B) The fractions showing maximum NLSBP activity were subjected to SDS-PAGE in 10% gels. The gels were stained with silver nitrate. The numbers on the lanes correspond to the fraction numbers in (A).


A
B
$^3H$-$E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
FRACTION NUMBER
[M] LYSINE
13 14 15
kDa
116
66
45
29

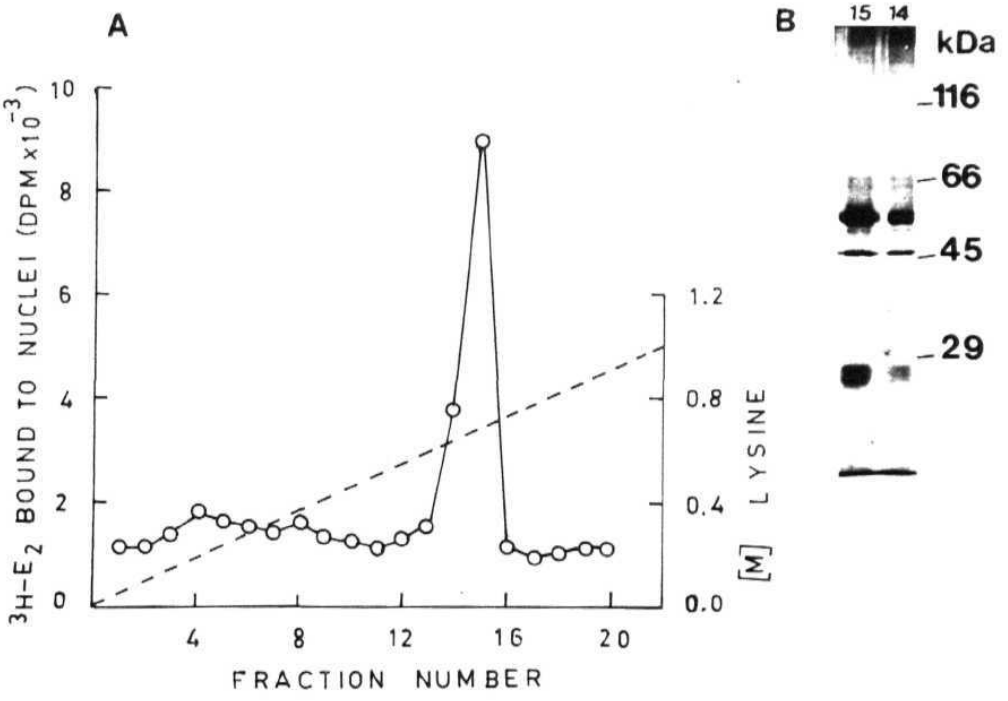

A
B
$^3H$-$E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
FRACTION NUMBER
[M] LYSINE
15 14
kDa
116
66
45
29

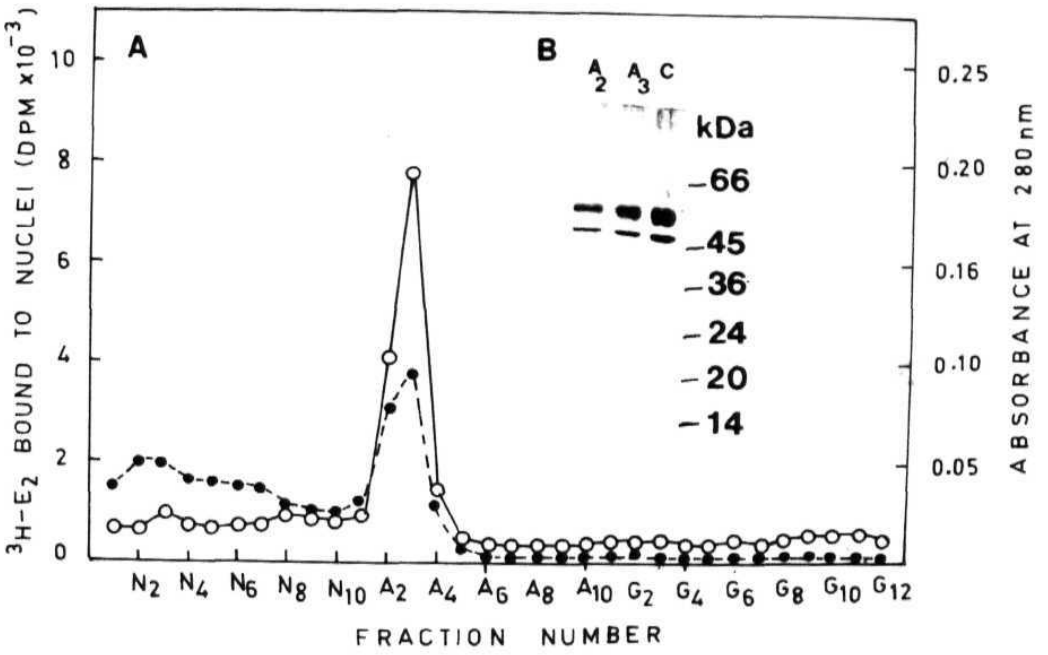
Figure 3.3. Anti-poly-aspartate IgG-Sepharose chromatography of the NLSBP.

(A) Goat uterine cytosol was chromatographed on a column of poly-lysine-Sepharose. The column was washed with 0.5 M lysine in TEMN buffer and eluted with 1 M lysine. The eluate was dialyzed against TEMN buffer to remove lysine and the dialysate was chromatographed over anti-poly-aspartate IgG-Sepharose (5 ml). The column was washed with TEMN buffer and eluted successively with 1 M NaCl ($N_1$ to $N_{10}$), 30 mM aspartate in TEM, pH 3.5 ($A_1$ to $A_{10}$), and 50 mM glycine-HCl, pH 2.5 ($G_1$ to $G_{12}$). The fractions (1.5 ml) collected were subjected to the NLSBP assay. The absorbance of the fractions at 280 nm was also measured (denoted by the broken line).

B) The protein in the fractions demonstrating the maximum the NLSBP activity in the figure 3.3A were precipitated with TCA and were subjected to SDS-PAGE followed by silver staining. In another experiment, the goat uterine cytosol was directly cliroinatographed on the poly-aspartate IgG-Sepharose and eluted as described above. The aspartate eluate was also subjected to SDS-PAGE (lane C).

Figure 3.4. The poly-aspartate antibody recognizes the NLSBP.

(A) NLSBP activity that eluted from the estrogen receptor-Sepharose($R_1$ and $R_2$), poly-lysine-Sepharose ($P_1$ and $P_2$) and anti-poly-aspartate IgG-Sepharose ($A_1$ and $A_2$) were subjected to western blot analysis. The non-specific adsorption sites on the nitrocellulose membranes were blocked with 3% BSA. The western blots were exposed to anti-poly-aspartate antibody (1:50) overnight. The blots were washed and re-exposed to HRPO-coupled anti-rabbit IgG (1:500) and were stained with 4-chloro, 1-naphthol and $H_2O_2$.


A
B
A2 A3 C
kDa
66
45
36
24
20
14
3H-E2 BOUND TO NUCLEI (DPM x10-3)
ABSORBANCE AT 280 nm
0
2
4
6
8
10
0.05
0.10
0.16
0.20
0.25
N2 N4 N6 N8 N10 A2 A4 A6 A8 A10 G2 G4 G6 G8 G10 G12
FRACTION NUMBER

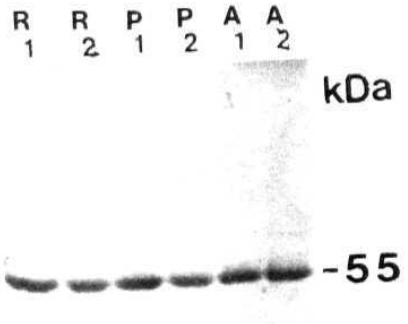

R1 R2 P1 P2 A1 A2
kDa
55

Figure **3.5A. SDS-PAGE** analysis of **tubulin.**

Tubulin was purified as detailed in the `methods'. 0.5, 2.5 and 5 μg tubulin was precipitated with TCA. The precipitate was washed with alcohol, air dried **and** was dissolved in sample buffer, and subjected to SDS-PAGE on 10% gels in lanes 1, 2 and 3 respectively. The gels were stained with silver nitrate.

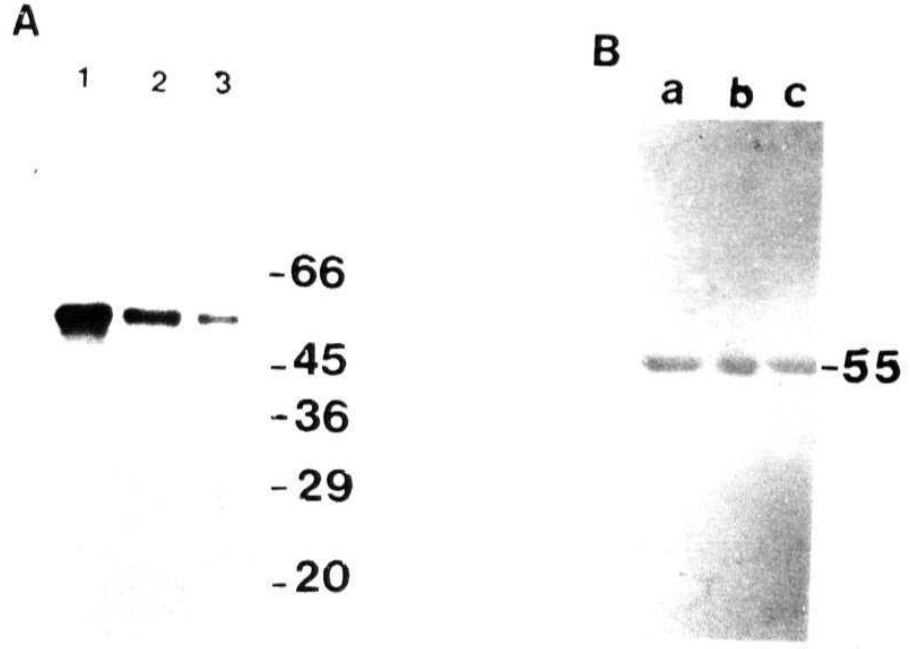
**5B. Western blot** analysis of **tubulin.**

Tubulin (0.5 μg) was subjected to SDS-PAGE on 10% gels and the proteins were transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 3% BSA and incubated overnight with anti-tubulin antibody (1:50). 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 $H_2O_2$ .

**Figure** 3.6. **Tubulin-Sepharose chromatography** of **the ER** nuclear **transport protein.**

(A) The **1** M lysine eluate from the poly-lysine-Sepharose column was dialyzed extensively in order to remove lysine and chromatographed over a column of tubulin-Sepharose (10 ml). The column was washed and the tubulin bound proteins were eluted with a linear NaCl gradient (50 mM to 1 M). Fractions (1.5 ml) collected were subjected to the NLSBP assay. The NaCl concentration of the fractions is denoted by the broken line.

(B) The fractions, that eluted from tubulin-Sepharose, demonstrating the NLSBP activity were subjected to SDS-PAGE on 12% gels. The gels were stained with silver nitrate. The lane numbers correspond to the fraction numbers in (A).


A
1
2
3
-66
-45
-36
-29
-20
B
a
b
c
-55

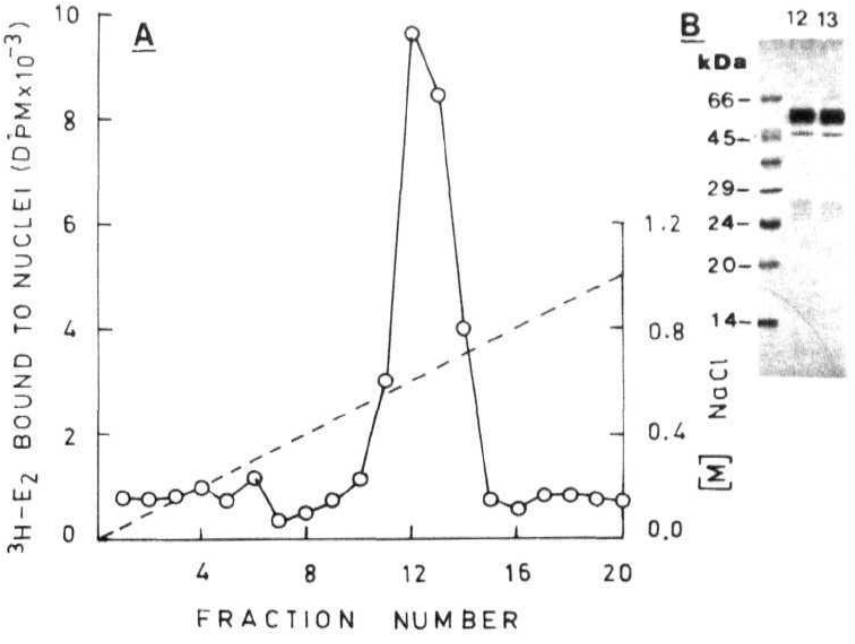

A
B
12 13
kDa
66-
45-
29-
24-
20-
14-
$^3H$-$E_2$ BOUND TO NUCLEI (DPMx$10^{-3}$)
[M] NaCl
FRACTION NUMBER
0
2
4
6
8
10
0.0
0.4
0.8
1.2
4
8
12
16
20

Figure 3.7A. SDS-PAGE analysis of pure actin.

Actin was purified as described in the 'methods'. The pure protein was subjected to SDS-PAGE on 10% gels. 2.5, 5, 10, 15, 20 and 25 μg actin was loaded in the lanes 1 to 6 respectively. The gels were subjected to silver staining.

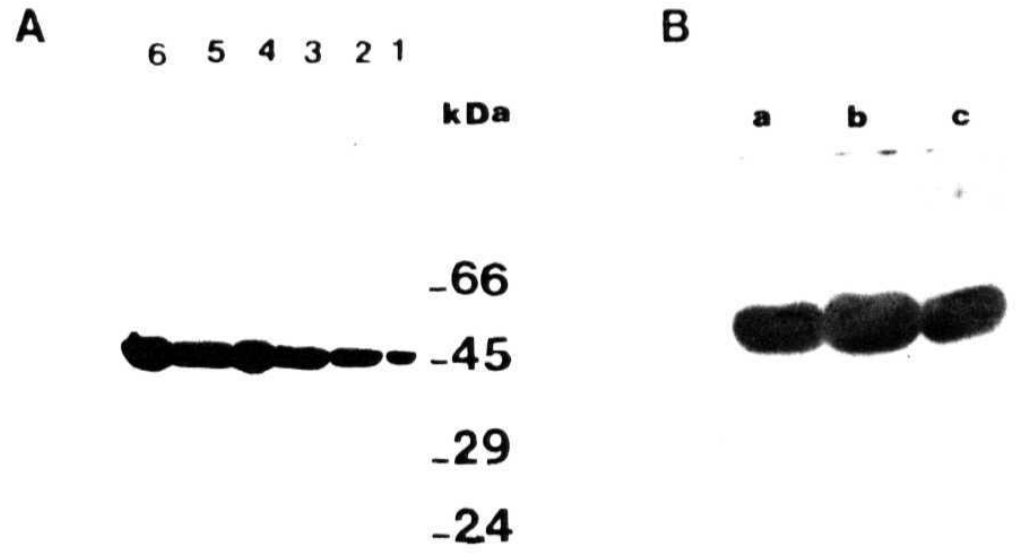
B. Western blotting analysis of actin.

Pure actin was subjected to SDS-PAGE on 12% gels and the proteins in the gel were transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated overnight with actin 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 3.8.** Actin-Sepharose chromatography of the NLSBP.

(A) The NLSBP activity that eluted from the poly-lysine-Sepharose column was dialyzed and chromatographed over a column of actin-Sepharose (10 ml). The column was washed with 10 mM ATP in TEMN buffer and eluted with a linear NaCl gradient (50 mM to 1 M). The eluate was collected as 1.5 ml fractions. The absorbance of the fractions at 280 nm was measured (broken lines) and were tested for the NLSBP activity. The NLSBP activity was expressed as $^3$H-estradiol bound to the nuclei.

(B) The fractions that demonstrated the NLSBP activity in (A) were precipitated with TCA. The precipitates were washed with alcohol and were subjected to SDS-PAGE on 12% gels. The lane numbers correspond to the fraction numbers in the figure 3.8A. The gels were stained with silver nitrate.


