ESTROGEN RECEPTORS OF THE GOAT UTERUS: EMPHASIS ON A RECEPTOR FORM THAT ENTERS THE NUCLEUS AS A CONSTITUENT OF HETERODIMER

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DECLARATION

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

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CERTIFICATE

This is to certify that Mr. Narayanan Karthikeyan, has carried out the research work embodied in the present thesis under my supervison and guidance for the full period prescribed under the Ph. D. ordinance of this University. I recommend his thesis entitled *"Estrogen Receptors of Goat Uterus: Emphasis on a Receptor form that Enters the Nucleus as a Constituent of a Heterodimer"* for the submission for the Degree of Doctor of Philosophy of this University.

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ABBREVIATIONS USED

ATP	Adenosine 5' triphosphate
BSA	Bovine serum albumin
DCC	Dextran coated charcoal
DDW	Double distilled water
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
E2	Estradiol 17p
EDTA	Ethylene diamine tetra acetic acid
ER	Regular estrogen receptor
E-RAF	Estrogen receptor activation factor
FITC	Fluoroscein iso thio cyanate
HAP	Hydroxylapatite
HRPO	Horse radish peroxidase
hsp 90	Heat shock protein 90
IgG	Immunoglobulin G
naER	Non-activated estrogen receptor
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethyl sulphonyl fluoride
POPOP	1,4, Bis (2,5 phenyl oxazyl) benzene
PPO	Diphenyl oxazole
SDS	Sodium dodecyl sulphate
ss-DNA	Single stranded DNA
TCA	Trichloro acetic acid

CONTENTS

CHAPTER I

INTRODUCTION

The macromolecular receptors involved in the early phases of steroid hormone action have been targets of experimental studies for the past three decades. **The** mechanisms by which receptors function, from the initial binding of the hormone to the molecular effects at the gene level, have been examined in great detail. Although the biochemical pathway is of a general category for steroid receptor function, selective differences do exist in the pathway of action of receptor molecules which produce the effects unique to the hormones they represent. It has long been estabilished that a major factor determining hormone response, is the specificity of ligand-receptor interaction. The cross-species specificity indicates that the receptors from different species are functionally similar (King, 1987).

The concept of steroid receptor activation developed along with the initial studies on the identification of estrogen receptors (ER) in the hormone target organs (Jensen and DeSombre, 1973; Shyamala and Gorski, 1969, Mohla *el al,* 1972). Sucrose density gradient analysis of the 3H-estradiol-receptor complex showed the presence of two species of cytosolic ERs, a 8S form in low salt gradients and a 4S species in high salt gradients. The rapid transformation of the inactive 4S form to the active 5S form was found to occur as a consequence of hormone binding to the receptor. The process of transformation of the inactive ER to the active ER has been referred to as 'estrogen receptor activation' (Grody *et al..,* 1982). The intermediate mechanisms involved in the receptor activation process have been subjects of intensive research.

INTERACTION OF STEROID RECEPTORS WITH THE HEAT SHOCK PROTEIN 90 (hsp 90)

In the absence of specific ligands, steroid hormone receptors are considered to exist

in an inactive state *in vivo.* Exposure of receptor *in vitro,* in isolated cells or hormone deficient animals, to the hormone under study results in rapid transformation of the inactive receptor to its active state. Before activation the receptors form large oligomeric complexes (7-10S) that are incapable of binding to DNA. This inactive state of the receptor was proposed to be maintained by the association of the receptor with other proteins. Several proteins have been identified. A 90 kDa protein was found associated with all the steroid hormone receptors (Ratajczak *et. ai,* 1988; Sabbah *et. ai,* 1989; Catelli *et. ai,* 1985a; Schuh *et. ai,* 1985 Carson-Jurica *et. ai,* 1989; Kost *et. ai,* 1989; Denis *et. ai,* 1987). It was identified by biochemical and immunological techniques (Joab *et. ai,* 1984; Catelli *et. ai,* 1985a; Sanchez *et. ai,* 1985) and also by cDNA sequencing (Catelli *et. al.,* 1985b) as the heat shock protein 90. The first concept that the untransformed oligomeric receptors contain hsp 90 came from the observations of Catelli *et. ai,* (1985). The non-transformed steroid receptors have a sedimentation coefficient of 8S which corresponds to an oligomeric complex of molecular mass of 250 to 300 kDa. A monoclonal antibody raised against the hsp 90 protein, BF4 was found to recognize all steroid hormone receptors of the 8S category. There is no disagreement in that when complexed with hsp 90, steroid receptors are unable to bind to DNA. Treatment of the receptor oligomeric complexes with salt or hormone *in vitro* results in the dissociation of the receptor-associated proteins, yielding a DNA binding receptor form. In addition, progesterone and glucocorticoid receptors were shown to form inactive complexes with hsp 90 when synthesized from mRNA in a cell free system (Dalman *et. ai,* 1989).

Chicken progesterone and human glucocorticoid receptors are capable of forming 8S complexes with hsp 90 derived from other species such as rabbit or monkey indicating that the interaction of the receptors with the hsp 90 is highly conserved through the

evolution of the steroid receptors (Howard *et. al.*, 1990).

Hollenberg and Evans (1988) have reported that the human glucocorticoid receptor has two regions of activation which can be blocked by its interaction with hsp 90 This result indicated that each molecule of the steroid hormone receptor interacts with two hsp 90 molecules.

The interaction of the receptors with hsp 90 is important not only in maintaining the receptor in an inactive configuration but also in maintaining an appropriate conformation required for the hormone binding activity of the receptor. A correlation was observed between the loss of hsp 90 from oligomeric glucocorticoid receptor complexes and the loss of the hormone binding activity (Bresnick *et. al.*,1989).

The role of hsp 90 in an endocrine perspective is a major unresolved proposition. It can be suggested that since hsp 90 has an 'unfoldase' activity in the transport of proteins across the membranes, it may modulate the newly synthesized receptors in such a way as to facilitate their entry into the nucleus. Binding of the ligand would bring about the conformational change leading to the exposure of the DNA binding regions of the receptors (Gustafsson *et. al.*, 1989). From these observations it is possible to assume that the hsp 90 plays a role in maintaining the steroid receptors in an unfolded form suitable for the passage of the receptors across the nuclear membrane (Pratt, 1990).

So far there are 3 functions of hsp 90 that have been proposed with respect to the steroid hormone receptors.

1). hsp 90 acts as an unfoldase enzyme.

2). hsp 90 stabilizes the receptor proteins.

3). hsp 90 plays a role in the transport of the receptor.

Recently to address the role of hsp 90 in hormone action, a strain of yeast was chosen in which hsp 90 expression was accessible for regulation. At low levels of hsp 90 the apo-receptors (unliganded receptors) seemed to remain mostly free of hsp 90, yet failed to enhance transcription of specific genes. Upon hormone addition, the receptors were activated but with markedly reduced efficiency. Thus hsp 90 did not seem to inhibit the receptor function solely by steric interference; rather, hsp 90 seemed to facilitate the subsequent response of the apo-receptor to the hormonal signal. This was the first biological evidence to show that hsp 90 acted in the signal transduction pathway for the steroid receptors (Picard *et. a/.,* 1990).

Besides hsp 90 several other proteins have been shown to be associated with the receptors. The avian progesterone receptor was identified to be associated with proteins of molecular mass 70,54,50 and 23 kDa in addition to the hsp 90. The 70 kDa protein was identified to be the hsp 70. hsp 70 was found associated with mouse glucocorticoid receptors (Smith *et. a/.,* 1990). Although other proteins have been identified in the oligomeric complex, the nature of these interactions and the evidence for the physiological significance of these proteins is not as convincing as it is for hsp 90 (Jurica *et. al.*, 1990). A 29 kDa protein was identified to be associated with the estrogen receptor and was recognized by the monoclonal estrogen receptor antibody (King, 1985). The p29 was examined to be quantitatively and qualitatively related to the estrogen-receptor complex. Immunoassay was proposed to be helpful clinically in steroid hormone dependent cancer (king, *et. al.*, 1986). This p29 differs from the hsp 90 in that the former was detected only in the ER positive cells (King, 1986).

IDENTIFICATION OF ESTROGEN RECEPTOR ACTIVATION FACTOR (E-**RAF)**

During the severities results from various laboratories (Notides and Nielson 1974, Thrower *el ai,* 1976, Yamamoto 1974, Yamamoto and Albert 1975) suggested that a DNA binding protein which is incapable of binding estradiol possessed the ability to convert the 4S estrogen receptor to the 5S receptor form. Thampan and Clark (1981) were the first to give direct experimental evidence for the existence of this protein in rat uterine cytosol. This protein was designated as the estrogen receptor activation factor (E-RAF). E-RAF was subsequently purified to homogeneity from the goat uterine cytosol (Thampan, 1987). In the goat uterine cytosol, the E-RAF was reported to exist as three molecular forms which were morphologically distinct but showed immunological cross-reactivity (Thampan, 1987; 1989). It was suggested that these three forms may be derivatives of one and the same protein as they showed identical tryptic and CNBr peptide maps (Zafar and Thampan 1993).

RECEPTOR SEQUENCE AND STRUCTURE

The molecular structures of the human ER (hER) and chicken ER (cER) have been studied extensively. The amino acid sequence revealed six regions of homology, A,B,C,D,E and F (Kumar *et ai,* 1987). Two of these regions, C and E, deserve special attention not only because they are highly conserved but also since they are the only two regions that are conserved between steroid receptor and non-steroid receptors (Green and Chambon, 1986). The region C (DNA binding domain), which is hydrophilic, has high content of cysteine, lysine, and arginine. This region has the sequences which are required for DNA binding. Deletion of this region resulted in the loss of the DNA binding capacity of the receptor (Kumar *et ai,* 1986). The attachment of the receptor to the major groove of the DNA is thought to be achieved via a projection from the DNA binding domain termed, as the "zinc finger". These are generated by the co-ordination of zinc ions with four cysteine residues (Green and Chambon 1987). Steroid receptors have two apparently unrelated zinc fingers encoded by separate exons (Huckaby *et. ai,* 1987) with four conserved cysteine residues. The two zinc fingers of ER were observeu to be present in the region C, which is analogous to the Xenopus transcription factor TF IIIA (Hartshorne *et. ai,* 1986). When hER region C was replaced with that of the human glucocorticoid receptor (hGR), the hybrid was shown to activate the glucocorlicoid responsive gene (Green and Chambon, 1987), suggesting that the region C determined the target gene specificity. Besides its involvement in the target gene specificity, it was also reported to be essential for activation of transcription, as demonstrated by deletion mutant experiments (Kumar *et. ai,,* 1988). Region E is more hydrophobic than the remainder of other regions. This region was identified to be the hormone binding domain (Kumar *et. ai,* 1986). A mutant of hER (HE15), which lacks the hormone binding domain, binds to DNA but activates transcription poorly (Kumar *et. ai,* 1986, 1987). Expression of the regions E and F, produces a protein which binds estradiol in a manner

similar to that of the intact receptqr, indicating that the hormone binding domain acts independently from the remainder of the receptor (Kumar *el. al.,* 1986). Webster *el. al,* (1988) demonstrated that the hormone binding domain could function independently as a transcription factor. The hormone binding domain is at the carboxy terminal, while the DNA binding domain is present near the amino terminal. Regions A and B are not conserved among the receptors and are present near the DNA binding domain (Kumar *et. al,* 1988).

The region D has 37 amino acids and functions as a hinge between the DNA binding and the hormone binding domains. Deletion and insertion experiments revealed no loss in the receptor function (Kumar *et. al.*, 1988).

NUCLEAR LOCALIZATION OF THE RECEPTORS

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

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

Immunoelectron microscopic localization of the estrogen receptor in rat uterine cells showed that the receptor was mainly nuclear but was also present in the cytoplasm (Vazquez-Nin *et. al.*, 1991). This is apparently a debatable topic as there is evidence in support of both the views. However, it has currently been accepted that the estrogen receptors are primarily localized within the nucleus

ESTROGEN RECEPTOR DIMERIZATION

The hormone binding domain of the steroid receptor has been shown to contain honnonally inducible transcription activation function (Giguere *et. ai,* 1986; Lees *et al.*, 1989; Rusconi and Yamamoto 1987; Webster *et. a/.,* 1988). It has been suggested that estradiol binding to the hormone binding domain of ER induces the formation of receptor dimers in solution (Fawell *et. al.*, 1990a, Linsteadt *et. al.*, 1986). The binding of the receptor to DNA or to the steroid responsive element as a dimer was proposed two decades ago by Yamamoto and Alberts (1972). The steroid responsive element (SRE) to which the receptor dimer binds have been shown to consist of inverted repeats in which the half sites are separated by 3bp. The consensus sequence of the repeats for estrogen responsive element (ERE) is T G A C C (Umesono and Evans 1989). The speculation that the nuclear receptors binds to the SRE as protein dimers originated from the observations that the responsive elements are palindromic sequences and have two fold rotational symmetry.

