INTRODUCTION
1.0 INTRODUCTION

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

1.1 STRUCTURE OF THE ESTROGEN RECEPTOR (ER):

The ER gene has been cloned, sequenced and the predicted amino acid sequence has been determined. The ER has 595 amino acids and a molecular mass of 66 kDa. The ER can be divided into six functional regions, from A to F.
The ‘A’ region is well conserved between chicken and human estrogen receptors and is required for transcriptional activation. The ‘C’ region is also very well conserved and contains the DNA binding domain. Deletion of this region results in the loss of DNA binding capacity of the receptor (Kumar et al., 1986). The DNA binding domain is composed of two highly conserved zinc fingers that set the receptor apart from other DNA binding proteins. These zinc fingers are generated by the co-ordination of zinc ions with four cystein residues (Schwabe et al., 1990). This region targets the receptor to the hormone response elements (Green et al., 1988). The ‘E’ region is also very well conserved and contains the ligand binding domain. This domain possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiological responses. In its simplest terms, the ligand binding domain can be thought of as a molecular switch that, upon ligand binding, shifts the receptor to a transcriptionally active state. The region ‘D’ has 37 aminoacids and function as a hinge between the DNA binding and the hormone binding domains.

The above mentioned receptor is the classical estrogen receptor. From our laboratory it has been shown that in addition to the classical ER, there exists another ER form, called the non activated ER (naER) which has the capacity to bind estradiol with high affinity while remaining incapable of binding to the DNA (Anuradha et al., 1994). This protein also has a molecular mass of 66 kDa, like the regular ER. The structural differences between the two proteins has been demonstrated by the non identical peptide maps that the two proteins display (Zafar and Thampan, 1993; Karthikeyan and Thampan, 1996). Karthikeyan and Thampan (1996) demonstrated that the naER is primarily localized in the plasma membrane and is specifically extracted into the medium when the membranes are
exposed to estradiol. The naER has been shown to have the capacity to dimerize with the estrogen receptor activation factor (E-RAF), a cytosolic DNA binding protein with no capacity to bind estradiol (Thampan and Clark, 1981; Thampan, 1987, 1989).

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

1.2 CELLULAR LOCALIZATION OF THE ER:

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

Welshons et al., (1984), by using immuno cytochemical techniques and enucleation experiments, proved that the unoccupied estrogen receptors were predominantly nuclear in localization.
Vazquez-Nin et al., (1991) by using immunoelectron microscopic technique showed that the receptor was mainly nuclear but was also present in the cytoplasm.

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

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

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

1.3.1 hsp-90:

The heat shock protein (hsp) 90 family is a group of highly conserved stress proteins found ubiquitous in eukaryotes. hsp 90 is the most abundant constitutive hsp in eukaryotic cells, accounting for 1-2% of cytosolic protein and is associated with all steroid receptors (Baulieu, 1987; Pratt, 1990; Pratt et. al., 1992). hsp 90 exists in a stoichiometric ratio of 2:1 (hsp:SR) in the 9s non-transformed receptor complexes. There are reports of hsp 90 localization in cytoskeleton including actin, in membrane ruffles (Koyasu et al., 1986), microtubules (Redmond et al., 1989) and intermediate filaments (Czar et al., 1996). It has been proposed that cytoskeleton-associated hsp 90 may reflect potential protein targeting and
trafficking function of hsp 90 and its associated proteins. The hsp 90 binding of the ER prevents it from binding to DNA and at the same time maintains the receptor in a conformation required for hormone binding (Bresnick et al., 1989). The direct evidence for this came from the studies of Picard et al. (1990) who have shown that mutants having low levels of hsp 90 but normal levels of steroid receptors are not responsive to the steroid as the receptors do not bind the hormone efficiently.

1.3.2 Immunophilins:

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

1.4 NUCLEAR TRANSPORT OF PROTEINS:

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

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

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

1.4.2 *The nuclear pore complex:*

The nuclear pore complex is a supramolecular assembly that straddles the inner and outer nuclear membranes of all eukaryotes. It is ~0.13μm in diameter, ~0.07μm thick and has a relative molecular mass of about 125 megadaltons (Akey, 1989., Reichelt et al.,1990). The NPC has two main functions: it allows passive diffusion of ions and small molecules through nuclear pores with a physical diameter of ~9 nm and it mediates transport of protein and ribonucleoprotein particles through a gated channel with a functional diameter of upto 26 nm (Feldherr et al., 1984; Gerace, 1992).
1.4.2-1. The Structure of the Pore Complex:

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

The NPC consists of four separate structural elements:

a) the Scaffold, which includes the majority of the pore
b) the central hub or the transporter of the pore, which appears to carry out the transport of proteins and RNA

c) short thick filaments attached to the cytoplasmic side of the pore and
d) a nucleoplasmic basket (Fig. 1.1)

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

b) The central hub or transporter (360-380 Å) is a proteinaceous ring. Akey (1990, 92) predicted that the central transporter would consist of two irises of eight arms each. The two irises are predicted to be stacked atop one another and to
Figure 1.1 Structural model of the nuclear pore complex.