A
6 5 4 3 2 1
kDa
-66
-45
-29
-24
B
a b c

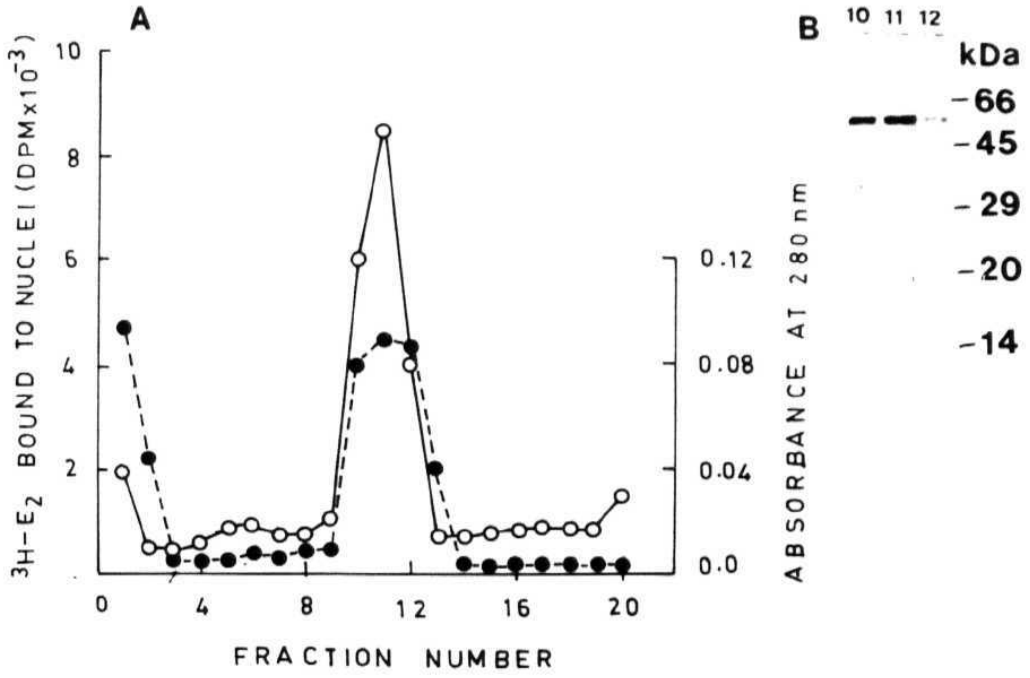

A
B
10 11 12
kDa
-66
-45
-29
-20
-14
$^3H$-$E_2$ BOUND TO NUCLEI (DPMx$10^{-3}$)
ABSORBANCE AT 280 nm
FRACTION NUMBER
0
4
8
12
16
20
0.0
0.04
0.08
0.12
2
6
8
10

**Figure 3.9. Chromatography of goat uterine cytosol on actin-Sepharose.**

(A) Cytosol was chromatographed over a column of actin-Sepharose (10 ml). The column was washed and eluted as described in figure 3.8. The fractions (1.5 ml/fraction) collected were subjected to the NLSBP assay. The salt concentration in the fraction is represented by broken lines.

(B) The fractions with the NLSBP activity eluted from actin-Sepharose (as in the figure 3.9A) were subjected to SDS-PAGE on 10% gels. The lane numbers are those corresponding to the fraction numbers in the figure 3.9A. The gels were subjected to silver staining.

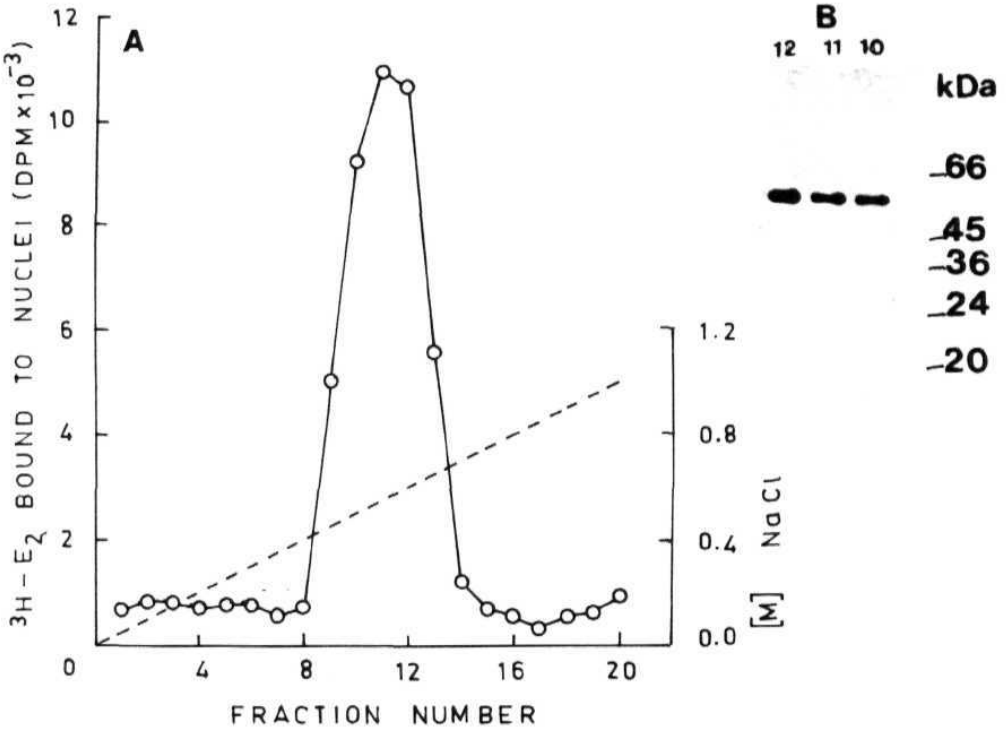

A
B
12 11 10
kDa
66
45
36
24
20
$^3H-E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
[M] NaCl
FRACTION NUMBER
0
2
4
6
8
10
12
0.0
0.4
0.8
1.2
4
8
12
16
20

Figure 3.10. Gel filtration analysis of the NLSBP.

The pure NLSBP was chroinatographed over a column of Sephadex G-100 (66 X 1.8 cm). The column was developed with TEM buffer containing 0.2 M NaCl and 2.5 ml fractions were collected. The fractions were subjected to the NLSBP 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 Rs (Stokes radius) determined for the column.

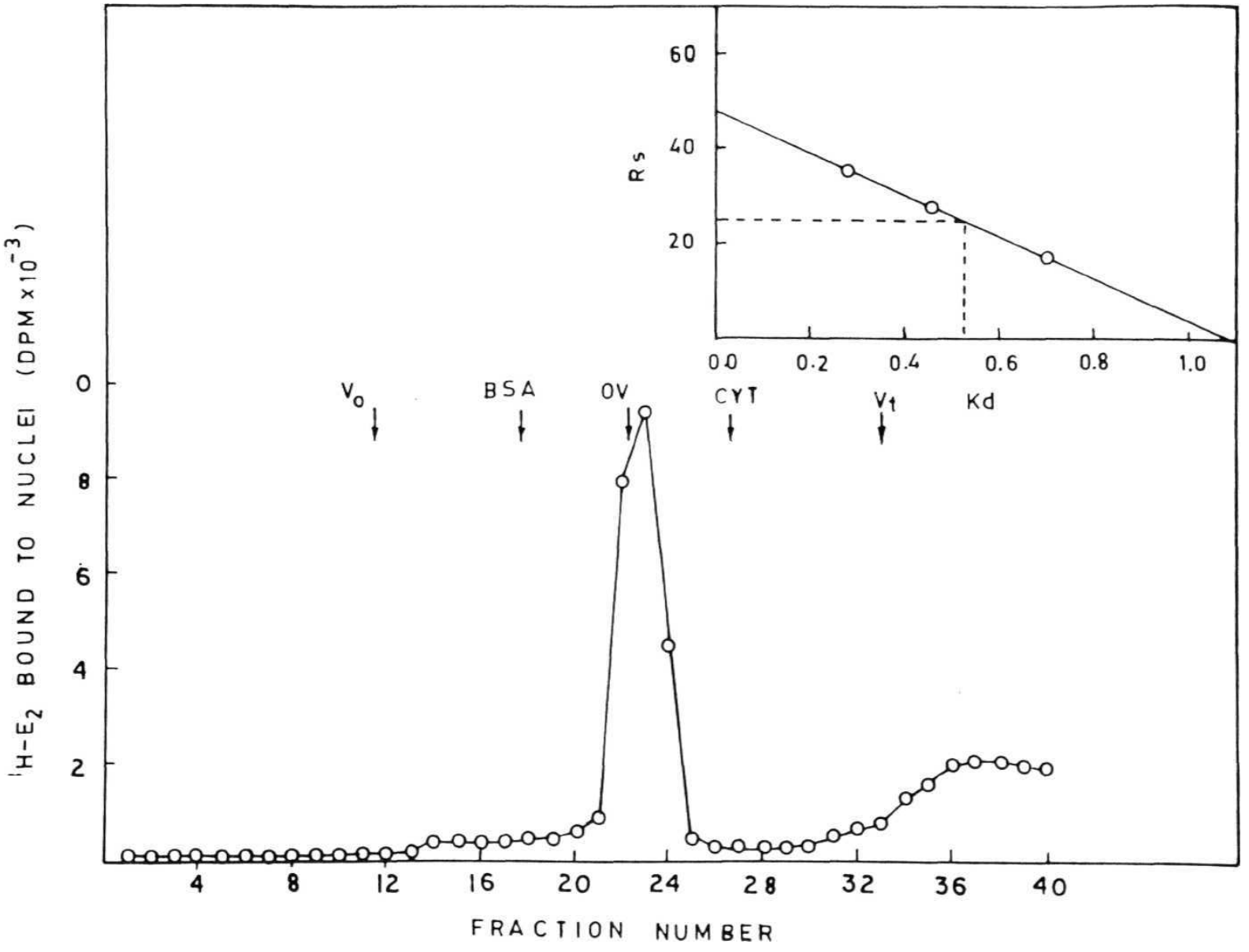

60
40
20
Rs
0.0
0.2
0.4
0.6
0.8
1.0
Kd
$V_o$
BSA
OV
CYT
$V_t$
0
8
6
4
2
$^3$H-$E_2$ BOUND TO NUCLEI (DPM x$10^{-3}$)
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER

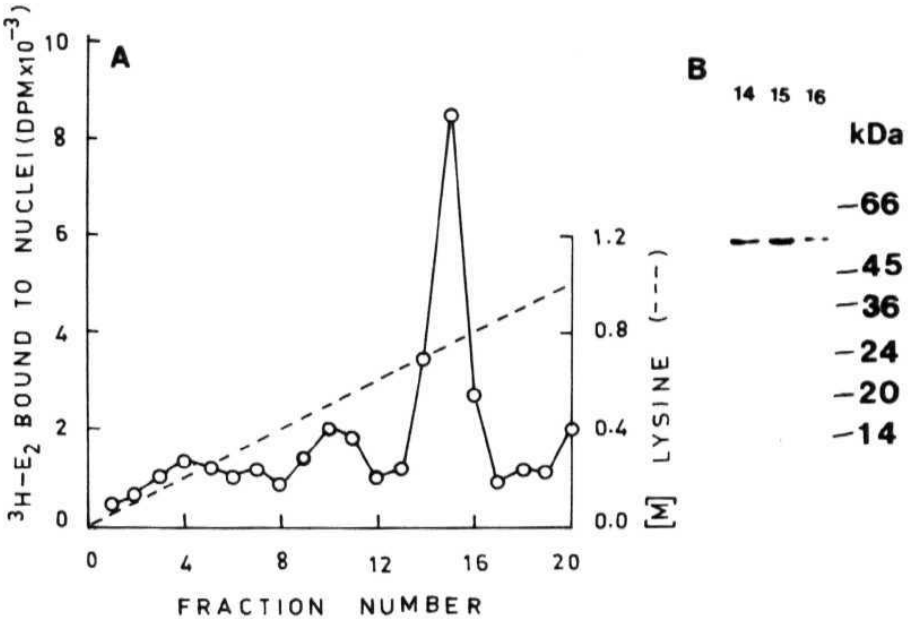
**Figure 3.11. NLSBP binds to the estrogen receptor-Sepharose in a lysine-dependent manner.**

(A) The NLSBP isolated from actin-Sepharose was dialyzed against TEMN. The dialysate was chromatographed over a column of estrogen receptor- Sepharose (10 ml). The column was washed with TEMN and eluted with a linear lysine gradient (0 to 1 M). The fractions (1.5 ml) collected were subjected to the NLSBP assay. The lysine concentration is represented by the broken lines.

(B) The fractions that were eluted from the estrogen receptor-Sepharose demonstrating NLSBP activity were precipitated with TCA. The precipitates were dissolved in sample buffer and were subjected to SDS-PAGE on 10% gels followed by silver staining. The lane numbers are the corresponding fraction numbers of (A).

**Figure 3.12. NLSBP** binds **to the** SV40 **T antigen NLS.**

The NLSBP isolated from actin-Sepharose was chromatographed over **a column** of SV40 NLS peptide cross-linked to BSA-Sepharose (3 ml). The column was washed and eluted with a linear lysine gradient (0 to 1 M). One ml fractions were collected. The TCA proteins were subjected to SDS-PAGE on 10% gels and the gels were stained with silver nitrate. The fraction numbers are given on the top of each lane.


A
B
$^3H-E_2$ BOUND TO NUCLEI (DPMx10$^{-3}$)
[M] LYSINE (---)
FRACTION NUMBER
14 15 16
kDa
66
45
36
24
20
14

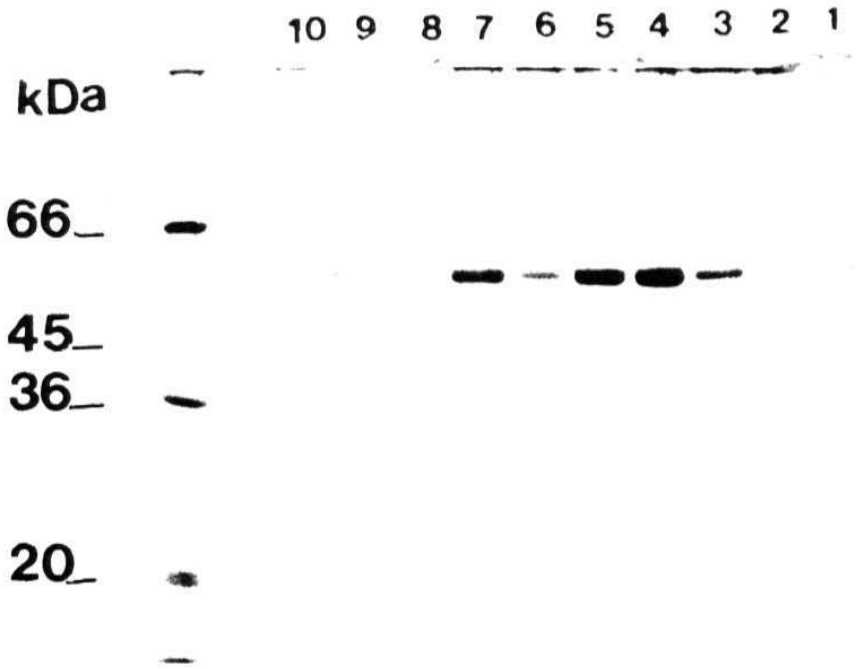
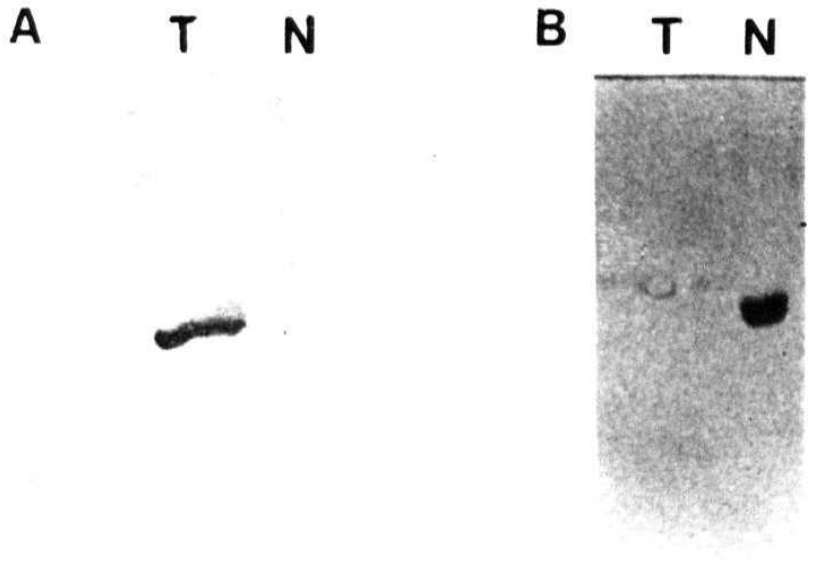

10 9 8 7 6 5 4 3 2 1
kDa
66
45
36
20

**Figure 3.13.** Evidences to show **that the NLSBP is** not **tubulin.**

Pure tubulin (T) and NLSBP (N) were subjected tc SDS-PAGE in duplicates. The proteins were transferred to nitrocellulose membranes. One of the blots was incubated with anti-tubulin antibody (1:50) (panel A) and the other was incubated with anti-poly-aspartate antibody (1:50) (panel B). The blots were re-exposed to HRPO-coupled anti-rabbit IgG (1:500) and were stained with 4-chloro 1-naphthol and $H_2O_2$ .

Figure 3.14. Western analysis of the NLSBP.