With the use of monoclonal antibodies, it was demonstrated that the receptor dimers are formed in the cytosol following heat transformation (Linsteadt *et. ah,* 1986). The studies carried out by Kumar and Chambon (1988) provided direct evidence for the formation of homodimers of human estrogen receptors as a requirement for its binding to ERE. The binding of glucocorticoid receptor to DNA as a homodimer was

also reported by Tsai *et. al.*, (1987) and Wrange *et. al.*, (1989).

Estrogen receptor contains both estrogen induced and constitutive dimerization domains. The binding of hormone favors the stable homodimer configuration and therefore it may be predicted that the hormone binding domain contains an efficient estrogen inducible dimerization domain. Whereas the DNA binding domain does nol have such a strong dimerization domain dimers might also be formed in the absence of the hormone binding domain. Since the dimer-DNA complexes were formed irrespective of the presence of the A/B region it was suggested that a weak constitutive dimerization domain must be present within the minimal region that is required for DNA binding (Green *el. al.*, 1988; Kumar and Chambon 1988).

Additional insight into the ER dimerization phenomenon has been obtained through the reports by Fawell *et. al.*, (1990b). Single amino acid substitution in the region E of the mouse ER prevented the receptor dimerization. Point mutation in this region abolished hormone binding, indicating that the steroid binding and the dimerization domains overlapped. Fawell *et. al.*, (1990b) made a comparison of the steroid receptors with uteroglobin, a progesterone binding protein secreted by the rabbit endometrium. X-ray crystallographic studies revealed that the uteroglobin monomers, comprising four irregular regions of a helix and a turns dimerize to form a globular protein with two fold symmetry and create an internal hydrophobic site to which single molecule of progesterone binds (Mornon *et. al.*, 1980; Morize *et. al.*, 1987; Bally and Delletre 1989).

Fawell *et. al.* (1990b) have identified a region within the steroid binding domain of mouse ER that is required for both DNA binding and dimerization. The mutant that failed

to bind estradiol dimerized and bound to DNA with high affinity *in vitro* suggesting that the steroid binding was not a prerequisite for DNA binding. These results contradicted the earlier reports on human ER expressed in HeLa celts where dimerization was found to be consequent to estradiol binding (Kumar and Chambon, 1988).

It was shown that the inhibition of ER binding to DNA by anti estrogen was mediated by impaired dimerization of the receptor (Fawell *et. ai,* 1990b). This result suggested that the anti estrogen-ER complex may have an altered tertiary structure (Geier *et. ai,* 1987). Mobility shift assays carried out in the presence of anti hormone-receptor-DNA complex and hormone-receptor-DNA complex showed that the anti hormone-receptor-DNA complex migrated faster than the hormone-receptor-DNA complex, indicating that the former has lower affinity for binding to the DNA when compared to the latter (El-Ashry *et. ai,* 1989). This concept was further supported by the results of experiments carried out by Baniahmad and Tsai (1993). They used a limited proteolytic digestion method in order to analyze the conformational change in the steroid receptor (estrogen, progesterone and androgen) upon binding either the hormone or the anti hormone. Distinct change was noticed in the pattern. Thus it was concluded that the natural ligand of a steroid receptor induces a dramatic conformational change in the receptor compared to the ligand-free form. The conformational change induced by the anti hormones, however, is different (Baniahmad and Tsai 1993). The conformational change was identified to take place in the C-terminus, hormone binding domain.

Results of the mobility shift assay and the proteolytic digestion experiments supported the hypothesis that anti hormones blocked transcriptional activation by inducing a different structure. This emphasizes the fact that a specific conformational change of the receptor has to precede the receptor-mediated transcriptional activation.

GENE REGULATION BY STEROID HORMONE RECEPTORS

Binding of the steroid to specific genomic loci was first demonstrated in Drosophila, where it was found that anti ecdysone antibodies bound to specific chromosomal puff sites at which transcription was stimulated by ecdysone (Groneineyer and Pongs, 1980). The hormone response is mediated by binding of the ligand to its receptor followed by the binding of the receptor-ligand complex to specific responsive elements (Yamamoto, 1985). The transcriptional control appears to be achieved through the interaction of trans-acting protein with cis-acting DNA promoter elements which may result in a negative effect (Ptashne, 1986). The consensus sequence for estrogen responsive element (ERE) (Beato, 1987; Umesono and Evans, 1989), has been shown below:

A G C T C A nnn T G A C C T

The palindromic nature of the estrogen responsive element suggests that the receptor binds to ERE as a dimer. Analysis of the estrogen receptor binding to ERE reveals that estrogen receptor binds to its responsive element as a homodimer (Kumar and Chainbon, 1988). However the mechanisms by which the receptor regulates gene transcription remains largely unclear. /// *vivo* transcription from a target promoter regulated by a transcription factor belonging to the steroid receptor superfamily was first described for COUP-TF (chicken ovalbumin upstream promoter transcription factor) (Sagami *et. al.,* 1986). This receptor stimulated the synthesis of RNA from an ovalbumin promoter in HeLa cell nuclear extracts. Kalff *et. al.,* (1990) and Freedman *el. al.,* (1989) showed that the rat glucocorticoid receptor DNA binding domain expressed in E.coli and progesterone receptor isolated from the rabbit uterus stimulated transcription of an MMTV promoter in a cell free system. Subsequently a cell free system was developed for other steroid receptors including the human glucocorticoid receptors (Tsai *et. a/.,*

1990) and mouse ER (Elliston *et. al.,* 1990).

A hormone dependent cell free transcription system was first reported by Corthesy *et. al,* (1988). The sole addition of estradiol stimulated RNA synthesis from a vitellogenin promoter in Xenopus liver nuclear extracts. Similar results were obtained with human and chicken PR expressed in Baculovirus system (Elliston *et. al.,* 1992). Thus analogous to the *in vivo* system, it is possible to activate gene transcription in a cell free system in both hormone dependent and receptor dependent manners.

The activation by estrogen of silent vitellogenin genes in Xenopus liver has proved to be a valuable model for understanding the mechanisms of steroid hormonal regulation of gene expression (Tata and Smith, 1979). Both triiodo thyronine and dexamethasone rapidly up-regulate ER mRNA in primary cultures of Xenopus hepatocytes (Ulisse and Tata, 1994). These results indicate the importance of interplay between different ligands of the steroid/thyroid hormone on the expression of the gene encoding one of the members of this superfamily (Ulisse and Tata, 1994).

The mechanism of transcriptional activation by steroid hormone receptors is largely unknown. The basal transcription factor TF IID (TBP) has been implicated as a target for transactivation (Greenblatt 1991). Recent results show that the binding of TF IIB appears to be the rate limiting step in the process of transcription activation in a system of HeLa cell nuclear extracts containing E.coli derived GAL4-AH (Lin and Green 1991). These results suggest that both TF IIB and TF IID may be targets for gene activation. It was shown that PR, GR and ER stabilize the formation of the pre-initiation complexes on the DNA (Klein- Hitpass *et. al.,* 1990; Tsai *et. al.,* 1990; Elliston *et. al.,* 1990). This suggests that the members of steroid receptor superfamily interact specifically with general

transcription factors including TF IIB and TF IID. This type of interaction may facilitate the process of transcriptional activation of responsive genes.

Although significant advances have been made several questions regarding the regulation of the gene transcription by steroid hormone receptors remain unresolved. One major question, how does the interaction with the basal transcription factor activate gene transcription?' is yet to receive a satisfactory answer.

NUCLEAR TYPE I AND TYPE II ESTROGEN RECEPTORS

For several years investigators have suggested that at least two forms of estrogen binding sites exist in the mammalian uterus (Erdos *et. al.,* 1969). One of the sites, the regular ER was studied extensively, was cloned and sequenced. This site was designated as the type I site. The other binding site designated as type II site received relatively very little attention.

Saturation analysis of estradiol binding in rat uterine nuclei using a wide range of tritiated estradiol concentration revealed a complex curve for specifically bound estradiol (Eriksson *et. al.,* 1978). The Scatchard plots analysis of the respective saturation binding data indicated the involvement of two binding sites. The type I site with high affinity for binding estradiol and the type II site with a relatively reduced affinity for estradiol. The type II was shown to have considerably higher estradiol binding capacity than the type I site. The presence of the type II in the nucleus interfered with the measurement of the type I sites.

The nuclear type II sites appeared to be different from the cytosolic type II. They differed in their Scatchard plots and the respective patterns of saturation binding of estradiol (Clark *et. al.,* 1979).

Based on the data available the differences between the type I and the type II sites may be presented as given below:

1). Type I is a DNA binding protein where as the type II is a non-DNA binding protein. The type II remains bound to the nuclear proteins, especially the RNA polymerases.

2). The type I differs from the type II in the estradiol binding parameters. The type I requires lower concentration of estradiol when compared to the type II in order to saturate the binding sites.

3). Type I displays a saturation curve which is a parabola and a linear Scatchard plot while type II displays a sigmoidal saturation curve and a curvilinear Scatchard plot.

Eventhough a partial purification of the nuclear type II estrogen receptor was reported recently (Markaverich and Gregory 1991), its purification to homogeneity was achieved for the first time in our laboratory (Karthikeyan and Thampan, 1994a).

The functional significance of the type II site is still not clear. The nuclear type II sites in the rat uterus was found to be associated with the nuclear matrix (Clark and Markaverich, 1982). This preliminary information suggested that the type II sites were possibly involved in DNA replication (Pardoll *el. al.*, 1980).

It was observed earlier (Thampan, 1989) that two distinct classes of nuclear estrogen receptors were associated with the rat uterine nuclear fractions containing RNA polymerases. Based on the data published by Clark and his colleagues, it appeared that the receptor activities associated with RNA polymerases possessed both type I and type II characteristics (DNA and non-DNA binding nature). But, predominantly the receptors were identified to be of the type II category (Thampan 1989). This result

further supported the concept that the type II sites interact with the nuclear proteins and not to DNA.

There are many instances to show that regulation of transcription can be afforded by phosphorylation of general transcription factors and also of subunits of RNA polymerases (Corden *et. al.*, 1990). The C-terminal domain (CTD) of the large subunit of the RNA polymerase II, can be phosphorylated at multiple sites (Edwards *et. al.,* 1990; Kolodziez *et. al.,* 1990). It has been proposed that phosphorylation of this subunit may be needed to trigger conversion of an initiation complex into an elongation complex (Laybourn and Dames 1990). It was also shown that the CTD bound to the TF IID subunit TBP and also that this interaction was blocked by phosphorylation of the CTD (Usheva *et. al.,* 1992). This provided a potential mechanism for releasing the polymerase from the initiation complex before the start of transcription.

NON-ACTIVATED ESTROGEN RECEPTOR:

Experimental results originated from our laboratory (Anuradha *et. al.,* 1994) showed the presence of a distinct class of cytoplasmic estrogen receptors which was designated as non-activated estrogen receptor (naER). The naER was identified to be a tyrosine kinase. It is a high affinity estrogen binding protein with no capacity to bind to DNA. Its high affinity for binding estradiol and its specificity for estrogens supported the view that naER is an estrogen receptor. This naER is different form proteins bearing similar names in the literature (Sica *et. al.,* 1976; Atrache *et. al.,* 1985; Redenilh *et. al.*, 1987) in that it does not get activated' during salt-dependent or temperature dependent reactions. It gains access to the DNA only upon dimerization with the estrogen receptor activation factor (E-RAF), a DNA binding protein localized in the cytoplasm. In short, it is a `never activated' receptor unless it dimerizes with the E-RAF. The regular receptor,

on the contrary, fails to dimerize with the E-RAF (Thampan, 1987).

The physical characteristics of naER were compared with those of the regular ER. It has a molecular mass of 66 kDa, sediments at 4.2S and has a Stokes radius of 360A. CNBr peptide map analysis revealed that the primary structure of naER was distinctly different from that of the regular estrogen receptor (Anuradha *et. al.,* 1994).

HETERODIMERS IN THE STEROID RECEPTOR SUPERFAMILY:

Receptor heterodimers have been identified in the actions of thyroid hormone, retinoic acid and vitamin D3 and also in the viral onco-proteins fos and jun which act as transcription factors.