A structural model of the nuclear pore complex as presented by Forbes, 1992 (with a portion in the front of the pore cut to show the details). The globular components of the cytoplasmic ring (CR) of the pore are connected to the spokes (S) as are the globular components of the nucleoplasmic ring (NR). The spokes are separated by 9 nm channels and support a central transporter (T). The transporter consists of two rises 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 (BR) extends from the nucleoplasmic ring of the pore.
open sequentially, each like the diaphragm of a camera, to let a nuclear protein or RNA through (Akey and Goldfarb, 1989). Elucidation of the structure of the central transporter of the nuclear pore has been derived from analyzing nuclear pores by rapid freezing technique (Akey and Goldfarb, 1989., Akey 1990; 91; 92). This rapid freezing technique has allowed retention of a structure (the central plug) that was often lost in previous studies. The transporter can be resolved within individual pores into one of the four recognizable and distinct forms. Each form has an eightfold symmetry and it is assumed that they represent sequential intermediates in nuclear import in the following order: 1) a nuclear protein (K) first binds at the periphery of the transporter ring; 2) it then moves to the central channel where it docks and induces the channel to open; 3) in response to the import signal, the first iris opens, lets a protein pass into the pore; 4) the second iris would then open, further passage would ensue; 5) the first iris closes, followed by the second, and translocation is complete.

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

Recent electron microscopic evidence indicates that the pore also is in contact with important accessory structures. Individual pores appear to be connected to one another by the nuclear lamina and by an additional set of pore-connecting fibers (Stewart and Whytock, 1988; Allen and Douglas, 1989). On the
cytoplasmic face of the pore thick fibers (~33Å0 diameter) that extend into the cytoplasm have been observed by a low voltage scanning electron microscopy (Ris, 1991). Several strong indications are available that such filaments exist (Scheer et al., 1988) and are a staging area for nuclear proteins to bind prior to transport (Richardson et al., 1988). On the nucleoplasmic side of the pore, a large basket-like structure has also emerged from the scanning electron microscopic studies of Ris (1990; 1991). This basket disassembles in the absence of Ca\textsuperscript{2+} and reforms when Ca\textsuperscript{2+} is added (Jarnik et al., 1991; Jarnik and Aeby, 1991). This basket structure appears to consist of eight filaments extending from the nucleoplasmic ring of the pore.

1.4.2-2. Nuclear pore complex proteins:

The nuclear pore complex could be considered as an organelle composed of a unique set of proteins necessary for transporting macromolecules across the nuclear envelope. Association of the nuclear pore complex proteins with both the nuclear membrane and the underlying lamina has made it difficult to isolate the pore complex in pure form. The first pore complex protein to be identified was the abundant high molecular weight concavalin- A reactive glycoprotein gp 120, which is located on the pore margin, largely inside the perinuclear cisternae. Subsequently a series of nucleoporins were discovered by virtue of their N-acetyl glucosamine containing O-linked carbohydrate moieties that make them reactive to wheat germ agglutinin (WGA). Evidence for the pore complex location of a newly identified polypeptide might include a) immunogold labeling, b) co-fractionation with pore-complexes enriched fraction, c) punctuate immuno fluorescence staining of the nuclear periphery.
Identification of approximately 20 genes encoding nucleoporins in yeast and a handful of others in higher eukaryotes has led to the classification of these proteins into several different groups based on sequence motifs contained within their primary aminoacid sequences. Two classes of phenylalanine/glycine (FG) repeats, GLFG and FXFG subtypes, have been identified. A number of nucleoporins contain one or the other or a combination of these repeats. Thus far, the in vivo function of the FG repeat regions has not been elucidated. It has, however, been shown that some soluble nuclear transport factors interact with these FG repeat domains in vitro (Iovine et al., 1995; Rexach et al., 1995). Thus, the FG repeat domains may play a role in targeting the soluble transport factors to the nuclear pore.