0.4, 0.8, 1.2, 1.6 and 2.0 μg NLSBP was subjected to SDS- PAGE on 10% gels. The proteins were transferred to nitrocellulose membranes. The membranes were incubated with anti-poly-aspartate antibody (1:50). The membranes 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
T
N
B
T
N

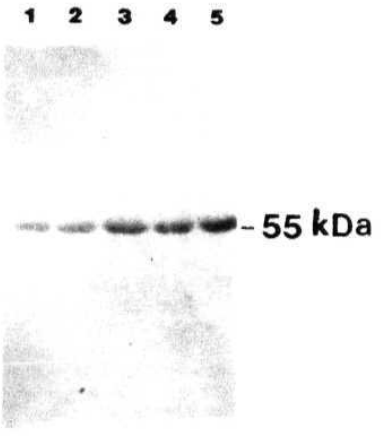

1 2 3 4 5
55 kDa

**Figure 3.15. Anti-poly-aspartate** antibody blocks nuclear **transport.**

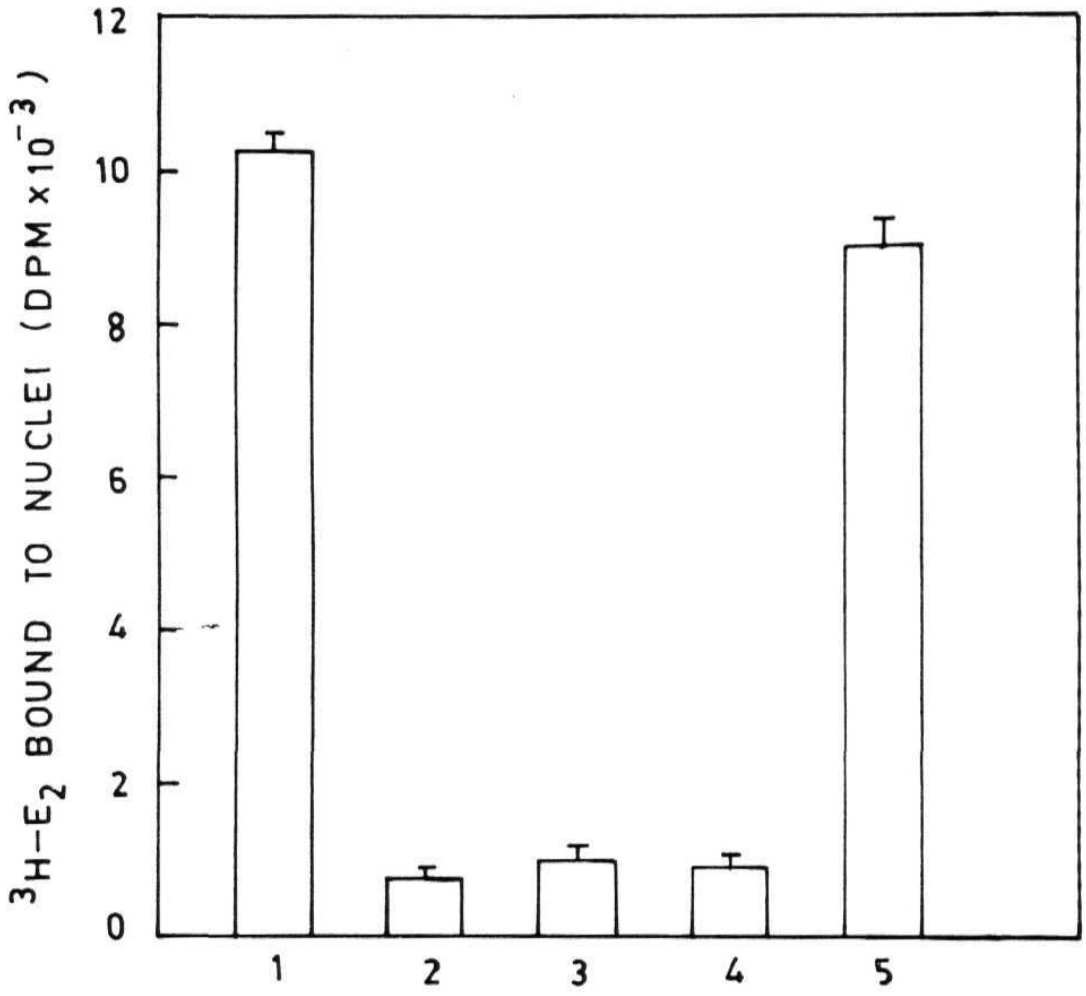
The NLSBP assay was performed as described in the `methods' (1) but with the following changes.

(2) To the assay mixture anti-poly-aspartate antibody was added in the ratio 1:10.

(3) The incubation mixture was the same as (2) but with **10** fold excess of ER.

(4) The incubation conditions were the same as in (2) but with 10 fold excess of **nuclei.**

(5) The incubation was performed as in (2) but with 10 fold excess of NLSBP.


$^3H-E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
12
10
8
6
4
2
0
1
2
3
4
5

# MECHANISM OF ER TRANSPORT INTO THE NUCLEUS.

# 4.0 MECHANISM OF THE NLSBP MEDIATED NUCLEAR TRANSPORT OF THE ER.

In the previous section (3.0), the purification of the NLSBP which mediates the transport of ER into the nucleus has been described. Some of the observations described in this section were made during the development of the NLSBP assay, prior to the purification of the NLSBP. These experiments were repeated using the pure NLSBP in order to rule out interferences due to the contaminants in the crude preparation. These results gave an insight to the mechanism of transport of the ER by the NLSBP and are described here in detail.

The mechanism of transport of many karyophilic proteins into the nucleus can be separated into two major steps. One, the recognition of the NLSs on the nuclear proteins by the NLS binding proteins (NLSBPs) and the targetting 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 ai,* 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.

## 4.1 THE TRANSPORT OF ESTROGEN RECEPTOR INTO THE NUCLEUS IS SATURABLE.

The NLSBP assay was performed using the media containing increasing concentrations of pure NLSBP (0.05 to 1.0 μg). The NLSBP and the $^{3}H$-$E_2$-ER complexes were incubated with nuclei in the assay buffer at 30°C for 30 minutes. The $^{3}H$-$E_2$-ER complexes transported into the nuclei was measured as described in the `methods'. There was a near-linear increase in the transport observed in the presence of a concentration of NLSBP upto 0.5 μg NLSBP. The transport was found to be saturable with 0.5 μg NLSBP. No further increase in the transport was noticed in the presence of a NLSBP concentration beyond this level (Fig 4.1).

It is possible to draw two conclusions from this experiment.

1) The NLSBP is an absolute requirement for the nuclear transport of ER.

2) The transport is saturable, suggesting that there is yet another limiting factor, probably associated with the nucleus.

The requirement of cytosolic factors in addition to the NLSBPs were observed for nuclear transport in some in vivo studies (Newmeyer and Forbes, 1990; Moore and Blobel, 1992). In an experiment designed to examine a similar requirement for the ER transport, cytosol was added to the incubation mixture and the assay was carried out as described before. There was no further increase in the nuclear transport observed in the presence of cytosol (data not shown). This is in agreement with the observations made by other groups using in vitro assays (Markland *et al.,* 1987; Imamoto-Sonobe et al., 1988; Kalinich and Douglas, 1989; Silver *et al.,* 1989; Parnaik and Kennady, 1990).

## 4.2 TRANSPORT OF THE ER INTO THE NUCLEUS IS RAPID AND TEMPERATURE DEPENDENT.

The kinetics of the ER transport into the nucleus was studied in the presence of NLSBP. The NLSBP assay was performed in duplicates using 0.5 μg NLSBP at both 4°C and 30°C. The transport was terminated after 5, 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 with ethanol.

The nuclear transport of the ER at 30°C was very rapid. About half of the ER present in the medium was transported within five minutes and almost all the transport was completed within a duration of 10-15 minutes. No transport of ER was noticed at 4°C following incubation for 30 minutes (Fig 4.2). Even after two hours of incubation at 4°C it was not possible to observe any change in this pattern (data not shown).

## 4.3 TRANSPORT OF THE ER INTO THE NUCLEUS IS ATP DEPENDENT.

During the early development of the NLSBP assay, an absolute requirement of ATP in the assay medium for the NLSBP-mediated transport of ER into the nucleus was observed. The NLSBP assay was performed as described before but the assay buffer was prepared with different concentrations of ATP.

The transport of ER in the presence of NLSBP was performed with 1, 2, 4 and 8 mM ATP in the assay buffer. As a control, one set was incubated without ATP. There was no transport of ER in the absence of ATP and an almost linear increase in the ER transport was observed in the presence of ATP concentrations

upto 4 mM. Further increase in ATP concentration did not cause a corresponding increase in the transport (Fig 4.3).

When NLSBP assay was conducted in the presence of 1 mM ATP, 40 creatine phosphate and 4 μg creatine phosphokinase, the ER-transport increased to the levels found in those incubated with 4 mM ATP. This result and the previous one in which no transport was observed at 4°C suggested that ATP was hydrolyzed and the energy released was utilized in the ER transport. When the incubation was performed with just 1 mM ATP negligible transport was seen, but when supplemented with the ATP re-generating system (creatine phosphate-creatine phospho-kinase) an enhanced transport of ER was observed.

## 4.4 TRANSPORT OF THE ER REQUIRES AN INTACT MEMBRANE.

Goat uterine nuclei were isolated as described in the experimental section. Two preparations of nuclei were made, one set of nuclei was prepared by treating nuclei with 0.05% Triton X-100, which solubilizes the nuclear membranes. The other set was prepared without any detergent treatment. For clarity, the first set is referred to as N-M (nuclei without the nuclear membranes) and the second set as N+M (nuclei with intact membranes). The NLSBP assay was performed with 0.5 μg NLSBP in the presence of 4 mM ATP at 30°C for 30 minutes with both N-M and N+M nuclei. No transport was seen in the sets which were incubated with N-M nuclei (Fig 4.4). Only the nuclei with the intact membranes were transport competent, suggesting that there is a secondary recognition site in the nuclear membrane.

## 4.5 A NUCLEAR MEMBRANE/PORE COMPLEX-BOUND PROTEIN IS ESSENTIAL FOR THE NUCLEAR TRANSPORT OF THE ER.

Triton X-100 treatment of the nuclei does not interfere with the NPC structure and function (Aaronson and Blobel, 1974). It solubilizes the nuclear membranes and removes the membrane bound proteins at the same time. Therefore, the loss of nuclear transport of ER noticed could not be ascribed to the destruction of the transport apparatus (the NPC) but to the absence of some factor (protein?) which was removed along with the nuclear membranes by Triton X-100.

In order to examine this possibility the following reconstitutive NLSBP assay was performed. The goat uterine nuclei were washed with 0.05% Triton X-100. The Triton X-100 solubilized material was dialyzed extensively in order to remove the detergent. The dialysate was concentrated and the proteins were resolved on a Sephadex G-100 column (66X1.8 cm). The column was developed with TEM buffer containing 0.2 M NaCl. Fractions (2 ml) collected were assayed for the NLSBP activity with the following modifications.

Goat uterine nuclei were isolated and were treated with 0.05% Triton X-100 (N-M). The N-M nuclei were washed again to remove the detergent as described in the experimental section. $^{3}H$-$E_2$-ER complexes were incubated with N-M nuclei in the presence of pure NLSBP (1 μg) 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-NLSBP complex to detergent treated nuclei (N-M) (Fig 4.5). These fractions were subjected to SDS-PAGE. Upon silver staining, 3-4 bands of molecular mass of ~14 kDa were observed (Fig 4.6).

To examine if any of these fractions themselves possessed the capacity to transport ER, the experiment mentioned above was repeated in its entirety, except that the incubation was performed in the absence of the NLSBP. There was no transport seen with any of these fractions (data not shown). This suggested that the ~14 kDa fraction was the secondary recognition site for the ER during the transport and probably recognized the ER-NLSBP complex only.

## 4.6 FLUORESCENCE ASSAY OF THE NLSBP-MEDIATED ER TRANSPORT INTO THE NUCLEUS:

The NLSBP assay does not distinguish between binding of the ER to the nuclear membrane and transport of ER into the nucleus. To confirm NLSBP-mediated transport of ER into the nucleus, the fluorescence assay for ER transport was performed as described in the methods (2.6).

Goat uterine ER was purified and conjugated with FITC following the procedure of Chard *et al.* (1987). Goat uterine nuclei (N+M) were incubated with FITC-ER either in the presence or absence of the NLSBP. The transport of the ER into the nucleus was found to be absolutely dependent on the presence of the NLSBP in the assay medium (Fig 4.7B). No transport of ER was observed in the absence of the NLSBP (Fig 4.7A). Upon addition of the NLSBP to the incubation medium transport of ER was seen within five minutes (Fig 4.7C). The ER transport was completed within 10-15 minutes of incubation in the presence of the NLSBP (Fig 4.7D and 4.7E).

The nuclear transport of ER required an intact nuclear membrane as was observed in the above experiments using the NLSBP assay. To confirm this, the fluorescence assay was performed. N-M nuclei were suspended in the NLSBP assay buffer. The nuclei were incubated with 5 μg FITC-ER alone (Fig 4.8A), or with 0.5 μg NLSBP (Fig 4.8B) at 30°C for 30 minutes. The transport was observed in a Leitz fluorescence microscope. No transport of ER was seen in the absence of the NLSBP. When the NLSBP was added, the FITC-ER was seen bound to the nuclear periphery, visualized as "rim-staining". Upon addition of ~0.5 to 1 μg of the 14 kDa protein(s), entry of the ER into the nucleus was observed within 2 to 3 minutes (Fig 4.8C).

To study the ATP dependence of the ER nuclear transport, N+M nuclei were incubated with FITC-ER (5 fig) and NLSBP (0.5 |ig) in the NLSBP assay medium containing no ATP for 30 minutes at 30°C. In the absence of ATP, the FITC-ER was seen bound to the nuclear membrane ("rim-staining") (Fig 4.8D). When ATP was added to the above incubation medium, entry of FITC-ER into the nucleus was rapid and could be observed within 2-3 minutes (Fig 4.8E).

## 4.7 NLSBP IS PRESENT BOTH IN THE CYTOPLASM AND IN THE NUCLEUS.

Goat uterine cytosol and nuclei were isolated as described in the methods. 25 μg of the cytosolic proteins (C), the Triton X-100 wash fraction of the nuclei (T) and the total nuclear proteins (N) were resolved by SDS-PAGE in mini gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with anti-poly-aspartate antibody (1:50) Two proteins of molecular mass 66 kDa and 55 kDa were detected in the cytosol, nuclear membrane (Triton X-100 wash fraction) as well as the nucleus by the poly-aspartate antibody

(Fig 4.9) suggested that the 55 kDa is present in the nucleus as well as in the cytosol. The presence of the 55 kDa protein in the nuclear membrane (the Triton X-100 wash fraction) probably represent the fraction of the NLSBP "in transit" while transport of NLSBP-ER into the nucleus was in progress.

## 4.8 THE NLSBP IS A DNA BINDING PROTEIN.

The NLSBPs mediate the transport of nuclear proteins and may themselves co-transport into the nucleus. They may remain in the nucleus or move out transporting another protein out of the nucleus. While the NLSBPs are in the nucleus, they might possibly be anchored to the nuclear matrix or to the DNA.

To determine whether the NLSBP interacts with the DNA, the pure NLSBP preparation obtained by actin-Sepharose chromatography was dialyzed free of salt and chromatographed over a ss-DNA-Sepharose column (3 ml), the flowthrough was recycled and the final flowthrough was collected. The column was washed with TEMN buffer and the DNA-bound protein was eluted with an increasing salt gradient (50 mM to 1 M NaCl). The eluate was collected as 1 ml fractions. All the fractions including the flowthrough were subjected to SDS-PAGE, followed by silver staining. The NLSBP eluted from the ss-DNA-Sepharose with ~0.25 M NaCl. The NLSBP was also seen in the flowthrough fractions owing probably to overloading (4.10).

The NLSBP bound to the ss-DNA-Sepharose albeit with a low affinity. The question that arose here was whether the binding to ss-DNA-Sepharose was specific for DNA or was it because of the charge of the column contributed by the phosphates. The latter possibility was examined through chromatography of NLSBP on phosphocellulose. The pure NLSBP preparation was dialyzed

against TEMN buffer and was chromatographed over a phosphocellulose (5 ml) column. The flowthrough was recycled several times onto the column in order to optimize binding. The final flowthrough fractions were collected. The phosphocellulose column was washed with TEM buffer and the proteins bound to the column were eluted with a linear salt gradient (0 to 1 M NaCl). The eluate and the flowthrough were subjected to SDS-PAGE. The NLSBP did not bind to phosphocellulose and was seen only in the flowthrough fractions (Fig 4.10). These results suggested that the binding of NLSBP to DNA was not due to the phosphates residues.

## 4.9 ATPASE ACTIVITY OF THE NLSBP.

The NLSBP mediated transport of the ER requires ATP. The energy produced by ATP hydrolysis may be used to trigger the transport of the ER. It was, therefore, of interest to examine if the NLSBP in itself possessed any ATPase activity. Increasing concentrations of the NLSBP (0.2-2 μg) were incubated with 6 mM ATP at 30$^{o}$C 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 that was directly proportional to the amount of NLSBP added to the medium (Fig 4.11). this suggested that the NLSBP is an ATPase; the energy released as a result of ATP hydrolysis is probably required for the transport of ER into the nucleus.

## 4.10 ATPASE ACTIVITY OF THE NUCLEI IS ENHANCED BY NLSBP.

The ATPase activity associated with the 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; '88). The nuclear ATPase was inhibited by quercetin, an inhibitor of $Mg^{++}$-dependent ATPase and not by ouabain, a Na-K dependent ATPase inhibitor (Thampan, 1988). This ATPase may play an important role in the cytoplasm-to-nucleus protein transport as well. The nuclear transport of ER required ATP hydrolysis as was seen in the previous experiments. The NLSBP itself demonstrated ATPase activity. It was of interest to see if the ATPase activity of the nuclei was enhanced by the NLSBP.