Thyroid hormone receptors belong to the same super family of steroid, vitamin D and retinoic acid receptors (Evans, 1988; Beato 1989). There are two major classes of thyroid hormone receptor isoforms, TRa and TRβ, encoded by separate genes (Evans 1988; Lazar *el. al.,* 1990). These isoforms bind to specific DNA sequences in target genes called thyroid hormone responsive elements (TRE) as monomers and dimers (Forman *el. al.,* 1992; Baniahmad *el. al.,* 1990; Holloway *et. al.*, 1990; Lazar *et. al.*, 1991). TR binding to TRE can be enhanced by heterodimerization with thyroid hormone receptor auxiliary proteins (TRAP) (Murray et.al., 1989; Darling *et. al.*, 1991). Retinoic acid receptors (RAR) and retinoid-X-receptor (RXR) also heterodimerize with thyroid receptor and enhance the binding of the latter to TRE (Yu *el. al.*, 1991; Zhang *el. al.,* 1992) Physiological concentrations of triiodo thyronine (T3) decrease the binding of TRa and TRb homodimers to TRE, but does not affect the TR-TRAP heterodimer binding to TRE (Yen, *et al.*, 1992b). In a similar way triiodo thyronine decreases the binding of T3 receptor homodiemrs or

retinoic acid receptor homodimers but not the T3-receptor-retinoid X receptor heterodimer to TRE (Yen *et. al.,* 1992a).

The three genes which encode the high affinity retinoic acid receptors (RAR) have been identified and termed as a, β and *y* (Ginguere *et. al.,* 1987; Petkovich *et. al.*, 1987; Brand *et, al.,* 1988; Krust *et. al.,* 1989; Zelent *et. al.,* 1989). Recently another class of retinoic acid receptors have been identified, which display low affinity for retinoic acid (Manggelsdorf *et. al.,* 1990; 1991), referred to as retinoid X receptors a (RXRa). The binding of retinoic acid receptor to its responsive element requires a co-regulator termed as RXRp, that increases the DNA binding capacity and the transcriptional activity of RAR RXRa also heterodimerizes with thyroid receptor and vitamin D receptor increasing both the DNA binding and transcriptional activity (Yu *et. al.,* 1991)

Vitamin D3 binding to its responsive element requires an additional nuclear protein, nuclear accessory factor having a molecular mass of 59-64 kDa. The accessory factor-dependent formation of DRE-vitamin D heterodimer complex was independent of exogenous vitamin D3 (Ross *et. al.*, 1992; Liao *et. al.*, 1990).

fos and jun, the products of nuclear proto-oncogenes form a non covalent association that was reported by several research groups (Rauscher *et. al.,* 1988; Sassone-Corsi *et. al.,* 1988). Cross family dimerization was also reported in these transcription factors by Hai and Curran (1991).

naER-E-RAF COMPLEX: THE ONLY KNOWN **HETERODIMER IN ESTROGEN ACTION:**

The naER has no capacity to bind to DNA. It gains access to DNA only when it forms a heterodimer with E-RAF, a DNA binding protein (Anuradha *et. al.*, 1994). This

heterodimer may exists as a system parallel to the regular ER homodimers in the mammalian uterus. The naER was identified to be a tyrosine kinase, while E-RAF was recognized as a serine kinase (Anuradha *et. al.*, 1994). The total disappearance of the protein kinase activities when the two protein dimerize indicates the necessity for the constituents of the heterodimer to dissociate within the nucleus. Theoretically, a phosphorylated protein has less affinity for DNA when compared to the non phosphorylated protein. The question, where does the protein kinase activities of the two proteins manifest? is yet to be answered. Two possibilities exists: 1). As independent entities they are probably involved in the phosphorylation of certain cytosolic proteins. 2). The constituents of the heterodimer dissociate, after reaching the nucleus, so that each protein could carry out its own independent protein kinase activities. The data presented in this thesis favors the latter possibility. The E-RAF was already shown to have the capacity to enhance transcription *in vitro* (Thampan 1989). The exact functional significance of this heterodimer is yet to be understood.

STEROID HORMONE EFFECTS ON THE PLASMA MEMBRANE

The identification of nuclear estrogen receptors and the receptor mediated pathway of steroid hormone action is not sufficient to account for all the known effects of steroids. Baulieu and co-workers (Baulieu *et. at.,* 1978), have, for example, demonstrated that progesterone and other steroid molecules are able to promote the maturation of Xenopus laevis oocytes, although these cells do not have any steroid receptors. They suggested that progesterone may trigger its action by interacting with the cell membrane. It thus appeared that, in addition to the receptor-mediated mechanism, steroids may also operate through other mechanisms and in particular through an effect on plasma membrane.

It appears that most of the steroid effects on membrane properties are not recognized as the direct effects of the steroids, but merely as receptor-mediated actions (Duval *et. ai,* 1983). The various effects of steroids on the membrane composition and functions has been reviewed by Duval *et. ai,* (1983). The fact that steroids may influence the biophysical properties of membranes was pointed out by Mueller, Rudin and their colleagues (1962, 1968). They showed that the experimental bilayers had physicochemical properties similar to the barrier structure of cell membranes. Among the direct effects of steroids on the membrane may be the effects on cell membrane fluidity (Lawrence and Gill 1974). Heap *et. ai,* (1970) showed changes in the membrane permeability to potassium ions, under the influence of certain steroids, especially testosterone.

There is some evidence that steroids particularly glucocorticoids alter membrane sugar synthesis. Ramachandran *et. ai,* (1981) described a system wherein stimulation of fucose and manose incorporation into membrane glycoproteins was achieved following treatment of HeLa cells with dexamethasone.

Little is known about the regulation of synthesis of membrane proteins by steroids. Geheb and co-workers (1981) showed that aldosterone enhanced the synthesis of lour to six membrane proteins in toad bladder cells, this induction was inhibited in the presence of actinomycin D. Manipulations of steroid levels have also been shown to induce a modulation of numerous peptide hormone receptors including those of prolactin, thyrotropin releasing hormone, gonadotropins and others (Duval *et. ai,* 1983).

Aldosterone has been recognized as one of the factors controlling the sodium transport in vertebrates (Edelman and Marver 1980). Johnson and co-workers (1982)

have shown that administration of dexamethasone to adrenalectomized rats induced a significant increase in the urinary potassium excretion without simultaneously altering sodium excretion (Johnson *et. al.,* 1982) Estradiol in addition to its effect on uterine water permeability, was shown to increase sodium uptake (Spaziani and Szego 1989).

Several years ago Baulieu and co-workers (Baulieu et.al., 1978) demonstrated that progesterone and other steroids could promote the maturation of Xenopus laevis oocytes by increasing the intracellular calcium concentration in these oocytes (Moreau *et. al.,* 1980; Pietras and Szego, 1975). There is also evidence to show that in several other tissues for the occurrence of steroid mediated calcium transport (Pietras and Szego, 1975). A decreased calcium uptake by myometrial cells in the presence of diethylstilbestrol (DES) was demonstrated by Batra and Bengtsonn (1978). Pietras and Szego (1975), on the contrary, reported a very rapid stimulation of calcium uptake by endometrial cells in the presence of estradiol. The exposure of membranes to estradiol led to the entry of heterologous substances (Szego and Pietras, 1984). This suggested **that a** mechanism, closely linked to a receptor internalization, may be responsible for the entry of these heterologous substances.

Recently Morley and co-workers (Morley *et. al.,* 1992) investigated the effects of steroids on the intracellular calcium ion concentration, in chicken granulosa cells. They observed an immediate 4 to 8 fold increase in intracellular calcium after the exposure of 10-7 M estradiol. The striking rapidity of this estrogen induced internal Ca^{+2} mobilization was observed to be consistent with the activation of a cell surface receptor which is different from the conventional slow acting, gene-stimulating nuclear estrogen receptor. Morley *et.al.,* observed that the estrogen mediated calcium mobilization was not blocked by actinimycin D or cycloheximide. This indicates that the estrogen triggered

Ca^{+2} response was not related to the conventional gene activating nuclear estrogen receptor (Morley, *et. ai,* 1992).

Many membrane enzyme activities are affected by steroid treatment. Na-K-dependent ATPase activity, which represents a major system of transport for sodium and potassium ions is decreased by estrogens in the rat uterus and liver, but increased by aldosterone in the kidney cells. Calcium dependent ATPase activity was shown to be reduced in the rat liver membranes following castration (Duval *et. ai,* 1983).

The various effects that steroids exert on membrane properties showed that in many cases, they represent `classical' steroid actions. Adaptation of the organisms could also be achieved through the proliferation or differentiation of the cell types. This process which is in part under the control of hormonal factors could promote the emergence of cells with different membrane phenotypes.

DEBATE ON THE KINETICS OF ESTRADIOL ENTRY IN THE UTERINE **CELLS**

The mechanism of entry of estrogens into uterine cells is currently ill defined. It would appear that steroids, due to their non-ionic, lipophilic nature, can freely diffuse through cell membranes. Peck *et. ai,* (1973) in their studies on the uptake and retention of estradiol by the uterus and diaphragm concluded that the entry of estradiol into target cells occurred by passive diffusion Williams and Gorski (1973), however, demonstrated that a considerable amount of hormone adsorbed to the tissue and did not enter the cells.

Though it has been generally accepted that the steroids diffuse freely into cells, there are evidences to indicate that this may not be the case under in vivo conditions.

Szego and Pietras (1981) argued in an extensive review that in steroid concentrating cells steroids are internalized by binding to specific macromolecules on the cell membrane. This view was supported by the work of Baulieu and his colleagues (Milgrom *et. al.*, 1973). They demonstrated that the entry of estrogens into the rat uterus was sensitive to SH-blocking agents. However estradiol binding also depended, though to a much reduced degree, on the integrity of the SH groups. However, it was observed that estradiol binding by the solubilized receptor was unchanged by iodoacetamide, while the process was decreased when intact uterine horns were incubated with estradiol (Jensen *et. al.*, 1967).

Apart from these experimental observations, the entry process of estradiol and other steroids was noticed to be a saturable phenomenon and also specific for the hormone under study (Milgrom *et. al.*, 1973). The most probable explanation for this, is the existence of a protein mediated process regulating the passage of estrogen through the membrane. Occurrence of protein-mediated transport of glucocorticoids has been described in fibroblasts (Gross *et. al.*, 1970).

ARE THERE STEROID RECEPTORS ON CELL MEMBRANES?:

Steroid binding to isolated plasma membranes has been analyzed by numerous workers, but has led to unreliable and conflicting results. This was illustrated by Pietras and Szego (1979). One of the major problems encountered by this type of study is the development of a well defined plasma membrane fraction. This necessity was discussed by Pietras and Szego (1979). It thus appears that many of the reports devoted to membrane receptor determinations are not fully conclusive, because they often lack data on the membrane integrity and purity.

Nenci and co-workers (1980;1981) using a cytochemical approach with fluorescent estrogen analogous bound to serum albumin, have presented evidence in favour of the presence of estrogen binding sites in the plasma membranes of human breast cancer cells. These binding sites were identified only in the target cells and not in other cell types. These sites displayed identical characteristics as that of the estrogen receptors (in high affinity, saturable binding to the ligand, etc., Nenci *et. al.*, 1980; 1981). Similar results were also reported by other in the mammary tumor cells and Xenopus oocytes (Sadler and Mailer 1982). There are several reports which support the existence of such a surface receptor (Bression, *et.al.*, 1986; Pietras and Szego, 1977; 1979; 1980).

There has not been any convincing observations in other laboratories confirming these findings of Szego and co-workers. Further, no additional information has been generated by Szego's laboratory, in the recent past, substantiating her earlier findings.

SCOPE OF THE THESIS

Our laboratory has, for the past several years, been studying the role of a DNA-binding protein of the goat uterus, the estrogen receptor activation factor (E-RAF) in estrogen action. Experiments aimed at the isolation of the estrogen receptor that dimerized with this protein resulted in the purification of a form of estrogen receptor that did not possess the capacity to bind to DNA on its own. This protein was designated as the non-activated estrogen receptor (naER). In the heterodimer thus formed, E-RAF bound to the DNA while the naER bound estradiol Experimental evidences indicate that entry of one of these proteins into the nucleus is strictly dependent upon its dimenzation with the other.

Among the structural and functional characteristics of the naER there are many in which the naER resembles the regular ER while remaining distinctly different in many others. Experimental data belonging to both the categories are being presented in the thesis

During the studies aimed at the purification and characterization of the nuclear estrogen receptor of the goat uterus, it was observed that the nucleus contained, in addition to the regular nuclear estrogen receptor an alternative form that did not bind to the DNA. Experimental data are presented here to support a hypothesis that this nuclear estrogen receptor, the R-II, is a transformed variety of the cytosolic naER. The basic biochemical mechanisms involved in this transformation are being examined.