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

Since the nucleoporins are both the structural and functional components of the nuclear pore complex, it seems likely that many of them will serve general function and that some may play more specific roles in the transport of different classes of macromolecules. A number of genes encoding nucleoporins have been identified in a screen for S.cerevisiae mutants defective in the export of poly (A) RNA from the nucleus (Amberg et al., 1992). The nucleoporins Nup100p, Nup116p and Nup145p contain putative RNA binding domains. Some nucleoporins are also directly implicated in protein import. The nucleoporin that falls most readily into this class is Nsp1p. In vitro studies suggest that the protein
import defect observed in Nsp1 mutants arises from a decrease in the docking of substrate to the nuclear envelope as well as from an inability to translocate substrate across the pore (Schlenstedt et al., 1993).

There are some more evidences to indicate that some nucleoporins play a specific role in protein translocation.
1) co-incident labeling of nuclear pore complexes with WGA- gold and nucleoplasmin-gold particles suggests that nucleoporins interact directly with proteins prior to their passage through the pore (Akey and Goldfarb, 1989)
2) Antibodies that recognize a subset of nucleoporins block nucleoplasmin import and RNA export (Featherstone et al., 1988).
3) WGA blocks uptake of proteins into the nucleus (Yoneda et al. 1987; Dabauvalle et al., 1988). The effect of WGA is not due to the occlusion of the channel, because a dextran through the pore is not blocked (Finlay et al., 1987). Instead, pore complexes assembled in vitro without WGA-binding proteins are morphologically intact but are unable to import large proteins properly (Finlay and Forbes, 1990). These reconstituted pores fail to bind NLS bearing proteins. Addition of WGA-binding pore complex proteins restores binding and import.

Understanding precisely what role each nucleoporin plays in macromolecular transport will probably require a model of the entire pore complex. There are some limited approaches to assigning in vivo function to a particular class of nucleoporins in eukaryotes. One method consists of depletion of xenopus egg extracts of nuclear pore components using WGA or antibodies prior to nuclear envelope assembly (Finlay et al 1991; Newmeyer et al., 1986; Powers et al., 1995). Such an approach has been used to demonstrate that the vertebrate
nucleoporin complex p62,p58,p54 is required for docking of proteins at the nuclear pore (Finlay et al., 1991). These experiments are, however, restricted by the availability of specific tools to deplete each nucleoporin.

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

1.4.3 Nuclear localization signals/sequences:

Proteins destined to be targeted into the nucleus following translation in the cytoplasm contain specific signals in their primary sequences. The most extensively studied signals are termed Nuclear Localization Signals (NLSs) and, unlike other signal sequences, can be located anywhere in the primary sequence of the protein. These nuclear localization signals were first suggested by De Robertis et al. (1978) that the nuclear proteins must contain in their primary structure a signal that enables them to accumulate selectively in the nucleus. Two criteria define NLSs: deletion or mutation causes cytoplasmic accumulation of a normally nuclear protein and when fused to a non nuclear protein, the NLS directs the protein to the nucleus. In many cases both the criteria can be taken into account to identify the NLSs. NLSs have now been identified in a large number of nuclear proteins. There is no single consensus among the many NLSs that have been identified to date. However there are some general descriptive rules that NLSs 1) are typically short sequences, usually not more than 8-10 aminoacids,
2) contain a high proportion of positively charged amino acids (lysine and arginine) often associated with proline,
3) can reside in any exposed region of a nuclear protein
4) are not removed following localization and
5) can occur more than once in a given protein.

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

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

The presence of the N-terminal flanking region of the SV40 large T antigen NLS in association with its NLS dramatically enhances nuclear transport. This region is distinguished by a phosphorylation site (Rihs and Peters, 1989). This phosphorylation site was identified to be a casein kinase (CK) II site and a survey of other nuclear proteins shows that all nuclear proteins have CK II sites in the vicinity of the NLS at a distance of 10-30 amino acids. Phosphorylation might modulate the affinity of NLS for its receptor (Rihs et al., 1991).
Robbins et al. (1991) further showed that the nucleoplasmin NLS is composed of two interdependent regions of basic aminoacids: mutations in either alone have no effect on nuclear localization activity. The nucleoplasmin NLS is non functional only when both domains are mutated. A nucleoplasmin like motif is seen in several proteins such as p53, N1/N2, No38 and the steroid receptors.