The goat uterine nuclei were incubated, as described in the experimental section, for different time intervals either alone or in the presence of two concentrations of NLSBPs (1 μg and 2 μgs respectively). 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 the nucleus increased rapidly during the first 10 minutes of incubation and plateaud off upto 30 minutes. The ATPase activity of the nuclei was enhanced in the presence of NLSBP (Fig 4.12). By deducting the activity associated with the NLSBP the increase in nuclear ATPase activity was calculated. The increase in ATPase activity with 1 μg NLSBP was ~12.5% while that with 2 μg was ~27%. In another set, the goat uterine nuclei were incubated with 2 fig NLSBP and 20 jig/ml quercetin as mentioned above. The ATPase activity of the nuclei as well as that enhanced by NLSBP was also inhibited by quercetin (Fig 4.12).

## 4.11 THE NLSBP ATPASE ACTIVITY IS INHIBITED BY QUERCETIN.

As seen in the above experiment, the NLSBP ATPase activity was also inhibited by quercetin. In order to examine this further, 2 μg NLSBP was

incubated with increasing concentrations of quercetin (0 to 20 jag/ml) for 30 minutes at 30°C. The inorganic phosphate released was estimated. The ATPase activity was inversely proportional to the concentration of quercetin used in the incubation medium (Fig 4.13). Half-maximal inhibition of activity was observed with ~8μg/ml quercetin.

## 4.12 QUERCETIN INHIBITS THE TRANSLOCATION OF THE ER INTO THE NUCLEUS.

The ATPase activity of the NLSBP as well as that of the nucleus was inhibited by quercetin. It was of importance to see whether the inhibition of the ATPase activity inhibited the transport of ER into the nucleus. The goat uterine nuclei were suspended in NLSBP assay buffer containing no ATP. These nuclei were incubated with 5 μg FITC-ER and 0.5 μg of NLSBP at 30°C for 30 minutes. These nuclei showed "rim staining" (data not shown, but similar to Fig 4.8 D). ATP and quercetin were added to a final concentration of 4 mM and 10 μg/ml respectively and incubation continued for another 30 minutes. The nuclei continued to show only "rim staining" (Fig 4.8E). Quercetin inhibited the translocation of the ER into the nucleus.

## 4.13 DISCUSSION.

Mechanisms associated with the nuclear transport of ER have been examined in this chapter by a detailed analysis of the NLSBP assay. Since this assay does not clearly distinguish between peripheral binding of ER to the nuclear membrane and transport into the nucleus, it was decided to perform a fluorescence assay for nuclear transport of ER using FITC labelled ER. The nuclear binding or nuclear transport observed in the NLSBP assay reflects truly the ER that is transported into the nucleus. The NLSBP 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 NLSBP assay did not detect any binding activity. The peripheral binding was probably not strong enough to withstand the stringent washing conditions of the NLSBP assay.

The mechanism of ER transport into the nucleus can be divided into two distinct steps based on observations made by performing the NLSBP assay and the fluorescence assay for nuclear transport.

1) Peripheral binding, i.e., the binding of the ER to the nuclear membrane. This was observed as "rim staining" in fluorescence transport assay. This step was mediated by the NLSBP and did not require any ATP.

2) Translocation of ER into the nucleus. This step was ATP dependent and was mediated by the 14 kDa protein(s). Requirement of ~4 mM ATP in the assay medium, which can be substituted by 1 mM ATP and the ATP re-generating system creatine phosphate and creatine phosphokinase, and the absence of transport at 4°C in the presence of 4 mM ATP and quercetin, all suggested that probably ATP was hydrolyzed and the energy released was utilized in the translocation of ER into the nucleus. This was also confirmed by the observations that quercetin, an ATPase inhibitor, inhibited translocation of ER into the nucleus. Requirement of ATP for the transport of other nuclear proteins too has been demonstrated (Newmeyer *et al.*, 1986; Richardson *et al.*, 1988). The transport of ER is very rapid. Almost all of the ER is transported in 10 minutes.

Only nuclei with intact membranes were competent for transporting ER. The nuclei treated with Triton X-100 were incapable of transporting ER in the presence of NLSBP. Such a requirement of intact nuclear membrane for nuclear transport was also demonstrated independently by two groups

(Imamoto-Sonobe et al., 1988; Zimmer *et al.,* 1988). The ~14 kDa protein(s) fraction isolated from the Triton X-100 solubilized material when added to the Triton X-100 washed nuclei could restore nuclear transport of ER only in the presence of NLSBP. This 14 kDa protein might be present in the nuclear membrane close to the NPC, the transport apparatus of the nuclei. The 14 kDa protein probably recognizes the NLSBP-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 NLSBP is an ATPase and also enhances the ATPase activity associated with the nuclei. The ATPase activity associated with the NLSBP and that of the nuclei were inhibited by quercetin, a Mg dependent ATPase inhibitor suggesting that the NLSBP is probably a $Mg^{++}$dependent ATPase.

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

The role of the NLSBP, therefore, is to recognize ER and transport it from the cytoplasm to the nuclear periphery followed by binding to the membrane. The role of the 14 kDa protein(s) is to recognize the ER-NLSBP complex and promote the transport of the ER-NLSBP into the nucleus.

The NLSBP is also present in the nucleus as demonstrated by the crossreactivity of the 55 kDa nuclear protein to poly-aspartate antibody. This suggests that probably the NLSBP shuttles back and forth into the nucleus. NLSBP also enters the nucleus along with the ER and probably plays an important role in the export of ER or any other nuclear proteins. While the NLSBP is present in the nucleus, it probably anchors to DNA as it was observed that the NLSBP binds to DNA.

Based on the results of chapters 3 and 4, a model for the transport of ER to the nuclei has been proposed (Fig 4.14). The ER (hormone bound or free) binds to the NLSBP in the cytoplasm, the NLSBP-ER complex probably makes use of the microfilament or/and microtubular network of the cell to move, in the cytosol, towards the nucleus. The NLSBP-ER complex binds to the nuclear membrane and is recognized by the 14 kDa protein(s) present in the nuclear membrane near the pore complex. The 14 kDa protein(s) binds to the complex and presents it to the NPC. The transport through the pore complex is ATP dependent. The docking of the ER-NLSBP at the NPC probably results in a gating event that facilitates transport by the hydrolysis of ATP associated with the NPC. The NLSBP-ER docks at the NPC and stimulates the ATPase activity associated with the NPC. The energy required for the transport through the NPC and for the expansion of the pore is thus provided by the hydrolysis of ATP by the ATPase associated with the nuclei which is stimulated by the NLSBP.

The role of the microtubules and microfilaments in the transport needs to be confirmed through the development of an appropriate assay system.

**Figure 4.1. The NLSBP transport of ER into the nucleus is a saturable process.**

0.05 to 1.0 μg NLSBP was incubated with $^{3}H$-$E_2$-ER complexes and nuclei at 30°C for 30 minutes and the assay performed as described in the "methods". The $^{3}H$-$E_2$-ER transported into the nucleus was extracted with ethanol. The radioactivity in the ethanol extracts was measured. The NLSBP activity was expressed as $^{3}H$-estradiol bound to nuclei.

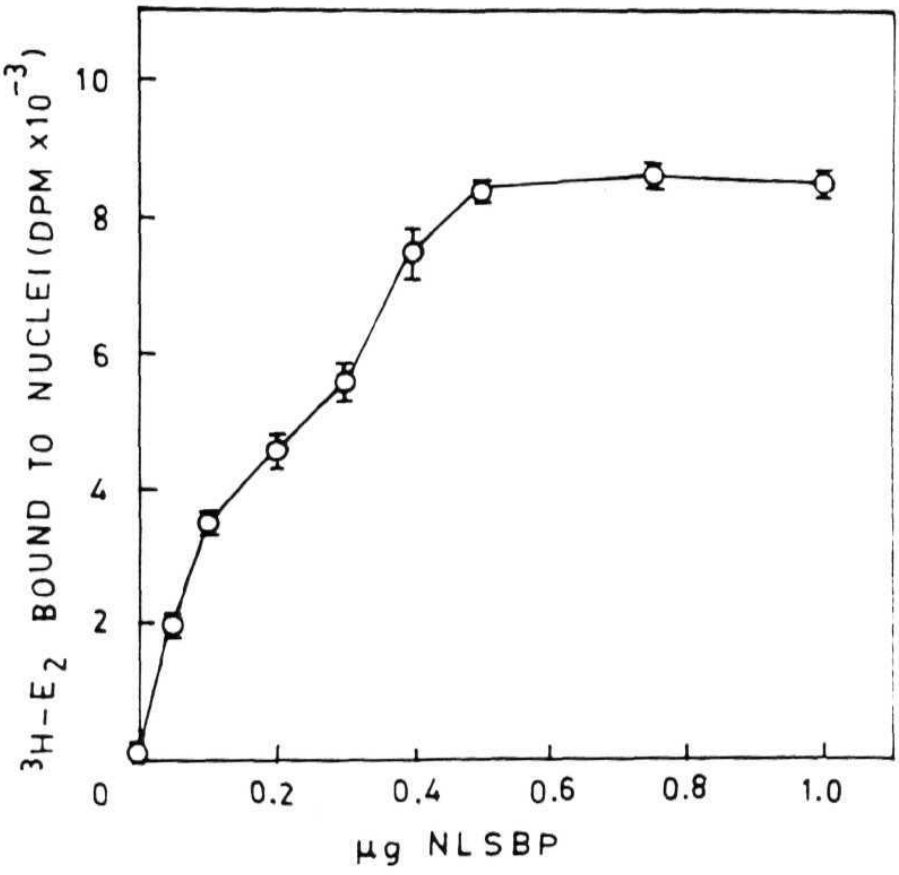

$^3H-E_2$ BOUND TO NUCLEI (DPM x10$^{-3}$)
10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
µg NLSBP

**Figure 4.2. Temperature dependence of the transport of ER into the nucleus.**

NLSBP (0.5 μg) was incubated with $^{3}$H-E$_{2}$-ER complexes and nuclei either at 4°C or at 30°C for different time intervals (0 to 30 minutes) and the assay was performed as described before. The $^{3}$H-E$_{2}$-ER transported into the nucleus was extracted with ethanol. The radioactivity in the ethanol extracts was measured. The NLSBP activity was expressed as $^{3}$H-estradiol bound to nuclei.

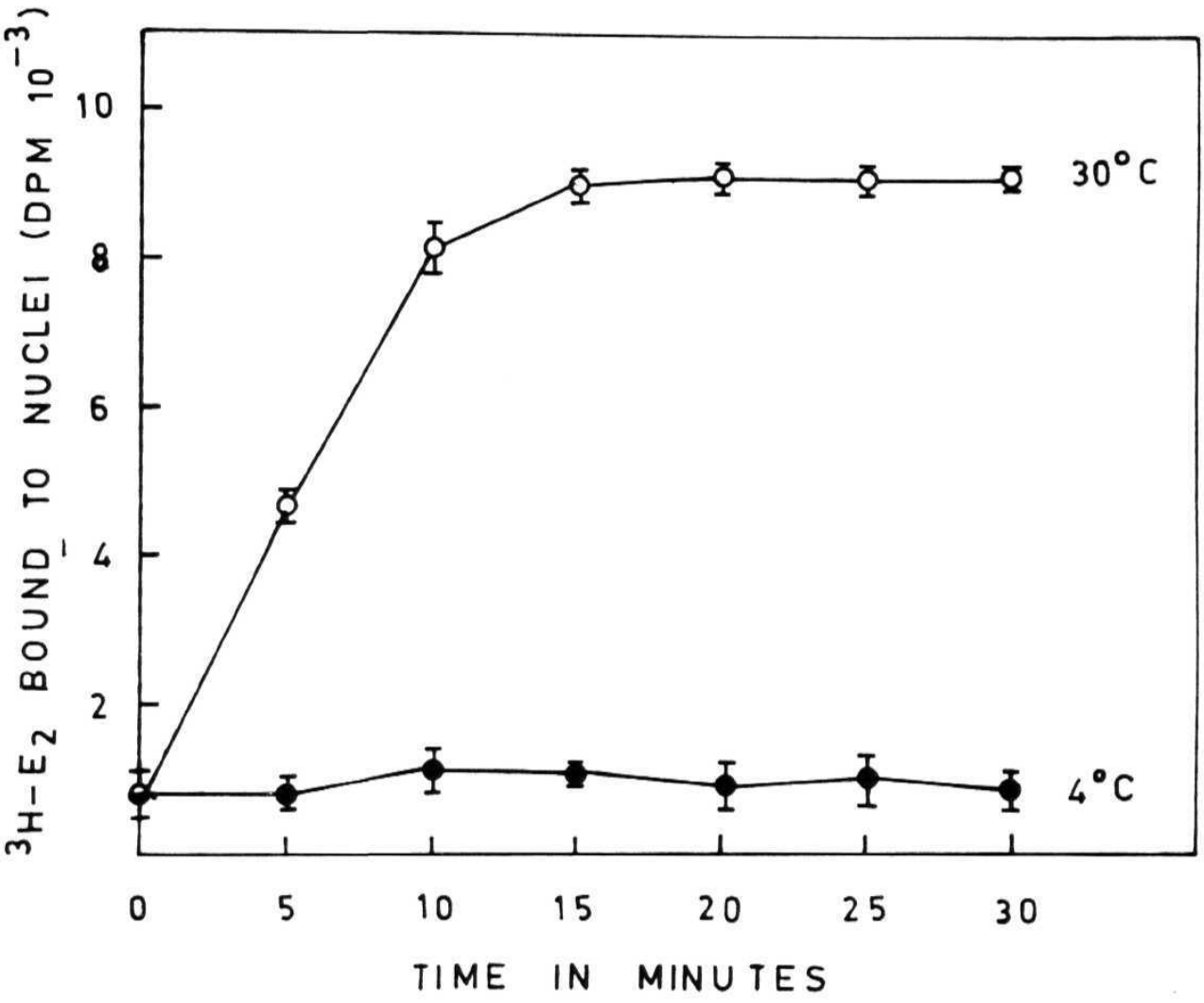

$^3H-E_2$ BOUND TO NUCLEI (DPM $10^{-3}$)
10
8
6
4
2
0
5
10
15
20
25
30
TIME IN MINUTES
30°C
4°C

**Figure 4.3. ATP-dependence of the transport of ER into the nucleus.**

NLSBP (0.5 jig) was incubated with $^{3}H\text{-}E_2$ -ER complexes and nuclei in the presence of 0, 1, 2, 4 and 8 mM ATP. One set of incubations was performed **with 1 mM** ATP, creatine phosphate (40 μM) and creatine phosphokinase (4 μg). **The** incubation was conducted at 37°C for 30 minutes. The $^{3}H\text{-}E_2$ -ER **transported into the** nuclei were measured as described **before. The activity was expresses** as $^{3}H$-estradiol bound to the nuclei.

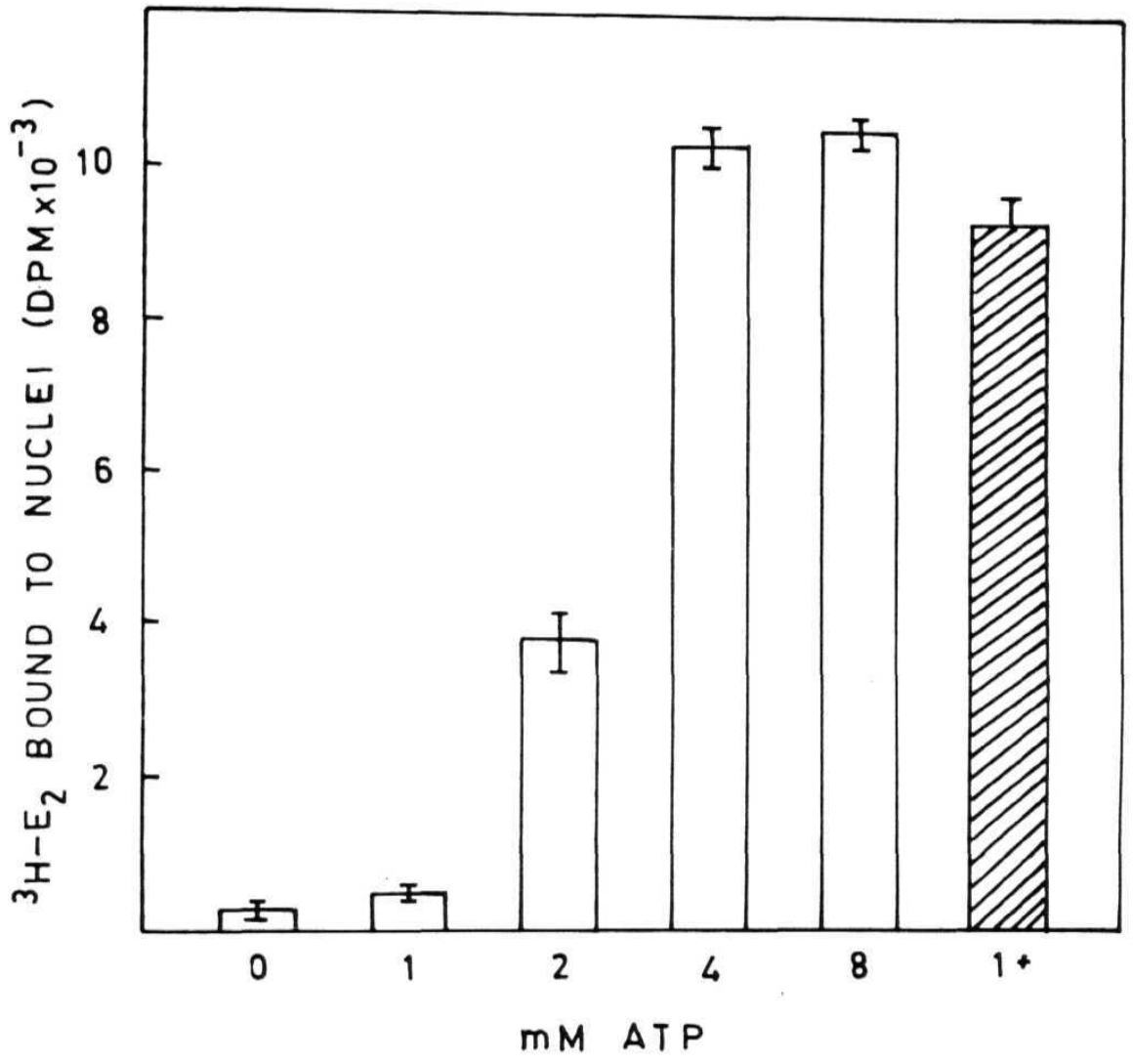

$^3H-E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
10
8
6
4
2
0
1
2
4
8
1+
mM ATP

**Figure 4.4.** Estrogen receptor **is transported** into only **those nuclei with intact** membranes.

The nuclei for this NLSBP assay were prepared as follows. One set of nuclei were treated with Triton X-100 (N-M). The other set of nuclei were not treated with **the** detergent (N+M). NLSBP (0.5 μg) and $^{3}H$-$E_2$ -ER complexes were incubated with both the sets of nuclei. The $^{3}H$-$E_2$ -ER transported into the nuclei was extracted with ethanol and the radioactivity was measured.