The experimental work presented here raises a question as to the nature of origin of this alternative form of estrogen receptor. Taking into consideration the immunological relationships and identical molecular size and mass of naER, E-RAF and ER and the identical affinity specificity of estrogen binding by the naER and the ER, it appears logical

to point out that the naER, the regular ER and the E-RAF could have evolved from a common pre-mRNA through alternative splicing. No attempt has been made in this thesis in order to examine the authenticity of the hypothesis. It is hoped that information on this matter will be made available sooner than latter

CHAPTER II

MATERIALS AND METHODS

MATERIALS

2,4,6,7 [3H]-estradiol-17β (sp.act.101Ci/mmol) was obtained from Amersham.γ-32p adenosine 5'-triphosphate (sp. act. 3000 Ci/mol) was purchased from Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre, Bombay. Adenosine 5'-triphosphate (ATP) was purchased from Boehringer Mannheim. Phenylmethyl sulphonyl fluoride (PMSF) and silver nitrate were obtained from Merck (Germany). Diethylstilbestrol (DES), quercetin, 2,5,-diphenyloxazole (PPO),1,4,-bis 2,5-phenyloxazoyl benzene (POPOP), dithiothreitol (DTT), estradiol-17p, testosterone, progesterone, dexamethasone, tamoxifen, Triton X-100, Tyrphostin 25, Nonidet P-40, 4-chloro-1-naphthol, phosphoaminoacids, Tween 20, bovine serum albumin (BSA), methyl αD-glucopyranoside, heparin disodium salt, fluorescein iso thio cyanate (FITC) isomer I, adenosine 5'-monophosphate, cytidine 5'-monophosphate and glycopeptidase F (N-glycopeptidase F; EC. 3.2.2.18 and 3.5.1.52) were purchased from Sigma Chemical Co., St. Louis, U.S.A.. Sepharose 4B, Sephadex G-100, G-50 and G-25 were purchased from Pharmacia Fine Chemicals, Sweden. p-amino benzamidine agarose was purchased from Pierce Chemical Co. DEAE cellulose (DE-52), cellulose cc31 and phosphocellulose were purchased from Whatman Biosys. Ltd., England. Nitrocellulose membranes were purchased from Schleicher & Schuell Inc., U.S.A. X-ray film was obtained from Konica and Solidex X-ray developer from May and Baker, Photochem, India; fixer was the acid fixing salt with hardener purchased from Kodak, India. Hydroxylapatite (Bio Gel HTP) was purchased from Bio-Rad. A mouse monoclonal antibody (Mab 17) and a polyclonal antiserum (SB 208) that specifically recognizes the estrogen receptor were a kind gift of Dr. Richard J. Miksicek (Dept. Pharmacol. Sci., State Univ. of NY, Stony Brook, NY). Mab 17 has been shown to recognize an aminoterminal epitope (34-48 amino acids) of the human estrogen receptor. The polyclonal antiserum recognizes multiple determinants (Neff, et.al., (1994) Mol. Endocrinol. 8; 1215-1223). Goat anti rabbit IgG, horse radish peroxidase coupled anti rabbit IgG, horse radish peroxidase and concanavalin A were obtained from Banglore Genei, Banglore. All other chemicals and reagents used were of analytical grade, obtained from local commercial establishments.

BUFFERS USED IN THE STUDY

Buffers **used in the isolation of nuclei:**

Buffer A; 50mM Tris-HCl, pH 7.6, containing 1mM $CaCl_2.2H_2O$, 2mM $MgCl_2.6H_2O$, 20mM KCl, 0.2mM phenyl methyl sulphonyl fluoride (PMSF) and 0.25M sucrose.

Buffer B: Same as bufter A except for sucrose which is 0.34M sucrose.

Buffers used in the purification of hsp **90:**

+MO buffer: 10mM potassium phosphate buffer pH 7.0 containing 10mM sodium molybdate, 12mM monothiglycerol and 0.2mM PMSF.

-MO buffer: Same as +MO buffer except that, the buffer did not contain any molybdate **salt.**

Buffers used in the receptor purifications:

TEM buffer: lOmM Tris-HCl, pH 7.6, containing 1mM EDTA, 12mM monothioglycerol and 0.2mM PMSF.

TEMN buffer: TEM buffer containing 50mM NaCl.

TCMN buffer: lOmM Tris-HCl, pH 7.6, containing 0.25M sucrose, 1mM CaCl2, 2mM MgCl2, 12mM monothioglycerol, 50mM NaCl and 0.2mM PMSF.

Buffers used in **the** purification of RNA **polymerase** II:

Buffer 1: 50mM Tris-HCl, pH 7.9, containing 20mM EDTA, 0.125% p mercaptoethanol, 25% glycerol, 0.2mg/ml soyabean trypsin inhibitor and 0.2mM PMSF.

Buffer 2: Same as buffer 1 except that the glycerol concentration was reduced to 10% and in addition it contained 0.2M ammonium sulphate.

Buffer 3: Same as buffer 1 except that 0.1mg/ml soyabean trypsin inhibitor was used and 0.25 mM DTT replaced Pmercaptoethanol.

Buffers used in **the** western blotting:

Towbin buffer: 25mM Tris buffer containing 192mM glycine and 20% methanol; pH was adjusted to 8.3 with Tris/glycine.

TN buffer: 10mM Tris-HCl, pH 7.6, containing 150mM NaCl.

TNT buffer: TN buffer containing 0.1% Tween 20.

Bradford's reagent:

10mg of coomassie Brilliant blue G-250 was dissolved in 5ml of ethanol. To this 10 ml of 85% (W/V) phosphoric acid was added. The resulting solution was made up to 100ml with DDW and was finally filtered through Whatman No. 1 filter paper.

TCMMN buffer: 10mM Tris-HCl, pH 5.0 containing 1mM each of $MgCl_2$, CaCl2 and MnCl2 50mM NaCI and 0.2mM PMSF.

Buffers used in plasma membrane isolation:

1). 1mM $NaHCO_3$ buffer pH 7.5.

2). Sucrose solutions of 0.25M, 2.0M, 63%, 49%, 41% and 37%, all made W/V in $NaHCO_3$ buffer pH 7.5.

METHODS

PROCUREMENT OF TISSUE:

Goat uteri were obtained from a local slaughter house, brought in ice to the laboratory and stored at -75°C until used in experiments.

PREPARATION OF GOAT UTERINE CYTOSOL:

Goat uterine cytosol was prepared as described by Van der Hoeven (1981). The uteri were minced finely. A 20% homogenate of the tissue was made in TEMN buffer using a Waring blender. The homogenate was filtered through glass wool and the filtrate was centrifuged at 15,000 X g for 15 min. The post-mitochondrial supernatant was collected. Polyethylene glycol· 6000 was added to the supernatant to a final concentration of 5%. The suspension was allowed to stir for 30min at 4oC. Following centrifugation at 15,000 X g for 15 min, the supernatant was collected and used as the cytosol.

The conventional method was used in the initial experiments for obtaining uterine cytosol. In this method, the post-mitochondrial supernatant was centrifuged at 1,05,000 X g for 2 hrs using a SW 28 rotor in a Beckman ultracentrifuge. The ensuing supernatant was collected.

ISOLATION OF NUCLEI:

The goat uterine nuclei were purified following the method described by Thampan (1985). Fresh uteri were used for the isolation of nuclei. The finely minced uteri were homogenized in 20 volumes of buffer A using a Polytron homogenizer (PT 45-80) at a setting of 4 (1 min X 5 with 5 min interval). The homogenate was filtered through

glass wool. The filtrate was centrifuged at 800 X g for 15 min. The pellet was treated with 0.1% Triton X 100 in buffer A. The pellet was washed twice with detergent free buffer A. The pellet was resuspended in 10 ml of buffer A, 5 ml of this suspension was carefully layered over 30ml of buffer B and centrifuged as before. The purified nuclear pellet suspended in buffer A, appeared to be free of any cytoplasmic contamination, when examined under a phase contrast microscope.

SOLUBILIZATION OF NUCLEAR MACROMOLECULES:

The nuclear pellet was suspended in TEM buffer containing 0.3M NaCl. This suspension was sonicated using a MSE sonicator fitted with a microtip (15 sec X 6 at a setting of 6 with an interval of 1 min). After sonication the material was centrifuged at 15,000 X g for 15 min. The concentration of NaCl in the supernatant was reduced to 0.05M following dilution with salt free buffer.

ISOLATION OF PLASMA MEMBRANE:

This was carried out following two different procedures.

PROCEDURE 1:

The method described by Yamashita and Field (1974) was followed. Buffers used in this procedure were: 1). 1mM $NaHCO_3$ buffer pH 7.5. 2). Sucrose solutions of 63%, 45%, 41%, and 37%, all made w/v in 1mM $NaHCO_3$ buffer pH 7.5.

Finely minced uteri were homogenized (20% homogenate) in the $NaHCO_3$ buffer using a Polytron homogenizer. The homogenate was kept in ice for 30 min. to allow cell lysis. The homogenate was filtered through nylon cloth of 80-120μ mesh and the filtrate was subjected to centrifugation at 1900 X g for 25 min. The resulting pellet containing

most of the plasma membranes and nuclei were suspended in 63% sucrose solution. The suspension was collected in centrifuge tubes (38ml capacity) of SW28 rotor in a Beckman ultracentrifuge). Sucrose solutions of 63% (12ml), 45% (9ml), 41% (11ml) and 37% (4ml) were layered successively over the sample. The tubes were centrifuged at 63,000 X g for 2hrs; plasma membranes with minimum contamination were obtained at the interphase between 37% and 41% sucrose solutions This band was collected and recentrifuged at 30,000 X g. The pellet was used for further analysis.

PROCEDURE 2:

The yield of the plasma membranes was very low from the procedure 1. Therefore another well cited procedure, described by Kidwai (1974), was followed. The buffers used in this method were: 1mM $NaHCO_3$, pH 7.5, containing either 0.25M sucrose or 2M sucrose.

The finely minced uteri were homogenized (20%) in 1mM NaHC03 buffer pH 7.5, containing 0.25M sucrose, using a Polytron homogenizer at a setting of 5 for 15 secs (x 5 with 60 secs intervals). The homogenate was filtered through a 80-120u nylon mesh. The filtrate was centrifuged at 7500 X g for 30 min. The resulting pellet was suspended in lmM NaHCO3 buffer containing 0.25M sucrose. This suspension was layered over a 0.25M to 2M sucrose density gradient. The tubes were centrifuged at 1,05,000 X g for 2.5hrs in a Beckman ultracentrifuge using a SW28 rotor. The plasma membrane formed a band at the interphase of the loading and the gradient. This band was collected using a Pasteur pipette, diluted to reduce the sucrose concentration and centrifuged again at 1,00,000 X g for 30min. The purified plasma membrane was collected as a pellet. This preparation was used in the subsequent hormone binding studies.

CYANOGEN BROMIDE ACTIVATION OF SEPHAROSE **4B AND LIGAND COUPLING TO** CNBr ACTIVATD SEPHAROSE 4B:

Cyanogen bromide activation of Sepharose 4B or agarose was carried out following the procedure described by March *et al* (1974). One volume of Sepharose 4B was washed with three volumes of double distilled water. The matrix was further washed with 1M sodium bicarbonate. The matrix was mixed with 1M sodium bicarbonate and was allowed to stir slowly. The speed of stirring was increased followed by the addition of 0.05 volumes of 2 mg/ml of cyanogen bromide solution in acetonitrile, all at once. After stirring the slurry vigorously for 15 min. it was transferred to a coarse sintered funnel and was washed with 10 volumes of 0.1 M sodium bicarbonate, pH 9.5. Ligand was dissolved in 1 volume of 0.2M sodium bicarbonate pH9.5. Coupling of the ligand was done for 24 hrs in the absence and 4 hrs in the presence of 1M glycine at 4oC. This matrix was washed with 20 volumes each of 0.1M sodium acetate, pH 4.0; 2M Urea and 0.1M sodium bicarbonate, pH 10.0. All the three solutions contained 0.5M NaCl. The gel was stored at 4oC as a suspension in the TEM buffer containing 0.04% sodium azide.