1.4.3-1 Steroid hormone receptor NLSs:

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

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

Ylikomi et al. (1992) chose to analyze steroid hormone receptor NLSs in their natural aminoacid sequence contest, because
1) the efficiency of NLSs is sensitive to variations in the protein context; thus a given NLS may become inactive when placed in a different environment even within the same protein (Roberts et al., 1987)
2) the β-galactosidase marker that is commonly used for heterologous nuclear targeting studies is a tetrameric protein, while steroid receptors are assumed to be
<table>
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<tr>
<th>(256-260) RKDRR</th>
<th>(265-273) KHKRQR</th>
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dimers (Kumar and Chambon, 1988) and the consequences of multimerization on NLS efficiency is unknown.

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

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

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

In the progesterone receptor, three p-NLSs, two of which are located within and directly adjacent to the second zinc finger, cooperate with each other and a weak hormone-inducible p-NLS in the PR HBD. No masking of p-NLSs by
the HBD was observed for ER and PR, while the ligand free glucocorticoid receptor HBD inhibited the activity of both homologous and heterologous NLSs.

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

1.4.4-1) NLS receptors:

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

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

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

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

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

1.4.4-3 NTF2:

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

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

1.4.5 Mechanism of nuclear transport:

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

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

Historically, nuclear protein import has been described as a two step process: an energy-independent binding at the nuclear pore followed by an energy-dependent translocation into the nucleus (Newmeyer and Forbes, 1988). Advances in our understanding of the import mechanism has led to the realization that this process can actually be divided into more distinct and specialized steps
1) recognition of the transport substrate in the cytoplasm,
2) targeting to the nuclear pore complex,
3) translocation through the nuclear pore,
4) release of the transport substrate at the nucleocytoplasmic face of the pore complex.

1.4.5-1) Recognition:

Proteins are targeted to the nuclear pore via an interaction in the cytoplasm between the NLS within the protein and a soluble NLS receptor. Recent advances have led to the isolation of a heterodimeric complex required for targeting NLS-containing proteins to nuclear pores. The first subunit is the importin-α of approximately 60 kDa. The second subunit is a protein of approximately 95 kDa termed importin-β. While both subunits of the NLS receptor can interact with NLS
sequences, the binding of importin-α is significantly tight. This finding in combination with the observation that importin-β interacts with repeats contained in several nucleoporins has led to the proposal of a model in which importin-α is primarily responsible for binding to NLS-containing proteins. Importin-β then targets the complex to the nuclear pore (Gorlich et al., 1995). Moroianu et al., (1992) showed that importin-α (Karyopherin-α) alone cannot bind to the nuclear envelope of digitonin-permeabilized cells while importin-β (Karyopherin-β) can bind without importin-α being present.

By immunofluorescence microscopy of methanol fixed cell, Moroianu et al.(1995) findings gave a very important information about importin α and β. Importin β was localized to the cytoplasm and the nuclear envelope and was absent from the nuclear interior. Recombinant importin-β can bind directly to nuclear envelope of digitonin permeabilized cells. In contrast, recombinant importin-α did not bind unless importin-β was present. Likewise in an import reaction with all recombinant transport factors (importin-α, importin-β, Ran and p10) import depended on importin-β. Localization of the exogenously added transport factors after a 30 minutes import reaction showed importin-β at the nuclear envelope and importin-α, Ran and p10 in the nuclear interior. In an overlay assay with SDS/PAGE resolved and nitrocellulose transferred proteins of the nuclear envelope, ³⁵S-labelled importin-β bound to atleast four peptide repeats containing nucleoporins-Nup358, Nup214, Nup153 and Nup98. This result indicates a division of labor, the α subunit of the importin heterodimer serves in NLS recognition, and the β subunit mediates docking to the peptide repeat containing nucleoporins. Most interesting is the finding that the importin-α enters the nucleus and is retained there, whereas importin-β does not enter the nucleus.
Immunofluorescence localization with anti-Karyopherin-β(importin-β) antibodies showed primarily cytoplasmic staining and a strong nuclear rim staining but no significant intranuclear staining.

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

1.4.5-2) Targeting to the nuclear pore:

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

Tubulin and actin coprecipitated with the estrogen receptor when goat uterine cytosol was immunoprecipitated with antibodies against estrogen receptor antibody (Zafar and Thampan, 1993). Similar result was also observed when the immunoprecipitation was carried out using antibodies against tubulin and actin. Tubulin and actin were also demonstrated to be associated with other steroid receptors. (Sanchez et al., 1988; Miyata et al., 1991). Nirmala and Thampan (1995a) showed that p55 bound to both actin and tubulin with very high affinity.
This interaction of both the ER and the p55 with the cytoskeletal elements of the cell suggests the possible involvement of cytoskeletal proteins in the transport of ER into the nucleus.