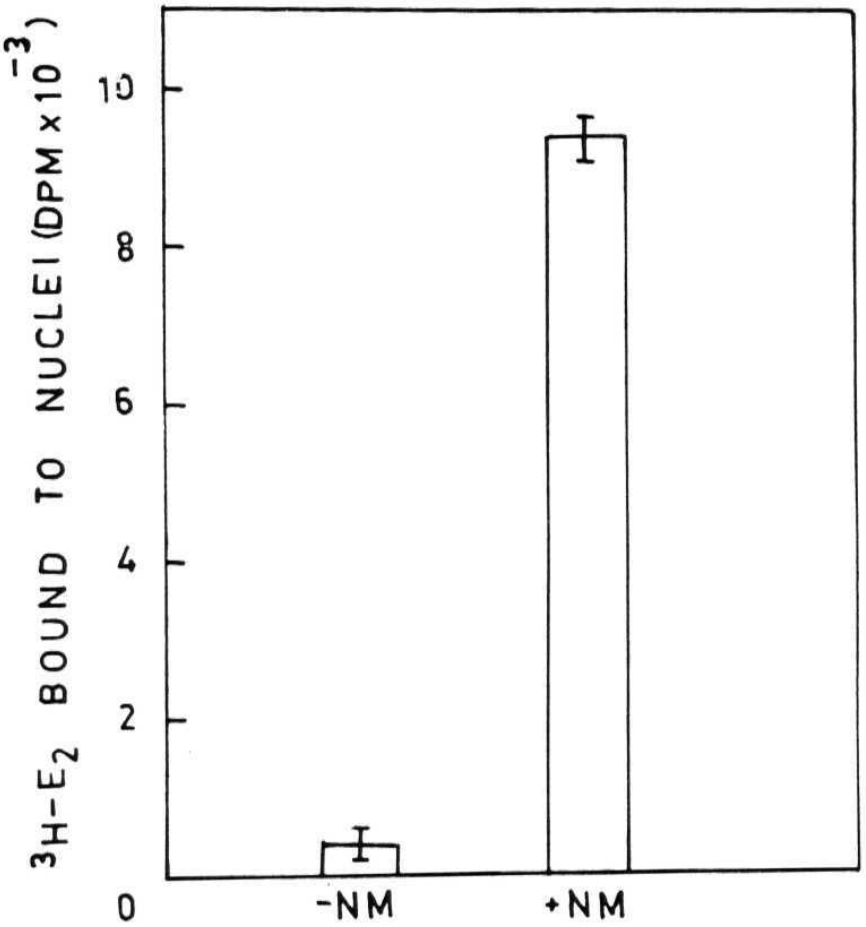

$^3H$-$E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$)
10
8
6
4
2
0
-NM
+NM

**Figure 4.5.** A nuclear membrane **protein** mediates **NLSBP dependent transport of ER into the detergent treated nuclei.**

**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 against TEMN buffer. The dialysate was concentrated and separated on a pre-calibrated column of Sephadex G-100. The column was developed with TEM buffer containing 0.2 M NaCl. The fractions (2 ml) collected were subjected to NLSBP assay with detergent treated nuclei (N-M). The absorbance of the fractions at 280 nm was also measured.

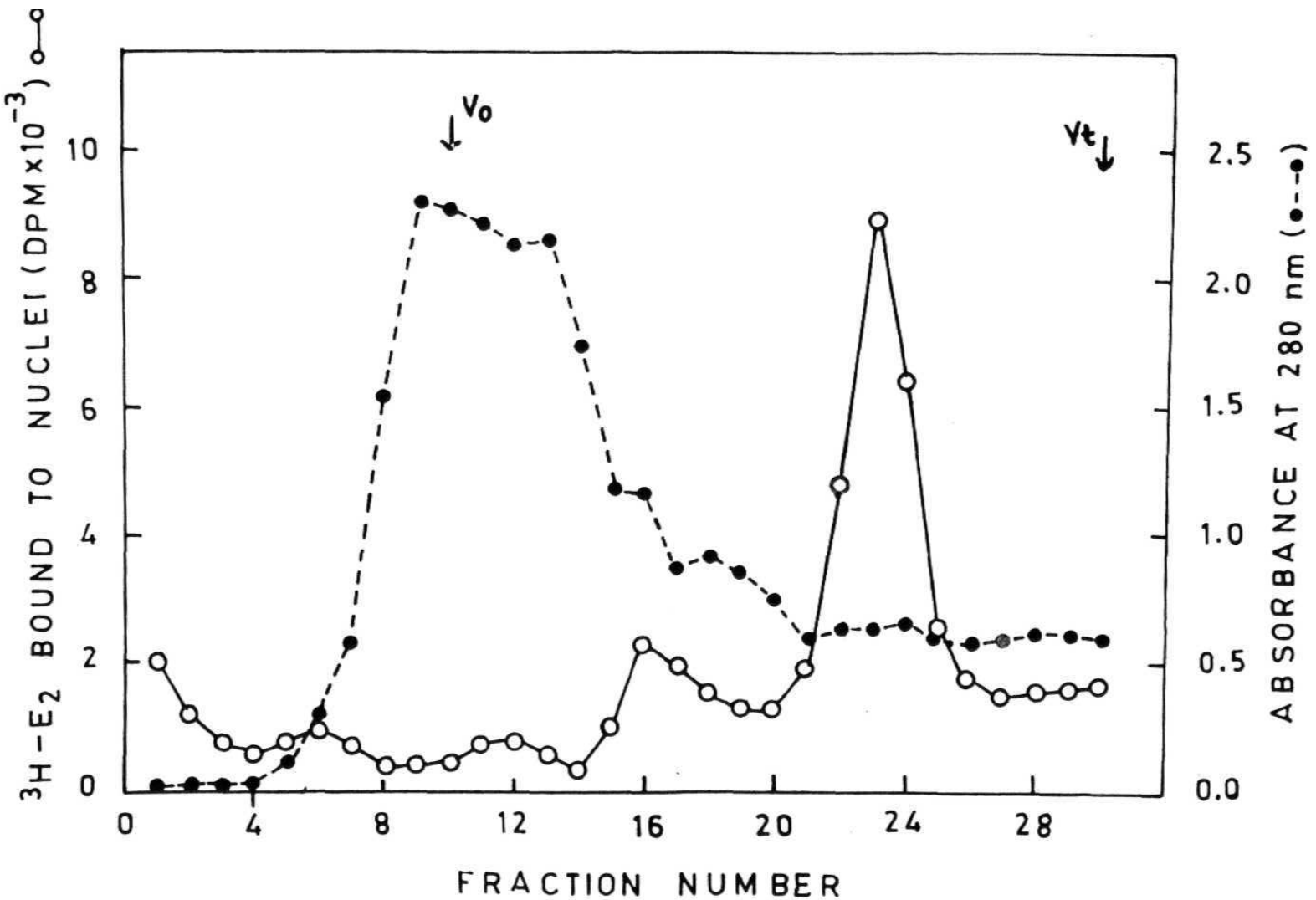

$^3H-E_2$ BOUND TO NUCLEI (DPM x $10^{-3}$) o—o
ABSORBANCE AT 280 nm (•--•)
FRACTION NUMBER
$V_o$
$V_t$
0
2
4
6
8
10
0.0
0.5
1.0
1.5
2.0
2.5
0
4
8
12
16
20
24
28

**Figure 4.6. SDS-PAGE analysis of the Sephadex G-100 fractions.**

The peak NLSBP activity fractions (from Sephadex G-100 column) were subjected to SDS-PAGE on 10% gels. The lane numbers correspond to the fraction numbers in the figure 4.5. The gels were stained with silver nitrate.

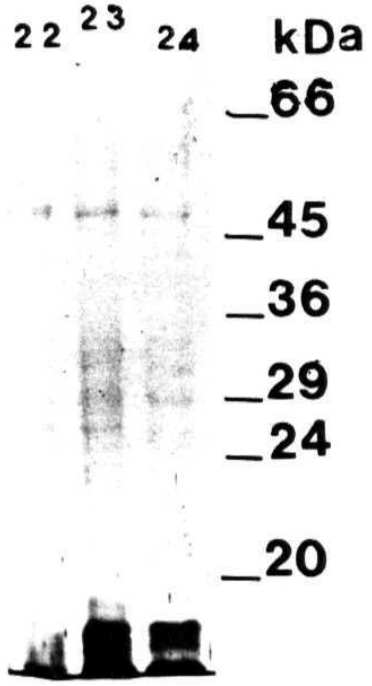

22
23
24
kDa
66
45
36
29
24
20

**Figure 4.7. Fluorescence assay for the ER nuclear transport.**

Goat uterine nuclei (N+M) were suspended in NLSBP-assay buffer containing 4 mM ATP and were incubated with FITC labelled ER (5 μg) at 30°C.

A) for 30 minutes without NLSBP (40X),

B) for 30 minutes with NLSBP (0.5 μg) (40X),

C) for 5 minutes with NLSBP (0.5 μg) (100X),

D) for 10 minutes with NLSBP (0.5 μg) (100X),

E) for 15 minutes with NLSBP (0.5 μg) (100X).

The FITC-ER transported into the nuclei was observed in a Leitz fluorescence microscope and was photographed using an Ilford 400 ASA film. (The magnification is given in the parentheses).

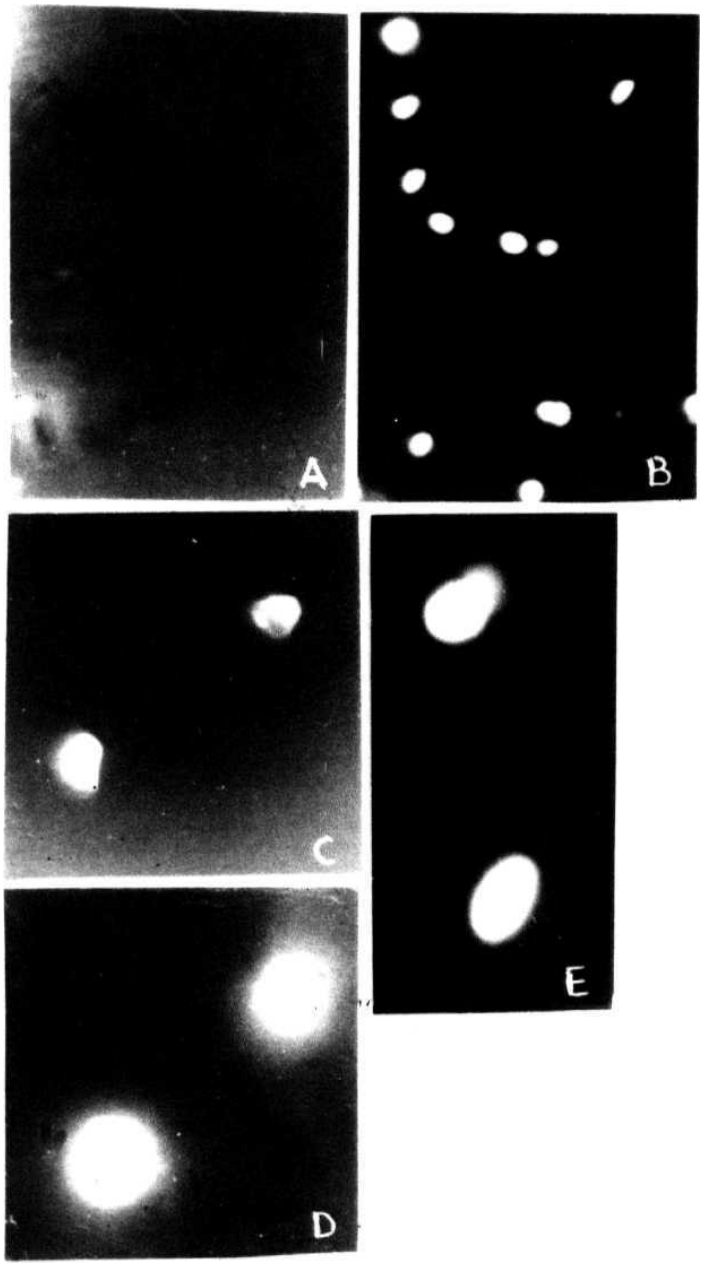

A
B
C
D
E

Figure 4.8. Fluorescence assay of NLSBP-mediated ER transport into the nucleus.

Goat uterine nuclei (Triton X-100 washed, i.e., N-M) were suspended in NLSBP assay buffer containing 4 mM ATP and incubated with FITC labelled ER (5μg) at 30°C.

(A) in the absence of NLSBP (100X),

(B) in the presence of 0.5 μg NLSBP for 30 minutes (100 X),

(C) in the presence of 0.5 μg NLSBP for 30 minutes and observed ~3 minutes after the addition of the 14 kDa protein(s) (100X).

(D) Goat uterine nuclei (with membranes intact i.e., N+M) were suspended in the NLSBP assay buffer (without ATP) and were incubated with FITC-ER (5 μg) and NLSBP (0.5 μg) at 30°C for 30 minutes (63/1.3X).

(E) To the above incubation was added ATP to a final concentration of 4 mM concentration and visualized about 2 minutes after the addition of ATP (63/1.3X).

(F) To a similar incubation medium as described in (D) ATP (4 mM) and quercetin (10 μg/ml) were added simultaneously to the medium and visualized 30 minutes later (100X).

The FITC-ER bound to the nuclei was examined in a Leitz fluorescence microscope and photographed using an Ilford 400 ASA film. (The magnification is given in the parentheses).

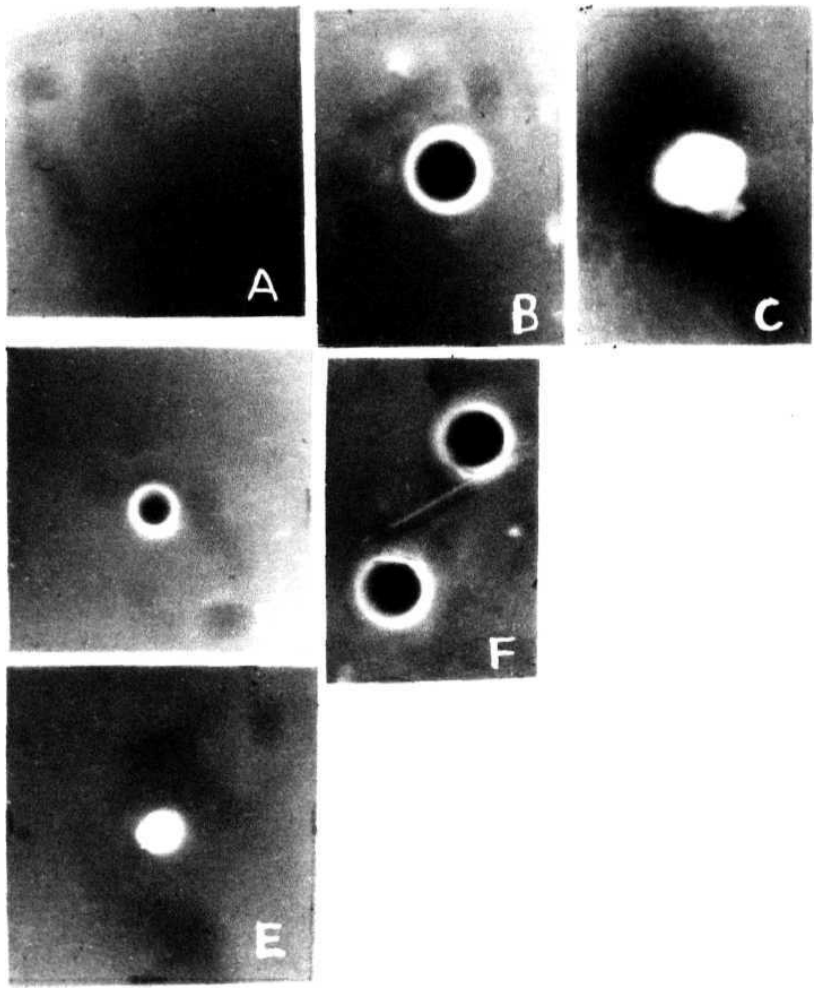
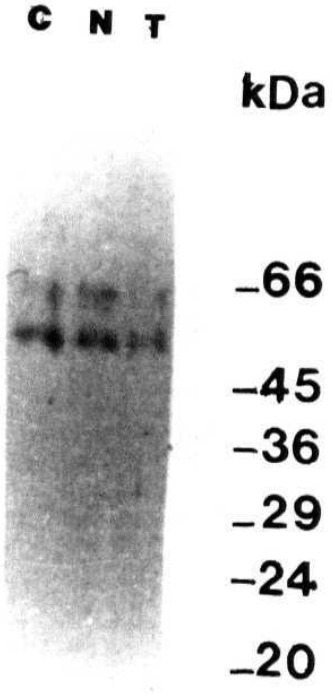

A
B
C
F
E

Figure 4.9. Western blot analysis of the cytosol, nuclei and nuclear membrane fractions for the detection of **NLSBP.**

Cytosol (C), nuclei (N) and the Triton X 100 wash fractions of the nuclei (T) (25 µg each) were separated on 10% gels. The proteins were transferred to nitrocellulose membranes. The membranes were incubated overnight with poly-aspartate antibody (1:50). The membranes were re-incubated with HRPO-coupled anti-rabbit IgG (1:500). The blots were stained with 4-chloro 1-naphthol and $H_2O_2$ .