PURIFICATION OF HSP 90 AND PREPARATION OF HSP-90 SEPHAROSE:

The heat shock protein 90 was purified following the procedure described by Sullivian *et al.*, (1985). Goat liver cytosol was made in the +Mo buffer. The liver cytosol was stirred with 0.1 volume of phosphocellulose equilibrated with +Mo buffer which was then filtered under vacuum. The filtrate was collected on heparin agarose equilibrated with +Mo buffer. The matrix was stirred for 20 min. After washing the matrix-bound the proteins were eluted with three volumes of -Mo buffer. The heparin-

agarose eluate was mixed with DEAE Sepharose equilibrated with -Mo buffer and stirred for 20 minutes. The matrix was pelleted following centrifugation and was washed with 20 volumes of -Mo buffer. After packing the matrix in a glass column the bound-proteins were eluted with -Mo buffer containing 0.3M KC1. The eluate was collected as 2ml fractions which were subjected to absorbance measurement at 280 nm. The peak absorbance fractions were pooled and concentrated.

Further purification was achieved by resolving the proteins on a precalibrated column of Sephadex G-100. The fractions which contained hsp-90 were pooled, dialyzed and lyophilized. The purified hsp-90 (judged by SDS-PAGE) was coupled to CNBr activated Sepharose 4B as described earlier.

PREPARATION OF SINGLE STRANDED DNA SEPHAROSE COLUMN:

ISOLATION OF DNA:

The DNA was isolated following the method of Marmur (1961). Nuclei were isolated from goat liver following the method of Thampan (1985). The purified nuclei were suspended in saline EDTA containing 25% sodium dodecyl sulphate. The mixture was incubated for 10 minutes in a water bath at 60OC following which it was cooled. Sodium perchlorate was added to the viscous suspension to a final concentration of 1M. The suspension was mixed thoroughly with equal volume of chloroform-isoamylalcohol (24:1 v/v) in a ground glass stoppered flask for 30 min. The emulsion was centrifuged at 5000 X g for 15 minutes The upper aqueous phase was withdrawn carefully. Two volumes of ethanol was layered gently on the aqueous layer in order to precipitate the nucleic acids. The precipitate was dispersed in saline citrate and subjected to extraction with chloroform-isoamylalcohol (25:1 v/v) until very little protein remained at the interphase. The DNA was reprecipitated with alcohol.

COUPLING OF DNA SEPHAROSE 4B:

The DNA was covalently coupled to CNBr activated Sepharose 4B as described by March et al. The DNA was denatured before coupling was done. It was dissolved in 0.5 M NaOH, following which it was heated in a boiling water bath for 15 min. Equal volume of 0.4M sodium bicarbonate solution, pH 9.5, was added. The denatured DNA was coupled to one volume of CNBr activated Sepharose 4B. After coupling the gel was suspended in TEMN buffer containing 0.04% sodium azide and was stored at 4°C.

PREPARATION OF CONCANAVALIN A SEPHAROSE 4B:

Commercially available concanavalin-A was coupled to CNBr activated Sepharose 4B. 10mg of Con-A was coupled to 15ml of CNBr-activated Sepharose 4B as described by March *et al.*, (1974) After coupling the gel was suspended in Tris-HCl pH 7.6 buffer containing 0.04% sodium azide.

PREPARATION OF ANTI PHOSPHOTYROSINE IgG SEPHAROSE:

Phospho tyrosine was coupled to BSA using the glutaraldehyde method described by Philanjanmeni *et.al.*, (1985). The detailed method was described under the immunological method's section. The purified IgG was checked for its specificity for phosphotyrosine by western blotting. 30mg of IgG was coupled to 15ml CNBr activated Sepharose 4B as described by March *et al.*, (1974). The gel was suspended in TEMN buffer containing 0.04% sodium azide.

SEPHADEX G-100 CHROMATOGRAPHY FOR STOKES **RADIUS CALCULATION:**

A Sephadex G-100 column (66 X 1.8cm) equilibrated with TEMN buffer containing 0.3 M NaCl was used for the determination of Stokes radius of the receptor protein. The column was precalibrated with proteins of known Stokes radii (BSA, ovalbumin and cytochrome C). The protein samples, concentrated to 100µl, were layered carefully over the matrix. The column was developed with TEMN buffer containing 0.3M NaCl. 1ml fractions were collected and assayed for the receptor activity. The Stokes radius was calculated as described by Siegel and Monty (1966).

PREPARATION OF HEPARIN-SEPHAROSE:

Commercially available disodium heparin (60 mg) was dissolved in 0.2M sodium bicarbonate buffer and was coupled to CNBr activated Sepharose 4B (30ml) as described earlier.

ELECTROPHORETIC METHODS

TRICHLOROACETIC ACID PRECIPITATION OF PROTEINS:

To one ml of protein (~100 µg) solution was added 500µl of 100% TCA to obtain a final 30% concentration of the acid. The tubes were left at -20oC for 15 min. The sample were thawed and the proteins were precipitated following centrifugation at 5000 X g for 15 min. The precipitate was washed twice with 100% ethanol to remove the acid. The precipitated protein was dissolved in the SDS sample buffer for electrophoresis.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS:

SDS-PAGE was performed as described by Laemmli (1970). The following solutions and buffers were made for gel polymerization and electrophoresis.

1. **30% Acrylamide solution:** 30g of acrylamide and 0.8g of methylene-bis-acrylamide were dissolved in double distilled water (DDW) and the volume was made upto 100ml.

2. **Lower Tris (4X1:** 1 5M Tris-HCl, pH 8.8 buffer containing 0.4% SDS.

3. **Upper Tris (4X):** 0.5M Tris-HCl, pH 6.8 buffer containing 0.4% SDS.

4. **Reservoir buffer (4X):** 0.1M Tris buffer containing 0.8M glycine; pH was adjusted with Tris/glycine to 8.2.

5. Gel running buffer (X); 175ml of the reservoir buffer (4X) was diluted to 700ml along with 7ml of 10% SDS.

6. APS solution: 2% APS solution was prepared fresh immediately before use.

10, 12 and 15% gels were used in the studies.

NON-DENATURING GEL ELECTROPHORESIS:

Native gel electrophoresis was performed as described by Davis (1964). The following solutions were made in order to polymerize a native gel.

1. Buffered TEMED: 36.6g of Tris buffer was dissolved in 90ml of DDW and the pH was adjusted to 8.9 with HC1. Following the addition of 0.46ml of TEMED the volume was made upto 100ml.

2. Acrylamide solution: Prepared as described for the SDS-PAGE.

3. Electrode buffer (10X): 6g of Tris was dissolved along with" 28.8g of glycine in DDW. The volume was made upto 100ml.

4. Gel running buffer (X): 1:9 dilution was made with the electrode buffer using DDW.

5. Sample buffer: To 1ml of Tris-HCl, pH6.8, 1ml of glycine and 1ml of DDW containing 0.25mg bromophenol blue were added and mixed.

A 6% gel was polymerized by mixing the following solutions: 9.9ml of acrylamide, 4.5ml of buffered TEMED, 18.18ml of 0.2% APS solution and 17.4ml of DDW. The solutions were mixed and the gel was polymerized. The stacking gel was not made.

Samples for native gel electrophoresis were concentrated and made salt free by dialysis against 10mM Tris-HCl, pH 7.6. ^{3}H estradioi binding to the native receptor was carried out by incubating the receptor overnight at 4oC with 20nM ^{3}H estradioi before the mixture was loaded onto gels. After performing electrophoresis at 4oC, the gel was sliced into 3mm pieces which were then transferred to scintillation vials for radioactivity measurement using a solvent system of toluene and Triton X-100 (composition given else where).

ELECTROPHORETIC CONDITIONS:

SDS-PAGE was performed at constant current (30mA) and native gel electrophoresis was carried out at constant voltage (120volts).

SILVER STAINING OF GELS:

The gels were stained with silver nitrate following the method of Blum *et.al.*, (1987). The gels were transferred to the fixative solution which contained 50% methanol, 12% glacial acetic acid and 100µl of 37% formaldehyde/200ml. The gels were fixed for lhr and were transferred carefully to a container having 50% ethyl alcohol solution, to remove SDS from the gel. After washing the gel for 30 minutes, the gels were incubated for one minute with sodium thiosulphate (200mg/lit). The gels were washed thrice, each for a duration of 2min, and were then incubated with a solution of silver nitrate (2g/lit containing 0.7ml of 37% formaldehyde) for a period of 30 minutes. The gel, washed thrice with DDW for durations of 2min each, was developed with a

solution of sodium bicarbonate (60g/lit containing 0.7ml of 37% formaldehyde).

The stained gels were dried using a BIO-RAD gel drier.

PROTEIN PURIFICA TION METHODS

PURIFICATION OF NUCLEAR TYPE 1 AND **TYPE II ESTROGEN RECEPTORS:**

Purification of nuclear type I and type II estrogen receptors were done following the methods described by Karthikeyan and Thampan (1994a).

PURIFICATION OF NUCLEAR TYPE **1** ESTROGEN RECEPTOR:

The purified nuclei were sonicated in TEM buffer containing 0.3M NaCl, in order to solubilize the macromolecules. The sonicated nuclei were centrifuged at 10,000 X g for 15 minutes. The supernatant was collected and diluted to reduce the NaCl concentration. This diluted material was chromatographed over a ss-DNA Sepharose (25ml) column equilibrated with TEM buffer. The flow-through fraction from the ss-DNA column was collected and used in the purification of type II estrogen receptor. The ss-DNA Sepharose column was washed with 5 volumes of TEMN buffer. The DNA-bound receptor was eluted with TEMN buffer containing 10mM ATP. The ATP eluate was dialyzed extensively in order to remove the ATP.

The dialysate was chromatographed over a Whatman DE-52 column (20ml) equilibrated with TEM buffer. The column was washed with 5 volumes of TEM buffer. The DE-52 bound receptor was eluted with TEM buffer containing 0.2M NaCl. The eluate was collected in 2ml fractions, and subjected to DNA cellulose binding assay as

described else where in this section

Final purification of the receptor was achieved upon performing a gel filtration on a Sephadex G-50 column (50 X 1cm) equilibrated with TEM buffer containing 0.3M NaCl. The peak fractions showing the receptor activity were pooled and layered over a column of Sephadex G-50. The column was developed with TEM buffer containing 0.3M NaCl. The void volume which contained the highly purified receptor, was collected.

PURIFICATION OF NUCLEAR TYPE II ESTROGEN RECEPTOR:

Sodium molybdate was added to the ss-DNA Sepharose flow through fraction (from the previous experiment) to a final concentration of 10mM. This material was chromatographed over a hsp-90-Sepharose column (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed with the same buffer and the hsp-90-bound receptor was eluted with TEM buffer containing zero molybdate. The eluate was collected in fractions, HAP biding assay was carried out as described else where.

The fractions that displayed peak activity were pooled and rechromatographed on a column of phosphocellulose (20ml) equilibrated with TEM buffer. The column was washed with TEM buffer and the phosphocellulose-bound receptor was eluted with a 0-1M NaCl linear gradient in TEM buffer. The fractions collected were assayed for receptor activity using the HAP method.

PURIFICATION OF NON-ACTIVATED ESTROGEN RECEPTOR:

The naER from the goat uterine cytosol was purified following the method described by Anuradha *et.al.*, (1994), with slight modifications. Goat uterine cytosol was

prepared as described earlier following the method involving the use of PEG 6000 The cytosol was mixed with Whatman DE-52 (25ml) equilibrated with TEM buffer containing 0.3M NaCl. The matrix was washed twice with TEM buffer containing 0.3M NaCl. The DE-52 bound receptor was eluted with TEM buffer containing 0.5M NaCl. The eluate was diluted with salt free TEM buffer to reduce the NaCl concentration to 0.05M. Sodium molybdate was added to this to a final concentration of 10mM. The material was chromatographed over a column of hsp-90-Sepharose (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed extensively and the hsp-90 bound receptor was eluted with TEM buffer free of molybdate salt. The eluate collected in fractions was subjected to HAP binding assay. The fractions containing peak activity were pooled and the pooled material was rechromatographed over a column of phosphocellulose (20ml) equilibrated with TEM buffer to achieve final purification of the receptor. The naER was eluted from the phosphocellulose column using a 0 to 1M NaCl gradient in TEM buffer. The eluate collected was subjected to HAP binding assay

PURIFICATION OF PLASMA MEMBRANE ESTROGEN RECEPTOR:

The plasma membrane was isolated as described earlier. The purified plasma membranes were suspended in TEM buffer containing 0.3M NaCl and 0.2% NP-40 (v/v). This suspension was stirred for 20min at 4oC. The material was diluted with salt free buffer to reduce the NaCl concentration This suspension was centrifuged at 10,000 X g for 15 min and the supernatant containing the solubilized proteins was collected.