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

1.4.5-3) Protein translocation into the nucleus:

Once transport substrates are targeted to the nuclear pore, they must undergo translocation through the pore complex. Mechanism of translocation through the transporter of the NPC was proposed by Akey (1990) in the double iris model based on electron microscopic studies of the NPC. The gating events results in the dilation of the first iris to allow the protein inside. The protein passes through the transporter and the first iris closes and the second iris open at the nucleocytoplasmic side to let the protein move into the nucleoplasm. WGA may prevent the dilation of the first iris by cross linking the subunits of the iris.
The factors which play role in the translocation step were identified to be 1) GTPase Ran: GTP hydrolysis by Ran seems to be important step in the nuclear transport (Moore and Blobel, 1994). In cell-free import assays by using either isolated nuclei or digitonin permeabilized cells ATP or ATP regenerating system can substitute the GTP and GTP hydrolysing factors (Ran) (Newmeyer et al., 1986; Richardson et al., 1988; Nirmala and Thampan, 1995b).

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

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

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

5) hsp 70: As with many other cellular transport processes, some studies have implicated the role of chaperone hsp 70 in nuclear protein import .(Imamoto et al., 1992; Shi and Thomas, 1992). The functional role played by hsp 70, however, remains unknown.
Nirmala and Thampan (1995a) showed that the p55-dependent translocation of the goat uterine ER into the nucleus is mediated by a 12-14 kDa protein(s) present in the nuclear membrane near the nuclear pore complex.

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

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

Nirmala and Thampan (1995 a;b) have shown that the estrogen receptor transport into the nucleus can be separated into two distinct steps: the p55-mediated transport and binding of ER to the nuclear membrane, followed by an ATP-dependent 12-14 kDa protein(s) mediated translocation of ER into the nucleus. The p55 has inherent ATPase activity and it is proposed that the energy released during this ATP hydrolysis is utilized in the nuclear transport of the ER.
From the observations mentioned above, it may be inferred that no single general mechanism can account for the transport of all the proteins. There exists selective differences in the transport of different proteins, probably indicating the specificity and selectivity that should be associated with the transport of individual proteins.

1.5 REGULATION OF NUCLEAR IMPORT:

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

NF-κB is a ubiquitous mammalian transcription factor whose activity is regulated at the level of its intracellular location. It is a tetrameric protein which
consists of two 50 kDa and two 65 kDa subunits. In stimulated B lymphocytes, NF-κB is nuclear, binds to the DNA and regulates the transcription of κ immunoglobulin light chain genes. However, in pre-B cells, where κ light chains are not expressed, NF-κB is cytoplasmic. The cytoplasmic form of NF-κB is associated with another protein, IκB, and this complex is unable to bind to the DNA. Phosphorylation disrupts the IκB-NF-κB complex and NF-κB enters the nucleus (Ghosh and Baltimore, 1990).

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

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

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

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

1.6 hsp 70:

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

1.7 hsp-25:

The biological roles of hsp 25 are unknown, however, it has been suggested that this protein may play a role in thermotolerance and regulation of gene expression. Moreover, like the high molecular weight hsp56, hsp 25 may be involved as a molecular chaperone.
SCOPE OF THE THESIS

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

In the absence of the hormone, estrogen receptor is predominantly nuclear in its localization (King and Green, 1984, Welshons et al., 1984). But exposure of cells to estradiol results in a significant and reproducible increase in nuclear accumulation of hER (Ylikomi et al., 1992). An estrogen-inducible p-NLS was found in the hormone binding domain (HBD) of the hER in addition to the three constitutive NLSs. The inducible and the constitutive ER pNLSs cooperate in the presence of estrogen and influence the nuclear translocation of ER when the cells are exposed to estradiol (Ylikomi et al., 1992). Nuclear co-translocation experiments indicated that in vivo the stability of ER dimers is hormonally controlled.
The experimental work presented in this thesis serves to understand the regulatory mechanism involved in the nuclear transport of estrogen receptor under the influence of estradiol. Consequently a couple of cytosolic proteins of molecular mass 28 and 73 kDa were identified and isolated apart from the p55. With the studies on these proteins what has been achieved is the knowledge that the estradiol-influenced receptor entry into the nucleus is highly regulated. The p28 antagonizes the ER-p55 interaction, apparently through the masking of the NLS on the ER. This inhibition is reversed by yet another, 73 kDa protein, the p73. Estradiol causes the release of p73 from ER and facilitates p28 dissociation from the NLS site on the ER. p55 occupies the NLS and mediates the transport. This, apparently is the main role played by the hormone in the nuclear transport of the ER.

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