Figure 4.10. The NLSBP is a DNA binding protein.

Pure NLSBP preparation, following actin-Sepharose chromatography, was chromatographed on a 3 ml column of DNA-Sepharose. The column was washed with TEMN buffer and eluted with a linear NaCl gradient (50 mM to 1 M). The fractions (1 ml) collected were subjected to SDS-PAGE and the gel was stained with silver nitrate. The flowthrough from the ss-DNA-Sepharose column (lane D) and the phosphocellulose column were subjected to SDS-PAGE. The gels were silver stained.


C N T
kDa
66
45
36
29
24
20

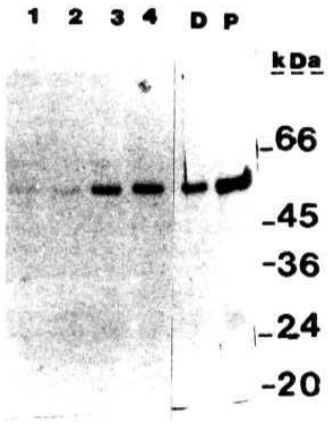

1 2 3 4 D P
kDa
66
45
36
24
20

Figure 4.11. ATPase activity of the NLSBP.

NLSBP (0.2 to 2.0 μg) was incubated with 6 mM ATP at 30°C for 30 minutes as described in the `methods'. The incubation was terminated by the addition of TCA. The Inorganic phosphate (Pi) released into the TCA soluble supernatant was estimated as described in the `methods'. The Pi released is expressed as μmoles Pi released/hour.

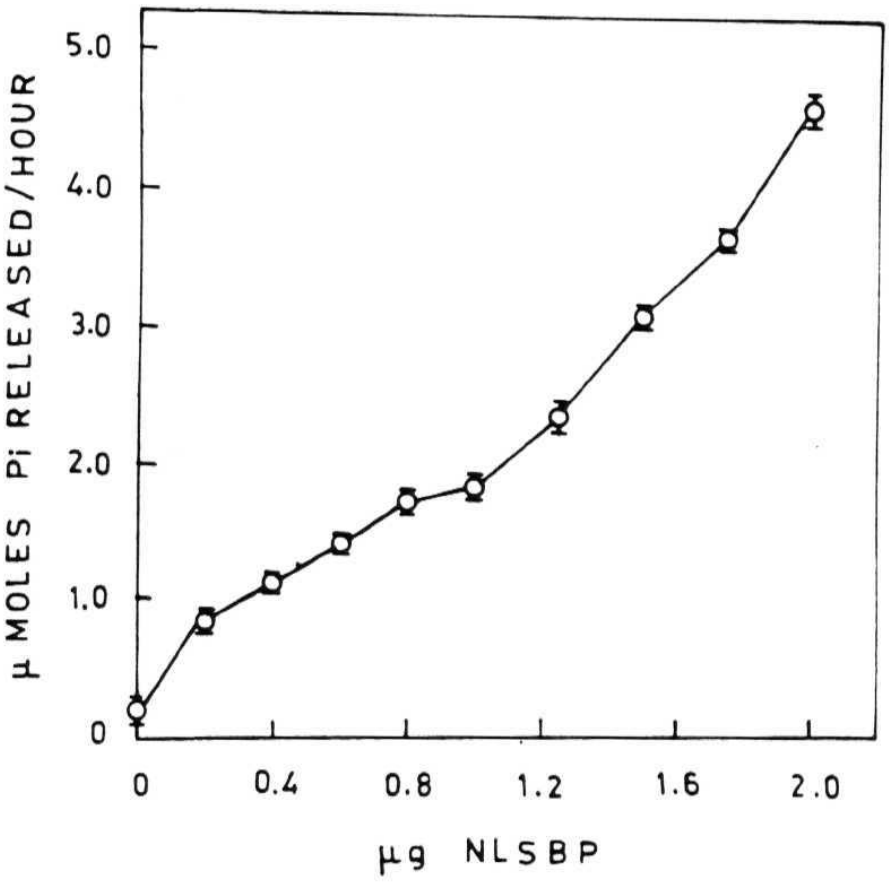

5.0
4.0
3.0
2.0
1.0
0
μ MOLES Pi RELEASED/HOUR
0
0.4
0.8
1.2
1.6
2.0
μg NLSBP

**Figure 4.12.** ATPase activity of the nuclei is enhanced by the NLSBP.

The goat uterine nuclei (N+M) were incubated for different time intervals (0 **to** 30 minutes) either alone (2) or in the presence of two concentrations of NLSBP - 1 μg (3) or 2 μg (4). In another set goat uterine nuclei were incubated with 2 μ **g** NLSBP and 20 μg/ml quercetin (1). The incubation was terminated by the **addition of** TCA. The Pi released into the TCA soluble supernatant was estimated and expressed as μmoles Pi released/mg DNA.

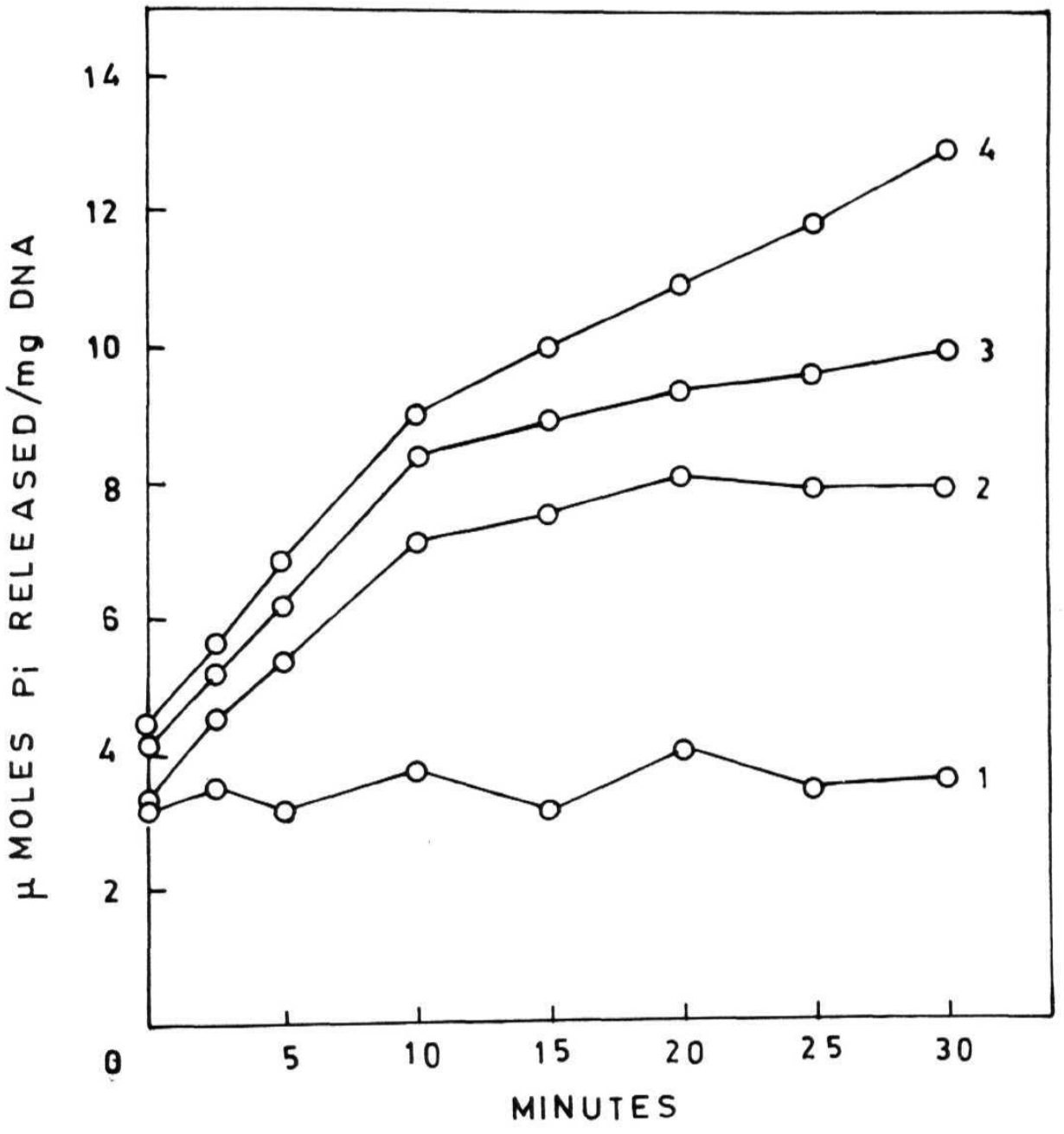

μ MOLES Pi RELEASED/mg DNA
14
12
10
8
6
4
2
0
5
10
15
20
25
30
MINUTES
1
2
3
4

**Figure 4.13. ATPase activity of the NLSBP is inhibited by quercetin.**

**NLSBP (2 μg) was incubated with increasing concentration of quercetin (0 to 20 μg/ml) for 30 minutes at 30°C. 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.**

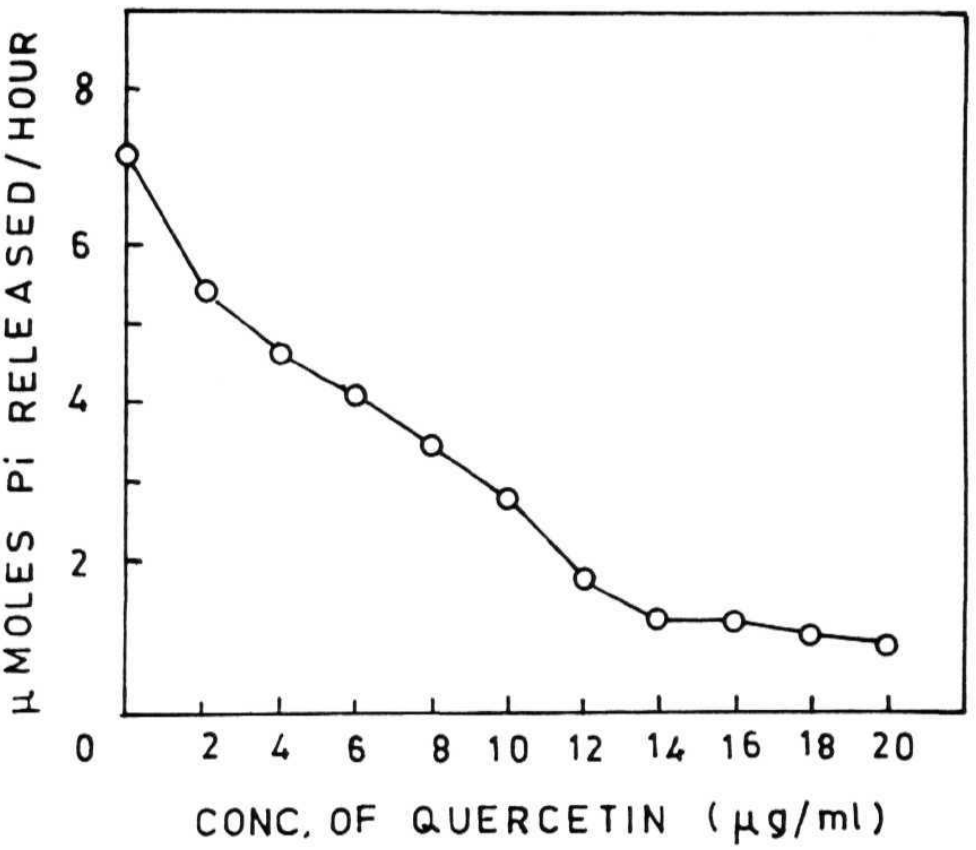
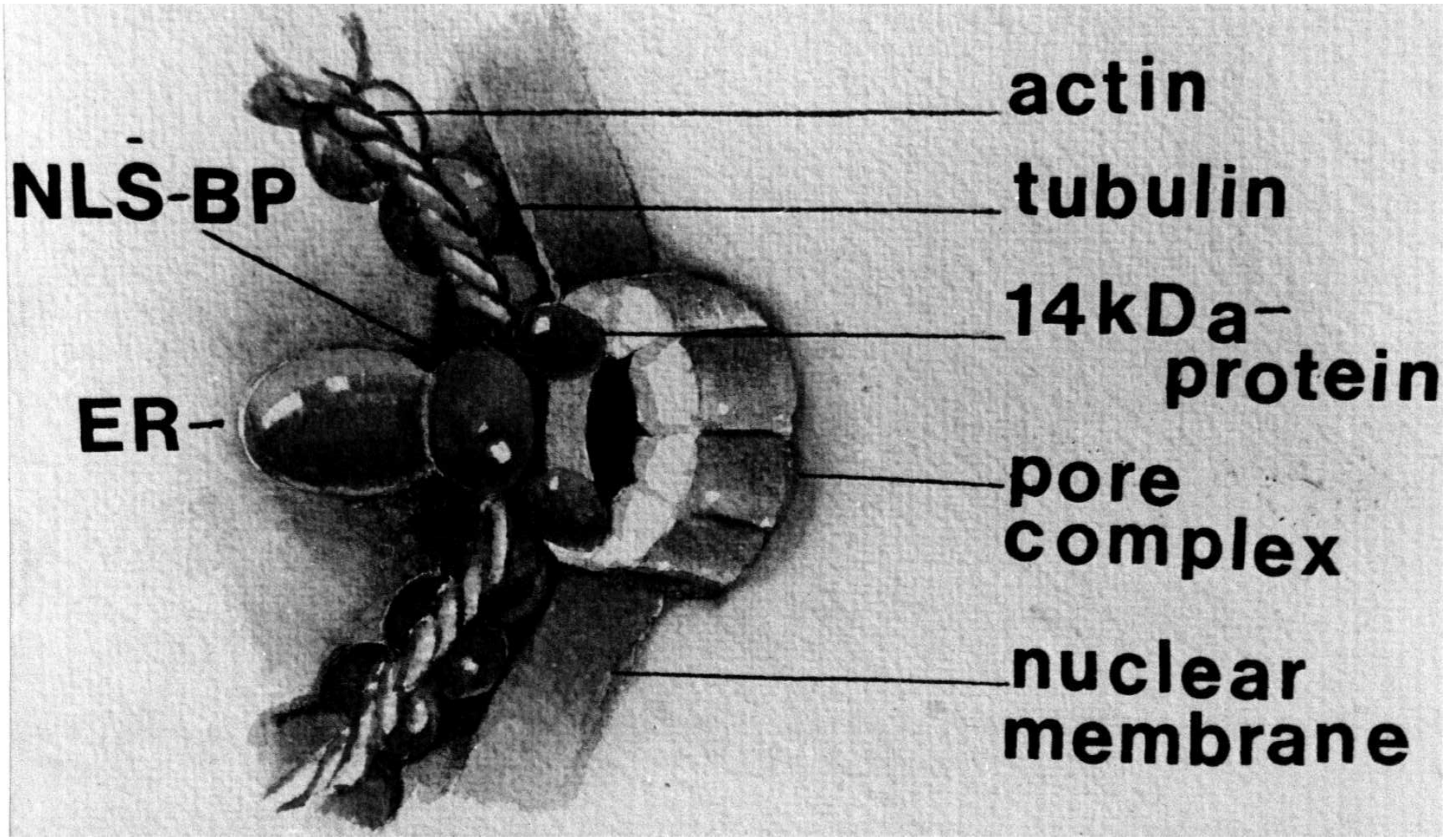

μ MOLES Pi RELEASED/HOUR
0
2
4
6
8
2 4 6 8 10 12 14 16 18 20
CONC. OF QUERCETIN (μg/ml)

**Figure 4.14. A model for the NLSBP mediated nuclear transport of the ER.**

NLS-BP: Nuclear localization sequence binding protein; Estrogen Receptor: ER.