The supernatant was chromatographed over a Whatman DE-52 column (25ml) equilibrated with TEM buffer. The column was washed with TEM buffer. The DE-52

bound receptor was eluted using a linear 0-1M NaCl gradient. The fractions collected were assayed for the receptor activity. The fractions which contained the peak activity were pooled, dialysed and chromatographed over a hsp-90 Sepharose column (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed with TEM buffer containing 10mM sodium molybdate. The hsp-90 bound receptor was eluted with TEM buffer containing zero molybdate. The fractions collected were assayed for the receptor activity using the HAP method. The fractions that contained peak activity were pooled and chromatographed over a phosphocellulose column (25ml) equilibrated with TEM buffer The column was washed with TEM buffer and the phosphocellulose-bound receptors were eluted with a 0-1M NaCl linear gradient in TEM buffer. The fractions collected were assayed for the receptor activity using the HAP method. The fractions which contained the receeptor activity were analysed on a 10% SDS gel to judge the purity of the receptor preparation.

PURIFICATION OF ESTROGEN RECEPTOR **ACTIVATION FACTOR II (E-RAF II):**

The E-RAF **II** was purified following the procedure described by Thampan (1987; 1989). Goat uterine cytosol was prepared using PEG 6000 in TEMN buffer as described earlier. The cytosol was mixed with p-amino benzamidine Sepharose equilibrated with TEM buffer in a beaker kept in an ice bath for 20min. The unadsorbed fraction was collected carefully by decanting the solution. This fraction was mixed with 20ml of Whatman DE-52 matrix equilibrated with TEMN buffer. The DE-52 unbound fraction was collected and was chromatographed on a column of ss-DNA Sepharose (25ml) equilibrated with TEM buffer. The column was washed with TEM buffer extensively. The bound proteins were eluted with TEM buffer containing 10mM ATP. The proteins in the eluate were precipitated using ammonium sulphate at 70% saturation. The

protein pellet was redissolved in a small volume of TEM buffer and was dialysed against TEM buffer. The dialysate was mixed with 25ml of 60% hydroxylapatite (HAP) equilibrated with 10mM sodium phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF The HAP suspension was left in an ice bath for 20min. The HAP pellet was washed twice with 150mM sodium phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF. E-RAF II was cluted using 250mM sodium Phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF. The eluate was dialysed against 10mM Tris-HCl pH7.6 and the resulting protein appeared as a single band of 66 kDa when subjected to electrophoresis in 10% gels.

PURIFICATION OF DNA DEPENDENT RNA POLYMERASE II:

Goat uterine nuclear RNA Polymerase II was purified following the method described by Kim and Dahmus (1988).

Goat uterine nuclei were purified as described earlier. The purified nuclei were suspended in buffer 1. Triton X-100 was added to a final concentration of 0.25%. The suspension was allowed to stir for 10min at 4oC; while stirring polyethyeneimine (2µl/ml of 10% aqueous suspension) was added in drops. Stirring was continued for another 15rnin. at 4oC. The suspension was centrifuged at 8000 Xg for lOmin. The pellet was recovered and resuspended in buffer 1. Following centrifugation at 14,600 X g for 15 min. the pellet was collected and suspended in buffer 2. Ammonium sulphate concentration in the suspension was made up to 0.2M. The suspension was centrifuged at 16,300 x g for 15 min and the supernatant was collected. The RNA polymerase II was precipitated following the addition of 0.25g/ml of ammonium sulphate. The mixture was stirred for 15 min and the pellet was collected following centrifugation of the

suspension at 25,000 X g for 30 min. The final pellet was suspended in buffer 3 having a final ammonium sulphate concentration of 0.12M.

The suspension was mixed in a beaker with a 50ml suspension of heparin Sepharose (prepared as described earlier) equilibrated with buffer 3 containing 0.12M ammonium sulphate. This was allowed to stir for 1hr at 4oC. The matrix was filtered under vacuum and was washed with three volumes of buffer 3 containing 0.12M ammonium sulphate. The heparin-bound enzyme was eluted with buffer 3 containing 0.7M ammonium sulphate. The eluate, collected through vacuum filtration, was diluted with buffer 3 to reduce the ammonium sulphate concentration to 0.1M. This diluted material was chromatographed over a 30 ml Whatman DE-52 column equilibrated with buffer 3 and the column was washed with two volumes of buffer 3. The DE-52 bound enzyme was eluted with buffer 3 containing 0.6M ammonium sulphate. Two ml fractions were collected and the absorbance at 280nm measured. The fractions showing the peak absorbance were pooled and dialysed. The enzyme was subjected to analysis on a 15% SDS gel and also on a non-denaturing gel.

IMMUNOLOGICAL METHODS

DEVELOPMENT OF ANTIBODIES IN RABBITS AGAINST ER / PHOSPHO TYROSINE - BSA CONJUGATE / RNA POLYMERASE II.

The purified proteins were mixed with equal volumes of Freund's complete adjuvant. The mixture was injected into rabbits of an inbred strain, at 6 sub-cutaneous locations and also into the foot pad. Four booster injections were given using the protein solution mixed with an equal volume of Freund's incomplete adjuvant at an interval of two weeks. Blood was drawn from the rabbit by cardiac puncture one

week after the fourth booster injection. The serum collected was stored at -20oc.

PURIFICATION OF **IMMUNOGLOBULIN:**

IgG was purified from the serum using the protein A agarose (Sigma) column adsorbtion method as described by Sambrook et.al., (1989). One ml of the swollen gel packed in a Pasteur pipette plugged with glass wool was used. The column was equilibrated with 100mM Tris-HCl pH 8.0. The crude serum was mixed with 0.1 volume of 1M Tris-HCl pH 8.0 and chromatographed over a column of protein A agarose. The column was first washed with 10 volumes of 100mM Tris-HCl pH 8.0 followed by 10 volumes of 10mM Tris-HCl pH 8.0. The protein-A bound IgGs were eluted using lOOmM glycine, pH 3.0. 500µl fractions were collected in microfuge tubes containing 50ul of 1M Tris-HCl, pH 8.0. The fractions were measured for their absorbance at 280nm. The peak fractions were pooled and the pH adjusted to 7.6. The material was dialysed against 10mm Tris-HCl, pH 7.6. The purified IgG fraction was stored in aliquots at -20oC.

PREPARATION OF **PHOSPHO TYROSINE-BSA** CONJUGATE:

Phospho tyrosine was coupled to BSA using glutaraldehyde, as described by Philajaniemi *et.al.,* (1987). 5mg of phospho tyrosine was dissolved in 3ml of 10mM sodium phosphate buffer, pH 7.4. This was mixed with 1mg of BSA in 1ml of lOmM sodium phosphate buffer pH 7.4. To the mixture was added, in drops, 300ml of 35% glutaraldehyde. The reaction was allowed to proceed for 24hrs in the dark following which it was stopped and the mixture was freeze dried. The lyophilized protein was dissolved in a small volume of TEMN buffer containing 0.3M NaCl and was chromatographed over a column of Sephadex G-25 in order to remove the uncoupled phosphotyrosine. The fractions containing the void volume from the column were

collected and dialyzed. Antibody in rabbits was raised against this protein conjugate as described. The IgG fraction was purified from the rabbit serum following chromatography on protein A agarose column as described earlier. The purified IgG was coupled to CNBr activated Sepharose 4B.

WESTERN BLOTTING:

Western transfer of proteins to nitrocellulose membrane, using a Bio-Rad Trans blot equipment, was done as described by Towbin *et.al.*, (1979) and Yonezawa *et.al.* (1988). Following SDS-PAGE, the proteins in the gel were transferred to nitrocellulose membrane (Schleicher & Schuell, Inc.) using a Trans Blot Electrophoretic transfer cell (BIO-RAD 170-3910). The transfer was done for 3hrs at 70 volts (~0.25A), using Towbin buffer. After transfer the nitrocellulose membrane was incubated with the blocking buffer (3% BSA solution in TNT buffer) at room temperature for 60min. The nitrocellulose membrane was washed (6x5min each) using TNT buffer. The nitrocellulose membrane was then incubated overnight with primary antibody (in 3% BSA solution). After incubation the filter was washed (6x5min each) with TNT buffer. The nitrocellulose filter was incubated with secondary antibody (horse radish peroxidase conjugated anti rabbit IgG, in 3% BSA solution) for 2hrs. After incubation the membrane was washed (6x5min each) with TN buffer. The membrane was stained using 4-chloro-1-naphthol as substrate (10% methanol, 90% TN buffer, 0.25% H_2O_2 containing 0.3mg/ml of 4-chloro-l-naphthol).

ENZYME ASSAY METHODS:

Enzyme assays were carried out to check the purity of the plasma membrane in the preparation. The enzyme activities measured were succinic dehydrogenase, glucose-6-phosphatase, 5'-nucleotidase and cytidine tri-phosphatase.

SUCCINIC DEHYDROGENASE (SDH):

SDH activity was assayed according to Arrigoni and Singer (1962). The reaction mixture contained the following constituents:

Reaction mixture;

100mM KH_2PO_4 buffer pH 7.6	--	0.2ml
100mM KCN	--	0.1ml
CaCl2 (4mM)	--	0.2ml
Dicholoro phenol indophenol (DCPIP)	--	0.3ml
Succinate (0.5M)	--	0.1ml
DDW	--	2.0ml
Total volume	—	2.9ml

The reaction mixture was prepared immediately before use. For each assay the mixture was pipetted out into the cuvette in the following manner.

1). Reaction mixture	-	2.9ml
2). Phenazine methosulphate (PMS; 10mM)	--	0.05ml
3). Sample (100µg protein)	--	0.05ml

The reaction was monitored at 600nm with 20 secs intervals and the rate of the reaction was calculated using a coefficient of extinction 16.9. The enzyme activity was expressed as units/mg protein/min, where one unit of the enzyme activity represented 1mmole of the dye reduced per min. The mathematical formula used in the calculation of the enzyme activity is give below.

µmoles of dye reduced per min/mg protein = O.D change X 1000 / 16.9 X mg protein X min

GLUCOSE 6-PHOSPHATASE:

Glucose 6-phosphatase activity (G-6-Pase) was measured according to Aronson and Touster (1974). The reagents used were:

1). Sodium glucose 6-phosphate 0.1M, pH 6.5.

2). Histidine, 35mM, pH 6.5. 3). Na-EDTA, 10mM, pH 7.0.

The enzyme activity was determined by measuring the rate of release of inorganic phosphate from glucose 6-phosphate. The assay mixture was made by mixing the reagents 1), 2), 3) and DDW in the ratio of 2:5:1:1.

50ul of the sample (25 µg protein) was mixed with 450ul of assay mixture and incubated for 30min at room temperature. After incubation 8% TCA (2.5ml) was added to stop the reaction. The inorganic phosphate released was estimated following Fiske-Subbarow's method (1925). The enzyme activity was expressed as mmoles of inorganic phosphate released /mg of protein/ min.

5' NUCLEOTIDASE:

5' nucleotidase enzyme activity was measured according to the procedure of Aronson and Touster (1974). The reagents used in the enzyme assay were:

1). 50mM Na-AMP, pH 7.0.

2). 0.5M glycine-NaOH buffer, pH 9.1.

3). 0.1M $MgCl_2$ solution.

The assay mixture was made containing reagents 1), 2), 3) and DDW in the ratio of 1:2:1:5. 50µl of the sample containing 25µg of protein was mixed with 450ul assay

mixture. Incubation was carried out for 30min at room temperature. The reaction was stopped by adding 2.5ml of 8% TCA. The inorganic phosphate released was estimated as described earlier.

The enzyme activity was expressed as mmoles of inorganic phosphate released/mg of protein/ min.

CYTIDINE TRIPHOSPHATASE (CTPase):

CTPase activity was estimated following the procedure of Perdue and Sneider (1970).

50ul of the sample (25ug of protein) was incubated for 30 min at 37oC with 1ml of assay mixture [20mM bicine (N,N,bis (2,hydroxyethyl) glycine), pH 7.8, containing 3mM CTP as substrate, 3mM $MgSO_4.H_2O$, and 250mM sucrose]. 8% TCA was added to stop the reaction. The inorganic phosphate released was estimated as described elsewhere. The enzyme activity was expressed as mmoles of inorganic phosphate released /mg protein/min.