The NLSBP-ER complex is shown here as interacting with the **14 kDa proteins, closely** associated with the nuclear pore complex and **the nuclear membranes. Tubulin** assemblies and actin polymers have been shown in **the background in** order to indicate their involvement in the NLSBP-mediated **nuclear transport of** the **ER.**


actin
tubulin
NLS-BP
14kDa-protein
ER-
pore complex
nuclear membrane

# UBIQUITINATION OF THE ESTROGEN RECEPTOR.

# 5.0 UBIQUITINATION OF THE ESTROGEN RECEPTOR

The half life of the mammalian estrogen receptor in the absence of the hormone is ~5 days. In the presence of estradiol, the ER levels fall within an hour. The normal levels of the ER are reached within 4 to 6 hours which can be blocked by actinomycin D or cycloheximide (SarfT and Gorski, 1971). Replenishment of receptors in the cytosol is contributed by the synthesis of new receptors and, to a small extent, by the recycling of the receptors that have moved out of the nucleus. Only 10% of the replenished receptors are contributed by recycling. The rest of the receptor pool is synthesized `*de novo*' Horwitz *et al* ., 1983).

The receptors, after binding the hormone bring about the transcription of the target gene and are degraded soon after. The ER has to be recognized and degraded selectively. The only known mechanism for selective degradation of proteins is the ubiquitin pathway. Thus it was important to examine whether the ER was also degraded by mechanisms belonging to the ubiquitin pathway. For this purpose, it was important to identify ER-ubiquitin conjugates in the cell.

During ubiquitination, as described in detail in the introduction (section 1.5), ubiquitin molecules are conjugated to a protein via an isopeptide bond between the C-terminal glycine of the ubiquitin and the ε-NH of an internal lysine of the protein under consideration. Such an interaction is not destroyed by SDS or β-mercaptoethanol. Thus ubiquitinated proteins are seen in SDS gels as a ladder of higher molecular weight species. The proteins can be mono-ubiquitinated or poly-ubiquitinated.

Using the above principle, I wanted to examine:

1. -whether the estrogen receptor was ubiquitinated; and
2. -whether estrogen receptor ubiquitination was influenced by esrradiol.

The experimental protocol to identify ubiquitinated ERs was to immunoprecipitate them with either the ER antibody or the ubiquitin antibody. The higher molecular weight species in the anti-ER immunoprecipitates or the anti-ubiquitin immunoprecipitates were to be analyzed for their cross reactivity with ubiquitin and ER antibodies respectively by western blotting. For this purpose, ubiquitin was purified and antibodies were raised in rabbits.

## 5.1 PURIFICATION OF UBIQUITIN.

Ubiquitin was purified from rat blood cells following the procedure of Parakh and Kannan (1992) as described in the section 2.13. This purification procedure makes use of a simple principle, the non-dialysability of ubiquitin (in a dialysis bag with a 12 kDa cut off). The erythrocyte extract was dialyzed against 20 ml of 25 mM ammonium acetate pH 4.5. The free ubiquitin moves out of the dialysis bag, as it is a ~8.5 kDa protein, along with the other proteins of <12 kDa molecular mass. The dialysis buffer was chromatographed on a column of CM-Sephadex (30 ml) equilibrated with 25 mM ammonium acetate pH 4.5. The flowthrough was recycled to optimize binding. The column was washed with the same buffer and the bound proteins were eluted with 250 mM ammonium acetate pH 7.2. The eluate was collected as 5 ml fractions and the measured for their absorbance at 280 nm. A single protein peak was observed (Fig 5.1).

The peak fractions were pooled and dialyzed against TEMN buffer (in a dialysis bag with a 3.5 kDa cut-off limit) in order to remove ammonium acetate.

The dialysate was lyophilized and an aliquot was dissolved in the SDS sample buffer; boiled for 10 minutes and resolved on 15% SDS gels. The protein appeared as a single band in the silver stained gels. The molecular weight of protein was calculated to be ~8.5 kDa (Fig 5.2A). The protein obtained was confirmed to be ubiquitin by western blot analysis using commercially available ubiquitin antibody. Ubiquitin was transferred to nitrocellulose membranes and the membranes were incubated overnight with anti-ubiquitin IgG. The blots were re-exposed to HRPO-coupled anti-rabbit IgG and were stained as described before. The anti-ubiquitin IgG crossreacted with the ubiquitin preparation (Fig 5.2B). Antibodies were raised against ubiquitin in rabbits as described in the methods. This antibody also demonstrated crossreactivity with the ubiquitin preparation (Fig 5.2B lane 2) and was used in the following experiments.

## 5.2 UBIQUITINATION OF ER IS ENHANCED BY ESTRADIOL *IN VITRO*.

Experiments were planned to identify the ER-ubiquitin conjugates in rat uteri and the influence of estradiol on the ER-ubiquitin conjugation *in vitro* was analyzed.

Rats (2 month old) were ovariectomized under light ether anaesthesia. Ten days after surgery the rats were sacrificed. The uteri of these rats were excised and the adjoining fat tissue was removed. The uteri were slit longitudinally and cut into small pieces. The uterine pieces were incubated in Dulbecco's minimal essential medium (DMEM) pH 7.6 containing 20 nM estradiol in a shaker water bath maintained at 37°C for 0, 15,30,60, 120, 180 and 240 minutes. A control incubation of uteri at 37°C for 240 minutes in the absence of estradiol

was performed. At the end of the incubation, the uteri were washed in cold TEMN buffer and were homogenized in the same buffer. The cytosol of these uteri was prepared following the procedure of Van der Hoeven (1981). The concentration of the protein in the cytosol was estimated by the Lowry's method (1951). Cytosol (1 mg) each of the samples was incubated in triplicates with anti-ER IgG (1:50) at 4°C overnight. To precipitate the antigen-antibody complex, the cytosol was incubated for an additional two hours with anti-rabbit IgG (1:500) at 4°C. The immunoprecipitates were pelleted by centrifugation at 10,000 X g for 10 minutes. The precipitates were washed with TEMN buffer and dissolved in SDS sample buffer. The samples were subjected to SDS-PAGE on 7.5% gels, in triplicates. One gel was stained with silver nitrate and the others were transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated overnight with anti-ER antibody and ubiquitin antibody separately. The nitrocellulose membranes were re-exposed to alkaline phosphatase-coupled anti-rabbit IgG and stained with BCIP/NBT as described before.

The silver stained gels of the anti-ER precipitated samples showed protein bands of increasing molecular weights with the increase in time of incubation with estradiol. With the increase in the high molecular weight species, the concentration of the 66 kDa protein (ER?) decreased upto 120 minutes. In the next two hours, the high molecular weight species increased slightly while the 66 kDa protein concentration returned to normal, suggesting *'de novo'* protein synthesis (Fig 5.3). The blot that was exposed to ER antibody, showed a similar protein profile. The high molecular weight bands crossreacted with the ER antibody, suggesting the ubiquitination of the estrogen receptor. The concentration of the ER (66 kDa protein) also decreased upto 120 minutes and normal levels were reached by 240 minutes. The higher molecular weight

species increased upon incubation with estradiol (5.4A) The blot that was crossreacted with the ubiquitin antibody also showed a similar pattern. A 66 kDa protein also crossreacted with the ubiquitin antibody. An experiment was performed to check whether the ER itself showed any crossreactivity with anti-ubiquitin. The ubiquitin antibody did not crossreact with the ER (data not shown). Thus this 66 kDa protein seen in the anti-ubiquitin stained blots could be another protein which co-precipitated with the ubiquitinated ERs. The ubiquitin antibody crossreacted with the high molecular weight species confirming that these high molecular weight proteins are indeed ubiquitinated ERs (Fig 5.4B). The intensity of staining with ubiquitin antibody increased with the increase in molecular weight of the protein, as was observed in the blots. This could easily be explained. The higher molecular weight bands represent protein complexes with a single molecule of ER conjugated with increasing number of ubiquitin molecules. The greater the number of ubiquitin molecules, the greater the intensity of staining with the ubiquitin antibody.

To demonstrate whether the increase in ubiquitin-ER conjugation in the presence of estradiol was specific, the following experiment was conducted. Ovariectomized rat uteri were incubated in DMEM for 60-120 minutes in the presence of 20 nM progesterone, testosterone and dexamethasone. The cytosol was prepared and immunoprecipitated with anti-ER IgG and crossreacted with the ubiquitin antiserum. No high molecular species were seen in the cytosol of the Progesterone, dexamethasone and testosterone treated uteri. (data not shown).

## 5.3 UBIQUITINATION OF THE ER IS ENHANCED BY ESTRADIOL *IN VIVO.*

The above experiment demonstrated that the ER was ubiquitinated *in vitro* and also that the ubiquitination was enhanced by the presence of estradiol. It was important to confirm this result *in vivo* and also examine the influence of **withdrawal** of estradiol on the ubiquitination of the ER.

Rats (2 month old) were ovariectomized under light ether anaesthesia and were sacrificed 6, 24, 48 and 72 hours after ovariectomy. As controls, two rats were sacrificed immediately after ovariectomy. After 10 days of ovariectomy, rats were implanted subcutaneously with estradiol-bees wax pellets and sacrificed 6, 24, 48 and 72 hours after implantation. As controls, 2 rats were sacrificed immediately after implantation.

All the uteri were excised and the adhering fat tissue was removed. The uteri were homogenized in TEMN buffer and the cytosol was prepared. The protein concentration of the cytosol was estimated by Lowry's method (1951). Cytosol (1 mg) was immunoprecipitated with either anti-ER IgG (1:50) or anti-ubiquitin IgG (1:50) as described before. The immunoprecipitates were subjected to SDS-PAGE in 7.5% gels in duplicates. One of the gels was stained with silver nitrate. Proteins in the other gel were transferred to nitrocellulose membranes and subjected to western analysis. The anti-ER immunoprecipitated samples were crossreacted with ubiquitin antibody and vice versa.

**The** silver stained gels of the anti-ER and anti-ubiquitin precipitated samples showed high molecular weight species (ER-ubiquitination) from 0

to 24 hours after ovariectomy. The high molecular weight species decreased after 48 hours of ovariectomy (Fig 5.5A and 5.6A) and crossreacted with both anti-ubiquitin (Fig 5.5B) and anti-ER (Fig 5.6B).

The higher molecular weight species increased along with the advance in time following hormone implantation, upto 24 hours, following which a decrease in the high molecular weight bands was noticed in both anti-ER precipitated (Fig 5.7A) and anti-ubiquitin precipitated (Fig 5.8A) gels. In both the gels, the high molecular weight species crossreacted with anti-ubiquitin (Fig 5.7B) and anti-ER antibodies (Fig 5.8B).

The higher intensity of staining with the higher molecular weight bands by the ubiquitin antibody was also observed in these experiments. The highest bands represent a single molecule of ER conjugated with several molecules of ubiquitin. As one comes down the ladder, the number of ubiquitin molecules conjugated to the ER would be lesser corresponding to the lesser intensity of the staining by anti-ubiquitin. The intensity of staining was directly proportional to the number of ubiquitin molecules conjugated to FR

## 5.4 DISCUSSION:

An increase in the molecular weight of the ER in the anti-ER and the anti-ubiquitin immunoprecipitated proteins in silver stained SDS gels was observed. These high molecular weight species cross-reacted with both ER and ubiquitin antibodies. These results suggested that these high molecular weight bands are ER-ubiquitin conjugates. This is the first report ever regarding ubiquitination of the ER or of any steroid receptor.

The ubiquitin-ER conjugation can also be referred to as ubiquitination of ER. The ubiquitination of ER was influenced by estradiol as observed by both *in vivo* and in vitro experiments. The withdrawal of estradiol *in vivo* decreased the ubiquitination of the ER. The increase in ubiquitination of ER in response to estradiol is specific as the other steroids, progesterone, testosterone, dexamethasone do not show any effect on the ubiquitination of ER (data not shown).

Based on these observations, I wish to propose that the ER is degraded by the ubiquitin pathway and that ubiquitination of ER is enhanced by estradiol. The conjugation of ER to ubiquitin precedes degradation of the estrogen receptor. Thus these results show that estradiol enhances degradation of the ER. This agrees with the earlier findings by Sarff and Gorski (1971) that ER levels in the cell decrease in the presence of estradiol.

Based on what is currently known about the enzymes involved in ubiquitination it may be predicted that ER is conjugated to ubiquitin by three enzymes, E1, E2 and E3. The ubiquitin-ER conjugates are then recognized by the ubiquitin degradation enzymes that degrade ER and releases ubiquitin for further use in the conjugation.

This assumption needs to be confirmed by the isolation of E1, E2 and E3 (which is probably specific for ER, an ER-ubiquitin ligase) and the demonstration of ubiquitination of ER *in vitro* in the presence of these enzymes. The work presented here is a basic study to identify the pathway by which the ER was degraded. Further work needs to be done to identify the different steps in the pathway.

Many other proteins have been examined for their degradation following the ubiquitin pathway, similar to the studies carried out as followed by me, i.e., immunoprecipitation with the protein antibody and cross-reaction with anti-ubiquitin. These included phytochrome, cyclin, p53 and Mat a2 repressor (Shanklin *et al* ., 1987; Glotzer *et al* ., 1991; Hochstrasser *et al* ., 1991; Scheffner *et al* ., 1993).

**Figure 5.1 CM-Sephadex chromatography of ubiquitin.**

**Rat** erythrocytes were lysed with double distilled water and the lysate was **dialyzed** overnight against 25 mM ammonium acetate pH 4.5. The dialysis **buffer** was chromatographed over a column of CM-Sephadex (30 ml), equilibrated with 25 mM ammonium acetate pH 4.5. The column was washed with the same buffer and eluted with 250 mM ammonium acetate pH 7.2. The eluate was collected as 5 ml fractions and the absorbance of the fractions at 280 nm was monitored.

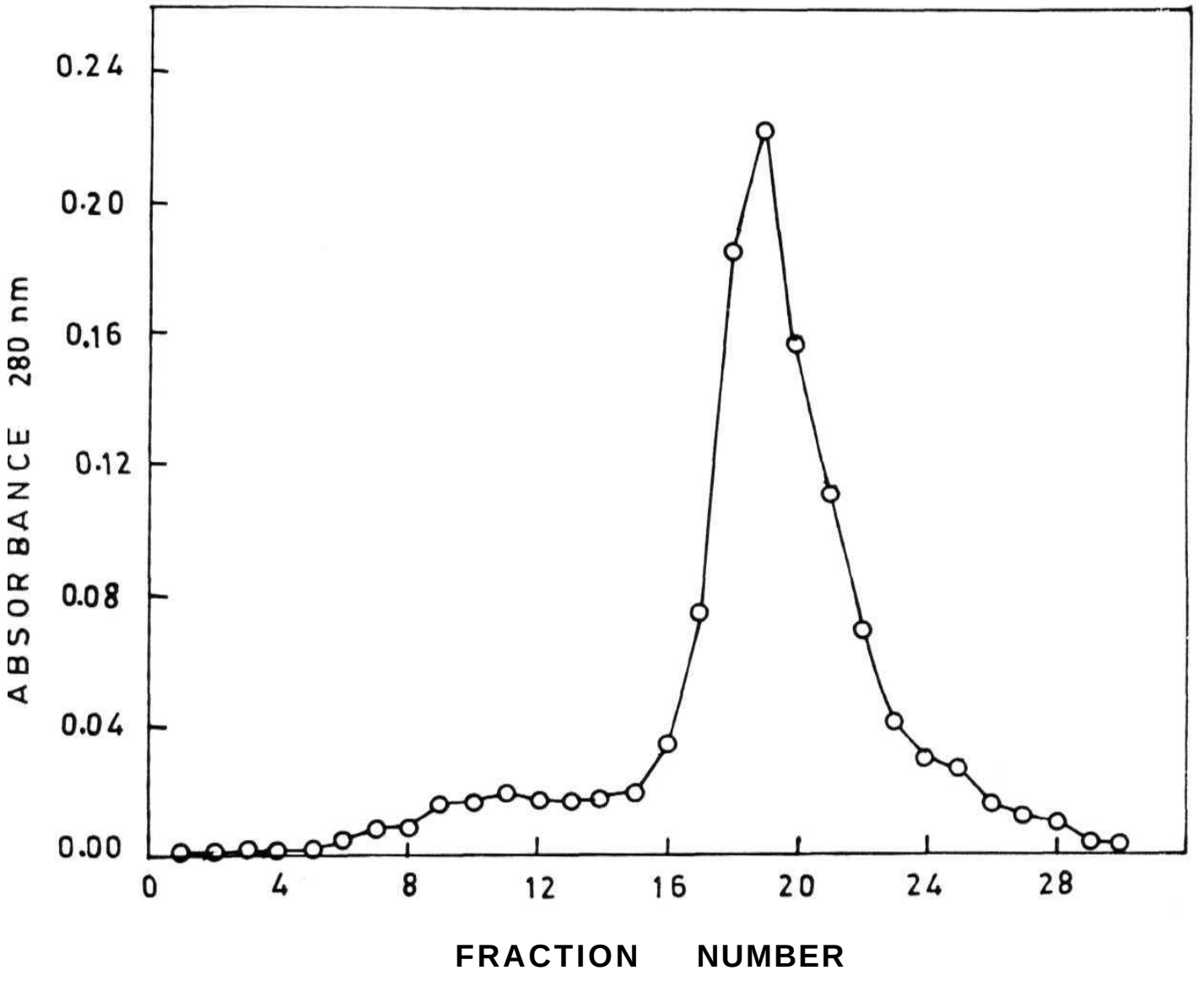
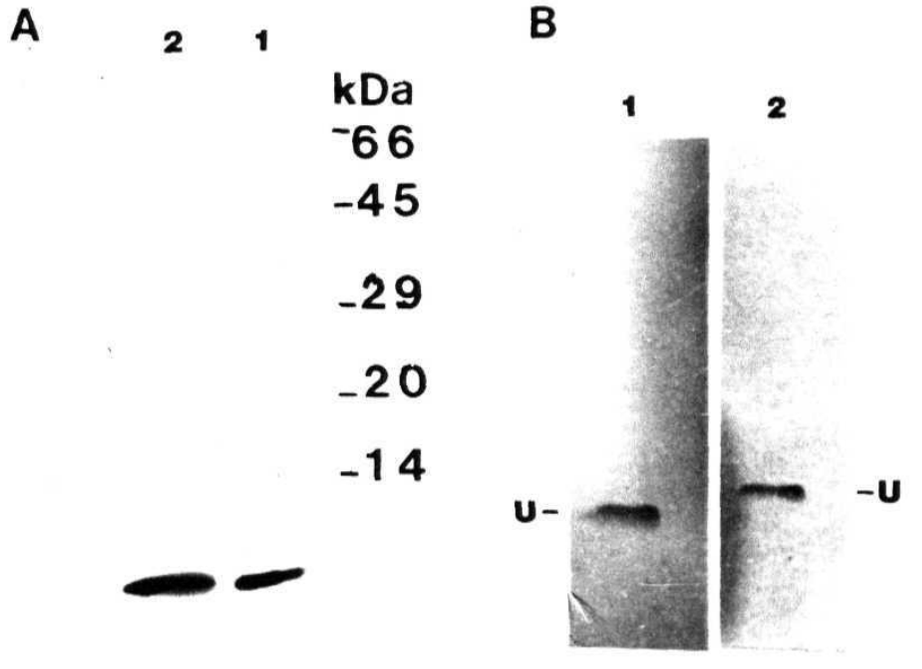

0.24
0.20
0.16
0.12
0.08
0.04
0.00
ABSORBANCE 280 nm
0
4
8
12
16
20
24
28
FRACTION NUMBER

**Figure 5.2A. SDS-PAGE analysis of ubiquitin.**

Ubiquitin was purified from rat erythrocytes as described in 5.1. The purified ubiquitin (2.5 jig and 5 μg) was subjected to SDS-PAGE (lanes 1 and 2 respectively) on 15% gels. The gels were stained with silver nitrate.

5.2B. Western blotting analysis of ubiquitin.

Pure ubiquitin was subjected to SDS-PAGE on 15% gels in duplicates. The protein in the gels were transferred to nitrocellulose membranes. One of the membranes was incubated overnight with the commercially available anti-ubiquitin antibody (1). The other was exposed to the antibody raised by me in rabbits against the purified ubiquitin (2). The blots were re-incubated with HRPO coupled anti-rabbit IgG. The blots were stained with 4-chloro 1-naphthol and $H_2O_2$ .


A
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1
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45
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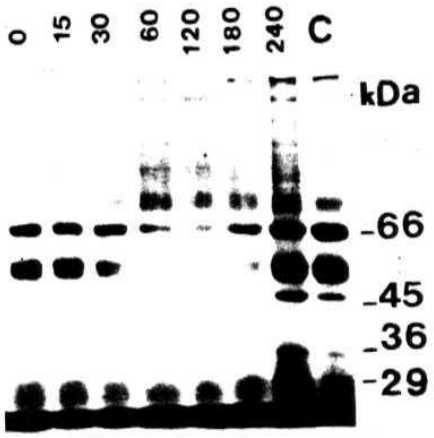
Figure 5.3 Ubiquitination of ER is enhanced by estradiol in vitro.

Ovariectomized rat uteri were incubated in DMEM at 37°C in the presence of 20 nM estradiol for 0,15,30,60,120,180 and 240 minutes respectively. One set of incubations was performed in the absence of estradiol for 240 minutes (C). The cytosol fraction was prepared in TEMN. 1 mg cytosol from each of these incubations was precipitated with anti-ER IgG (1:50). The immunoprecipitates were separated on 7.5% gels and stained with silver nitrate. The molecular weight markers (kDa) are indicated.

Figure 5.4. Western blot analysis of the ubiquitin-ER conjugates.

Ovariectomized rat uteri were incubated in DMEM at 37°C in the presence of 20 nM estradiol for 0 (A), 15 (B), 30 (C), 60 (D), 120 (E), 180 (F), and 240 (G) minutes respectively. One set of incubations was done in the absence of estradiol for 240 minutes (0). The cytosol (1mg) of these uteri was immunoprecipitated in duplicates with anti-ER IgG. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. One of the blots was incubated with anti-ER (1:50) (5.4A) and the other with anti-ubiquitin (1:50) (5.4B) overnight. The blots were washed and re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT.


0
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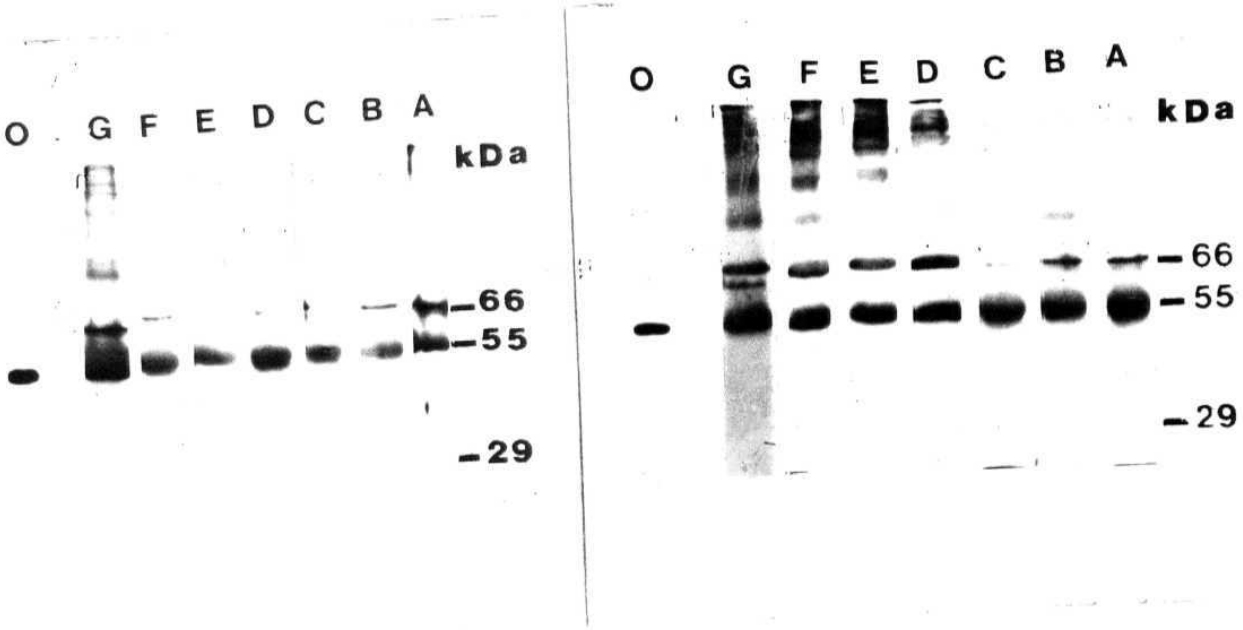
O
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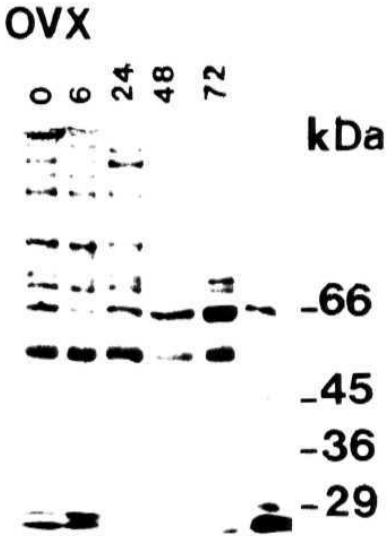
29

**Figure 5.5 Ubiquitination of estradiol** decreases **upon withdrawal of estradiol following ovariectomy.**

Rats were ovariectomized and sacrificed 0, 6, 24, 48 and 72 hours after ovariectomy. One mg uterine cytosol was incubated with anti-ER IgG (1:50) (5.5A) or anti-ubiquitin IgG (1:50) (5.5B) overnight at 4°C. Anti-rabbit IgG (1:200) was added to the above incubation mixture and was incubated for an additional two hours. The immunoprecipitates were obtained following centrifugation at 10,000Xg. The precipitates were subjected to SDS-PAGE on 7.5% gels and the gels were silver stained.

**Figure 5.6 Western blot analysis of the ubiquitin-ER conjugates.**

Rats were ovariectomized and sacrificed 0 (A), 6 (B), 24 (C), 48 (D) and 72 (E) hours after ovariectomy. One mg uterine cytosol was immunoprecipitated with anti-ER IgG (1:50) (5.6A) or anti-ubiquitin IgG (1:50) (5.6B) as described in the figure legend 5.5. The immunoprecipitates were separated by SDS-PAGE on 7.5% and transferred to nitrocellulose membranes. The blot containing the anti-ER immunoprecipitated samples was incubated with ubiquitin antibody (5.6A) and the other blot containing the anti-ubiquitin immunoprecipitated samples with ER antibody (5.6B) overnight. The blots were washed and re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT.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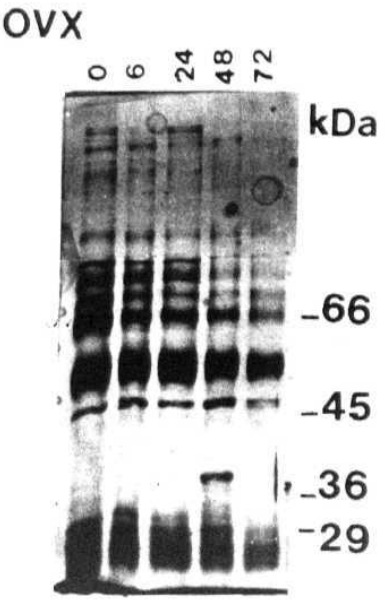
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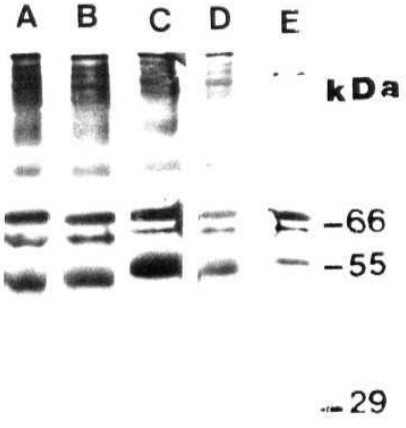
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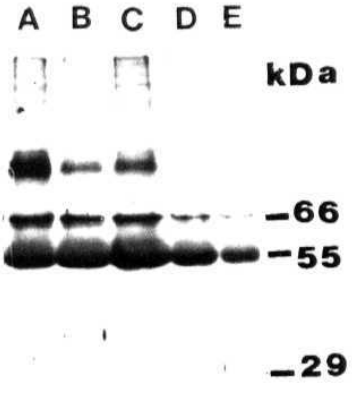
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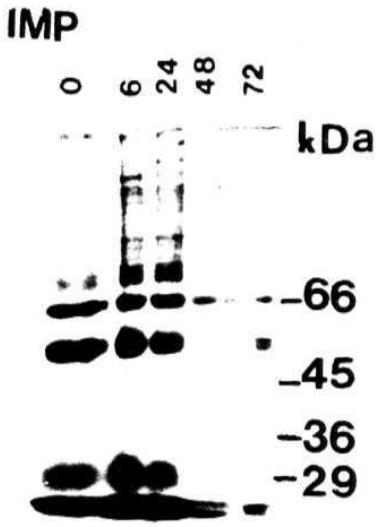
Figure 5.7. Ubiquitination of ER is enhanced by estradiol in vivo.

Ovariectomized rats were implanted with estradiol-bees wax pellets. Rats were sacrificed 0, 6, 24, 48 and 72 hours after implantation. The uterine cytosol (1mg) of all the experimental groups was incubated overnight with anti-ER IgG (1:50) (5.7A) or anti-ubiquitin IgG (1:50) (5.7B) at 4°C. Anti-rabbit IgG (1:200) was added to the above incubation mixture and incubated for an additional two hours. The immunoprecipitates were subjected to SDS-PAGE on 7.5% gels and silver stained.

Figure 5.8. Western blot analysis of the ubiquitin-ER conjugates following implantation with estradiol.

Ovariectomized rats were implanted with estradiol bees wax pellets. Rats were sacrificed 0 (A), 6 (B), 24 (C), 48 (D) and 72 (E) hours after implantation. One mg uterine cytosol was immunoprecipitated with anti-ER IgG (1:50) (5.8A) or anti-ubiquitin IgG (1:50) (5.8B) as described in the figure legend 5.7. The immunoprecipitates were separated by 7.5% SDS gels and transferred to nitrocellulose membranes as described in the methods. The blot 5.8A was incubated with ubiquitin antibody (1:50) and blot was incubated with ER antibody (1:50) overnight. The blots were re-exposed to alkaline phosphatase coupled anti-rabbit IgG (1:1000). The blots were stained with BCIP/NBT.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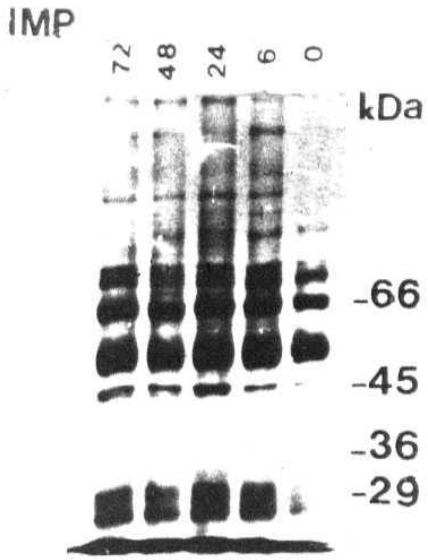
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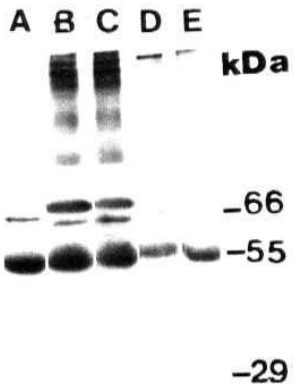
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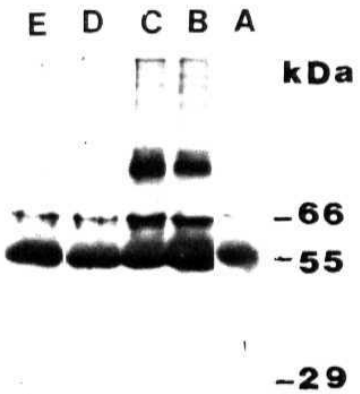
E D C B A
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# CONCLUSIONS

# CONCLUSIONS

1. **The** estrogen receptor nuclear localization sequence binding protein (NLSBP) is a 55 kDa protein isolated from goat uterine cytosol. This is the first report on the NLSBP-mediated nuclear transport of the ER, or any steroid receptor.

2. The transport of estrogen receptor from the cytoplasm to the pore complex **might be** aided by the microtubular and/or microfilament network as the NLSBP **binds to** both tubulin and actin.

3. **The** NLSBP recognizes ER and transports it from the cytosol to the nuclear membrane. The NLSBP-ER complex binds to the nuclear membrane. This step in transport is not ATP-dependent.

4. The NLSBP-estrogen receptor complex is recognized by a secondary site, a 14 kDa protein present in the nuclear membrane. The 14 kDa protein(s) translocates ER into the nucleus in a ATP dependent manner. The requirement for the 14 kDa nuclear membrane protein(s) in nuclear transport has been demonstrated for the first time. Earlier reports on nuclear transport have been concentrated on the role of cytoplasmic factors in nuclear transport. The role of a nuclear membrane component in nuclear transport of proteins has only been speculated so far.

5. NLS binding protein displays ATPase activity and also enhances the ATPase activity associated with the nucleus. This is a $Mg^{++}$-dependent ATPase as it is inhibited by quercetin.

6. Quercetin, an ATPase inhibitor, inhibits the translocation *of* ER into the nucleus. Energy released upon ATP hydrolysis is required in the translocation **step.**

7. Antibodies raised against poly-aspartate recognizes the NLS binding protein and inhibits the nuclear transport of the estrogen receptor.

8. Experimental evidences are presented here to show that the estrogen receptor is ubiquitinated `*in vitro*' and `*in vivo*'.

9. Ubiquitination of the estrogen receptor increases in the presence of estradiol and decreases when estradiol is depleted from the system. The influence of estradiol on the ubiquitination of ER is specific.

10. The ubiquitination of the ER presented in this thesis is the first report on ubiquitination of any steroid receptors.

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