INORGANIC PHOSPHATE ESTIMATION:

The inorganic phosphate was estimated following the method described by Fiske and Subbarow (1925). The reagents used in the estimation of phosphate were:

ANSA reagent

--7.312g of sodium metabisulphite

--0.25g sodium sulphite

--0.4g of 1,2,4 ANSA (1,2,4 aminonaphthol sulfonic acid)

Sodium metabisulphite, ANSA and sodium sulphite were ground together to a fine mixture. 1g of this was dissolved in 25ml of DDW. 500µl of sample in TCA was vortexed with lml of acid molybdate. 200µl of ANSA reagent was added and incubated for 10min. After incubation the absorbance was measured at 600nm. Standard values were obtained using inorganic phosphorus solutions in the 1 to 10µg range.

ESTROGEN RECEPTOR ASSAYS:

DNA CELLULOSE BINDING ASSAY:

DNA cellulose was prepared following the method of Alberts and Herrick (1971), using goat liver DNA and Whatman cc-31. 60mg alkali-heat-denatured DNA was mixed with 60mg of native DNA and 12g of cellulose cc-31. The slurry was dried overnight, lyophylized and finally suspended in TEMN buffer. The suspension was stored, in 3ml aliquots, at -75oC.

The DNA cellulose binding assay was carried out as outlined by Thampan (1987). The estrogen receptor was incubated overnight at 4oC with 20nM ^{3}H-estradiol, in a total volume of 200µl. The unbound hormone was separated from the bound using dextran coated charcoal (1% charcoal and 0.1% dextran suspended in TEMN buffer). The receptor-hormone complex was incubated with the DNA cellulose suspension for 30min at 30oC. The DNA cellulose pellet was washed twice with ice cold TEMN buffer. The labelled estradiol associated with the DNA cellulose pellet was extracted with 1ml of ethanol for the measurement of radioactivity.

HYDROXYLAPATITE BINDING ASSAY:

The HAP binding assay was carried out as described by Clark and Peck (1979).

Commercially available HAP (BIO-RAD) was used in the assay. 100µl of the

sample was incubated overnight at 4oC with 20nM final concentration of 3H-estradiol + 100 fold molar excess of diethylstilbestrol (DES). 250µl of a 60% HAP suspension in TEMN buffer was added and the mixture was incubated in ice for 15min. After washing twice with ice cold TEM buffer, the HAP pellet was extracted with 1ml ethanol. The radioactivity in the ethanol extract was measured.

SATURATION KINETICS OF ESTRADIOL BINDING TO RECEPTORS:

The saturation binding of the receptor was analyzed by incubating 100µl of receptor samples overnight with 2 to 40nM concentrations of 3H-estradiol + 100 fold molar excess of DES. HAP binding assay was carried out as described earlier. The saturation binding data was further analysed following the method of Scatchard (1949). The number of binding sites and equilibrium constant (Kd) were calculated from the Scatchard plot.

NON-ACTIVATED ER ASSAY:

100µl of receptor sample was incubated with 3H-estradiol giving a final concentration of 20nM, overnight at 4oC. The unbound hormone was removed following charcoal adsorption. The tubes were centrifuged at 5000 X g for 3min. The supernatant was added to the tubes containing E-RAF and was allowed to incubate at 4oC for 15min. DNA cellulose was added and the mixture was incubated at 30oC for 30min. The pellet was washed twice with ice cold TEMN buffer and the labelled estradiol associated with the DNA cellulose pellet was extracted with 1 ml ethanol for the measurement of radioactivity.

CYANOGEN BROMIDE FRAGMENTATION OF ESTROGEN RECEPTORS:

Peptide mapping was carried out using CNBr as described by Kasper (1970). Chemical degradation and enzymatic digestion of proteins were employed in the study of

peptide map comparison CNBr has a specificity for methionyl residues. The cleavage of the protein was carried out in 70% formic acid. 100µg of pure protein was dissolved in 10µl of 70% formic acid. A 50 fold excess of CNBr (solid) was added and the mixture was incubated at room temperature for 1 hr. 1ml of DDW was added to stop the reaction. The peptides were lyophylized and analyzed on a 12% SDS gels.

SUCROSE DENSITY GRADIENT ANALYSIS:

The purified receptor (300µl) was layered over 4.5ml of a 5 to 20% linear sucrose density gradient prepared in TEMN buffer containing 0.3M NaCl. The tubes were subjected to centrifugation in a Beckman ultracentrifuge (L8-80M) using a vTi-80 rotor at 2,60,000 X g for 2hrs. The fractions collected by gravity flow were subjected to estrogen receptor assays.

CARBOHYDRATE DETECTION AND ESTIMATION:

Carbohydrate moiety associated with the purified receptor preparation was detected using two procedures.

1). Concanavalin A Sepharose **chromatography:**

Concanavalin A (con A) was coupled to Sepharose 4B following the method described earlier. The column was equilibrated with TCMMN buffer. The purified proteins were chromatographed on a 3 ml column of con A Sepharose. The flow through volume from this column (including the washing volume) was collected in fractions. After washing the column the con A-bound proteins were eluted using the same buffer containing 0.3M methyl a-D-glucopyranoside. Fractions collected were analyzed for their absorbance at 280nm. This procedure was described originally by Lloyd (1970).

2). Glycoprotein staining on blots:

This was carried out according to Olden and Yamada (1977). The purified protein, subjected to SDS-PAGE, was western-transferred to nitrocellulose membranes. The membranes were first blocked with 3% BSA in TN buffer for 1hr, following which, they were washed with TN buffer for 30min (3X10 min each). The membranes were further incubated with con A (2mg/ml) for lhr. After washing for 25 min (5 X 5min each) with TN buffer, the membranes were incubated with HRPO (100 units/10ml) for 4hrs The membranes were washed for 30 min (3 X 10min each), following which they were stained with 4-chloro-1-naphthol.

Carbohydrate content in the receptor proteins were estimated according to Dubois et.al., (1956)

Standard glucose solutions of concentrations 1,2,5,10 and 20µg were made. 5% phenol reagent was prepared by dissolving 5g of phenol in 100ml of concentrated sulphuric acid.

To 2ml of the sample, containing 100µg of protein, was added 10ml of the phenol reagent. This was followed by the addition of 5ml of concentrated sulphuric acid. The vortexed samples were incubated at room temperature for 30mm. After incubation the absorbance in the samples at 490nm was measured. A standard graph was plotted with concentration versus absorbance. The carbohydrate content was calculated from the standard graph.

FLUORESCENT LABELLING STUDIES:

FITC LABELLING OF RECEPTORS:

Fluorescein isothiocyanate (FITC) labelling of proteins was carried out as described by Chard (1987). 1mg of protein was dissolved in 250µl of 0.15M

Na2PO4.2H2O, pH 9.5. 100µl of FITC solution in the same buffer (1mg/ml) was added to the protein solution. The pH of the mixture was adjusted to 9.5 using 1M Tris. This was incubated for 2hrs at room temperature. After incubation the unbound **FITC** was removed following chromatography of the mixture over a column of Sephadex G-25. The covalent coupling of FITC with the protein was confirmed by viewing the SDS gel under an UV lamp.

EXPERIMENTS TO STUDY THE INTERACTION OF THE RECEPTORS WITH RNA POLYMERASE II:

To study the receptor interaction with RNA polymerase II the following experimental methodology was used. The FITC labelled receptors were incubated with RNA polymerase II in TEMG buffer, over a period of time, 0,5,10,15,20,30,40,50 **and** 60 min. at 37oC. After incubation, the tubes were cooled in ice; sodium chloride was added to the protein mixture to a final concentration of 0.5M in order to dissociate the receptor-RNA polymerase complex. Anti RNA polymerase IgG was added to this material and the dissociated RNA polymerase was immunoprecipitated as described elsewhere. The fluroscence associated with the immunoprecipitated RNA polymerase **II** was measured.

PHOSPHORYLATION STUDIES:

1). Auto phosphrylation of receptors:

10µl of the receptor protein (10µg) in the protein kinase assay buffer (10mM Tris-HCl, pH7.6 containing 6mM MgCl2, 2mM CaCl2, 12mM monothioglycerol, 0.2mM PMSF and 10µM ATP) was incubated with 5mCi/5ml [γ-32P] ATP for 1hr at 30oC. After incubation the reaction was terminated by the addition of 5ml of SDS

sample buffer (4X). The phosphorylated proteins were subjected to SDS-PAGE and/ the dried gels were exposed to X-ray films.

2). Detection of phospho amino acids:

The purified protein was allowed to phosphorylate in the presence of [y-32P] ATP. After phosphorylation the proteins were precipitated with 5% TCA, washed twice with ethanol and suspended in 6N HC1 (50µl). Protein hydrolysis was carried out at 110°C for 16hrs. The hydrolysed proteins were dissolved in 10µl of butanol:acetic acid:water (65:15:25) and spotted on a Whatman No. 1 chromatography paper, along with 20nM each of phosphotyrosine, phosphoserine and phosphothreonine as standards. The amino acids were allowed to separate by ascending chromatography using the butanol, glacial acetic acid, and water mixture (65:15:25). The chromatograms were dried, sprayed with ninhydrin (0.2% ninhydrin dissolved in acetone containing 0.1% pyridine) and finally exposed to the X-ray films.

3). Identification of the RNA polymerase II subunits phosphorylated :

10mg of the receptor was incubated with RNA polymerase II (20mg) in the presence or absence of estradiol. The phosphorylation was allowed to proceed as described earlier. The proteins, after phosphorylation, were subjected to SDS-PAGE followed by autoradiography.

MISCELLANEOUS:

Quantitation of proteins:

The protein estimation was carried out as descried by Bradford *et.al.*, (1976). To 100µl of the sample, 1ml of Bradford's reagent was added, mixed and the colour developed was measured at 595nm after an interval of 5min. BSA was used as the protein standard.

Radioactivity measurement:

Samples (ethanol extracts) were added to 3ml of scintillation cocktail [5g PPO/ 1 and **POPOP/** 1 in toluene Triton X-100 (67:33)]. The radioactivity was measured in a Beckman liquid scintillation counter (LS1701). Correction for quenching was carried out using the external standardization technique. Efficiency for tritium was 50-54%.

Measurement of fluorescence:

The fluorescence in the sample was measured at an excitation wave length of 492nm and emission wave length of 520nm, using a JASCO spectrofluorimeter.

Two dimensional gel electrophoresis:

Two dimensional gel electrophoresis was carried out as described by OTarrell® (1975). The buffers used were given below:

1. **IEF sample** solution: 9.5 M urea, 2% (w/v) **NP-40**, 2% **ampholines** (comprised of 1.6% of pH 4-6 and 0.4% of pH 3.5-10) and 5% **β-mercaptoethanol**.
2. Acrylamide solution: 28.38% (w/v) acrylamide and 1.62% **bis-acrylamide** were dissolved in distilled water.
3. **Stock NP-40** solution: 10% (w/v) **NP-40** in distilled water.
4. Ampholines: Ampholines were used as 40% (v/v).
5. Ammonium persulphate solution: 10% (w/v) ammonium persulphate was dissolved in distilled water.
6. **Gel** overlay buffer: 8M urea in distilled water.
7. Anode electrode buffer: 0.01M phosphate buffer.
8. Cathode electrode buffer: 0.02M NaOH in distilled water (degassed and stored).
9. Sample overlay solution: 9M urea, 2% ampholines (comprised of 1.6% pH 4-6 and 0.4% **pH** 3.5-10)
10. Agarose solution: **1g** of agarose in 100ml of SDS sample buffer (without bromophenol blue).

Sample preparation:

Lyophilized proteins **(2μg)** were dissolved in IEF sample buffer **(50μl)**.

First dimension:

Isoelectric focusing gels were made in glass tubing (105 X 3 mm) sealed at the bottom with **parafilm**. The gel mixture (for 4 tubes) was made as given below:

Urea:		2.25g
Acrylamide:		0.67ml
NP-40 Stock solution (10%):		**1ml**
Water:		0.99ml
Ampholines	pH 4-6:	**200ul**
	pH 3.5-10:	**50μl**

@ OTarrell, PH., (1975) High resolution two dimensional electrophoresis of proteins. ***J.Biol.*** *Chenv* 250: 4007-4021.

The gel mixture was degassed. 10µl of 10% APS solution was added followed by 7 µl of TEMED. The gel mixture was loaded into the glass tubes using a syringe with a long narrow gauge hypodermic needle. The gel was overlaid with gel overlay solution. After polymerization of the gel, the overlay solution was replaced by IEF sample buffer. The IEF sample buffer was replaced after 30 minutes with 0.02M NaOH. The parafilm at the end of the tubes were removed and placed in the electrophoretic apparatus. The lower reservoir was filled with 0.01M phosphate buffer and the upper reservoir was filled with 0.02M NaOH. The gels were pre-run for i) 15 minutes at 200 volts; ii) 30 minutes at 300 volts and iii)30 minutes at 400 volts.

The samples were loaded, upon emptying the upper reservoir and the buffer on the top of the gel. The samples were overlaid with 10µl of sample overlay solution, then the tubes were filled with 0.02M NaOH. The upper tank was re-filled with 0.02M NaOH solution. The gels were subjected to electrophoresis at 400 volts for 12 hrs and then at 800 volts for 2 hrs.

The gels were extruded from the tubes using a syringe and a needle. The gel was equilibrated with 5ml of SDS sample buffer (without bromophenol blue) at room temperature for one hr.

Second dimension:

The second dimension gel electrophoresis was carried out as described by Laemmli (1970). 10% polyacrylamide gels of 3mm thickness were polymerized as described in this section. The upper gel was polymerized without any comb and to accommodate the IEF gel, little space was left. the IEF gel was carefully placed and was kept in position by pouring 1% agarose solution.

Electrophoresis and silver staining of the gel was carried out as described earlier.

CHAPTER III

A DNA BINDING (R-I) AND A NON-DNA BINDING (R-II) ESTROGEN RECEPTOR IN THE GOAT UTERINE NUCLEUS:

PURIFICATION AND CHARACTERIZATION

Several recent reports identify estrogen receptors of the mammalian estrogen responsive tissues, to be primarily localized in the nucleus even in the absence of the hormone (Clark *et. al.,* 1982, King and Greene, 1984; Mc Clellan *et. al.*, 1984; Welshons *et. al.,* 1984; Geier *et.al.*, 1987). Analysis of nuclear estrogen binding sites by [3H]-estradiol exchange assay revealed a complex picture with the involvement of at least two distinct classes of binding sites, the type-I and the type-II (Eriksson *et.ai,* 1978; Markaverich and Clark 1979). Nuclear type-I ER represents the classical ER displaying a saturation curve which is a parabola and yielding a Scatchard plot in the form of a linear component. Nuclear type-II ER, however, has a more complex binding function and displays a sigmoidal saturation curve and a curvilinear Scatchard plot (Clark *et.al.*, 1985; Markaverich and Gregory 1991). Little is known about the biochemical function of the nuclear type-II ERs. However, the presence of these receptors in the nuclear matrix suggests a possible role for them in DNA replication (Pardoll *et. al.,* 1980, Clark and Markaverich 1982).

The nuclear estrogen receptor, be it the type-I or the type-II has not been fully characterized due mainly to the lack of proper methods for the isolation and purification of the protein. This chapter presents the methods developed in our laboratory for the purification of two nuclear ERs (R-I and R-II) which, I believe are nuclear estrogen receptors type-I and type-II.

One factor that hindered the purification of the type II receptors by other investigators (Markaverich and Gregory 1991) was the resistance of these receptors for extraction with salt. The method presented by us for the solubilization of the receptors involves sonication of the nuclei in the presence of fairly low concentrations of NaCl, the time involved having been kept to the minimum.

RESULTS

PURIFICATION OF THE DNA BINDING FORM OF NUCLEAR ER (R-I):

The supernatant fraction recovered from the sonicated nuclear suspension was chromatographed over a ss-DNA Sepharose column, equilibrated with TEMN buffer The DNA binding form of the nuclear ER (R-I) binds to the column After extensive washing with TEMN buffer, the DNA-bound proteins were eluted selectively using TEM buffer containing 10mM ATP. This method has earlier been used successfully in the purification of goat uterine E-RAF (Thampan, 1987).

The ATP eluate was rechromatographed on a Whatman DE-52 column. After washing extensively with TEM buffer, the R-I receptors bound to the column were eluted with TEM buffer containing 0.2M NaCl. 2ml fractions were collected and subjected to DNA cellulose binding assay. A homogeneous peak of estrogen binding activity was observed (Fig. 1). When the peak fractions were analyzed on a 10% SDS-PAGE, two protein bands were observed with molecular mass of 66 kDa and 46 kDa (Fig.3C).

In order to achieve further purification, the pooled material was subjected to gel filtration using a Sephadex G-50 column, equilibrated with TEM buffer containing 0.3M NaCl. The void volume was collected which displayed the presence of ER (data not shown). No additional ER activity was detected in the post-void volume fractions.

The void volume from the Sephadex G-50, when subjected to electrophoresis in a 10% SDS-gel, showed the presence of a highly purified protein of molecular mass 66 kDa(Fig.3D).

PURIFICATION OF THE NON-DNA BINDING FORM OF NUCLEAR ER (R-II):

The basis for the assumption that this ER was a non-DNA binding form was the earlier observations that the ER activity extracted from the goat uterine nuclear RNA

polymerases fractions did not possess any capacity to bind to DNA (Thampan, 1989). Sodium molybdate was added to the ss DNA Sepharose flow through fraction to give a final concentration of 10mM, following which the material was chromatographed over a hsp90-Sepharose column equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed extensively with the same buffer. The bound R-II receptors were eluted with TEM buffer free of molybdate. The eluate was collected in 2ml fractions which were subjected to HAP binding assay for estrogen receptor.

A protein peak was noticed after >90% of the total bound proteins dissociated from the matrix (not shown in the Fig.2). A peak of ER activity which coincided with the protein peak was observed when the fractions were subjected to HAP assay for the **R-II** receptors (Fig.2). The fractions which displayed ER activity were pooled, precipitated and subjected to electrophoresis in the presence of SDS in a 10% gel. Two distinct protein bands were observed in the gel of molecular mass of 66 kDa and 55 kDa (Fig.3E).

PAGE UNDER NON-DENATURING CONDITIONS:

The purified receptor samples were incubated with [3H]-estradiol, overnight at 4oC. R-I and **R-II** were applied to two wells in a 6% acrylamide gel. The electrophoresis was carried out at 100 volts for 8 hrs (4oC). After electrophoresis one lane was silver stained while the other was sliced into 3 mm pieces; the radioactivity associated with the gel pieces was analyzed (Fig.4). While two peaks of R-I activity (Fig.4B), associated with two distinct silver stained bands, were observed in the native gel of **R-I** (Fig.3A), **R-II** showed the presence of only one peak of activity (Fig.4A) associated with the single stained protein band in the non-denaturing gel (Fig.3B). The two **R-I** protein bands of the non-denaturing gel, when subjected independently to SDS-PAGE, re-appeared as 66 kDa bands (result not shown). At the same time the R-II protein which appeared homogeneous in the native gel displayed the presence of two bands in the SDS gel (Fig.3E).

IMMUNORECOGNITION OF R-I AND R-II BY ANTI E-RAF IgG:

The antibodies raised against goat uterine E-RAF II was used in this study. Both the R-I and the **R-II** were found to cross react with anti E-RAF IgG (Fig.5).

SEDIMENTATION ANALYSIS:

The purified R-I and R-II receptors were subjected to sucrose density gradient analyses. The fractions collected were subjected to DNA cellulose binding assay for R-I (Fig.6A) and HAP assay for R-II (Fig.6B) receptors. Bovine serum albumin and ovalbumin were used as sedimentation markers. Sedimentation values calculated for the R-I and **R-II** receptors were 4.8 S and 3.8 S respectively.

GEL FILTRATION ANALYSIS:

The Stokes radii for the purified receptors were calculated as described by Siegel and Monty (1966). A Sephadex G 100 column was used for the determination of Stokes radii. The column was equilibrated with TEMN buffer containing 0.3M NaCl. Standard proteins, bovine serum albumin, ovalbumin and cytochrome c, of known Stokes radii, were used in the column calibration. The Stokes radii of the R-I and R-II were calculated to be 360A and 210A respectively (Fig.7).

BINDING PARAMETERS:

HAP binding assay was carried out in order to study the hormone binding parameters of the purified receptors. The receptor fractions were incubated with different concentrations of [3H]-estradiol (2 to 20nM for R-I and 4 to 40nM for R-II) + 100 fold molar excess of DES. The specific binding activity was calculated by deducting the non-specific binding data from the total. The kd values for R-I and R-II calculated from the Scatchard plot were 1nM and 2nM respectively (Fig.8).

CNBr FRAGMENTATION OF R-I AND **R-II:**

In order to compare the peptide maps of R-I and R-II, CNBr fragmentation of the proteins was carried out. After fragmentation of the proteins, the peptides formed

were lyophilyzed and were subjected to electrophoresis in the presence of SDS in a 15% polyacrylamide gel. A clear distinction in the nature of the CNBr peptides formed was noticed between the R-I and the R-II (Fig.9). The single dimensional peptide profiles were analyzed in an automated laser scanner using the software program (1988) of Bio-Med instruments.

EFFECT OF QUERCETIN AND DITHIOTHREITOL ON THE BINDING OF E2 TO R-I AND R-II RECEPTORS:

Reports have already been made to the effect that reducing agents like dithiothreitol have inhibitory effect on the E2 binding to the nuclear type II sites, while showing no effect on the nuclear type-I sites (Markaverich, *et. ai,* 1981). In order to investigate whether the receptors, purified, possessed the characteristics of the type I and type II nuclear ERs, additional experiments were carried out. R-I and R-II were incubated with different concentrations either of DTT or of quercetin in the presence of [3H]-estradiol + 100 fold molar excess of DES. Specific binding activity was measured using the HAP binding assay method. The medium used in this experiment was the TE buffer.

It was observed that while E2 binding to R-I remained unaffected under the experimental conditions metioned above the E2-R-II interaction was negatively influenced both by DTT and by quercetin. The minimum possible concentration of DTT that could bring about maximum inhibition (30%) in the hormone binding to R-II was 0.5mM while a 45% inhibition in hormone binding to R-II was brought about by quercetin at a concentration of350nM (Fig. 10).

DISCUSSION

Very simple and expedient methods have been developed for the isolation and purification of R-I and R-II receptors of the goat uterine nucleus. The main feature of the purification protocol for the R-I is the selective elution of the receptors from the ss-DNA-Sepharose column by 10mM ATP. The ATP binding to the receptor dissociates the receptor from the DNA which indicates that a certain amount of overlap may exist between the ATP binding sites and DNA binding sites on the R-I. A similar ATP elution of estrogen receptor activation factor (E-RAF) from the ss-DNA column was reported by Thampan (1987). Using this procedure a 55 % yield was obtained with a 5.2 X 103 fold purification over the crude R-I receptor in the nuclear sonicate. One of the major hindrances encountered in the purification of type-II receptor by earlier investigators was the resistance of the receptors to salt extraction. We have achieved more than 58% recovery of the R-II receptors by sonication. Several reports have appeared in the literature regarding the interaction of ERs with heat shock protein 90 (Catelli *et.al.*, 1985; Ratajczak *et. ai,* 1987; Pratt, 1990). The best method in our hands, for the isolation of R-II receptors from the nuclei has been the binding of R-II to a hsp90-Sepharose column in the presence of molybdate ions and the subsequent elution using a molybdate-free buffer.

The SDS-PAGE of the purified R-I receptor displayed a protein band of 66 kDa, while purified R-II receptor displayed two protein bands of 66 and 55 kDa. When the purified receptors were analyzed under nondenaturing conditions, R-I displayed two bands, while R-II displayed only one band. This observation serves to confirm the earlier reports that the DNA binding form of R-I is a homodimer (Miller, *et. ai,* 1985; Fawell, *et. ai,* 1990a; Fawell, *et. ai,* 1990b). The only difference noticed is that the homodimer is composed of two monomers having two different net charges on the protein. In the case of R-II the lower molecular mass noticed in the SDS-PAGE probably represent a partially proteolysed form of R-II. Purification of the R-II ER to

absolute homogeneity has subsequently been achieved (presented in chapter V).

The cross reaction of the receptors with anti E-RAF IgG may suggest one of the following.

a) The E-RAF and the receptors (R-I and R-II) are totally different proteins, but sharing common antigenic determinants.

b) The E-RAF may be the product of a mutated receptor gene or may be a product of the receptor gene that has undergone modifications resulting in the loss of the hormone binding property (a truncated ER).

c) The E-RAF and the receptors may belong to a common superfamily of transcription factors, which have retained common antigenic determinants during the course of macromolecular evolution.

The recent experimental data from our laboratory (Zafar and Thampan, 1994) serve to indicate that the alternative **`b'** is highly unlikely since the tryptic and CNBr peptide maps of the ER and E-RAFs displayed were totally non-identical

The effect of reducing agents on the estradiol binding to the receptor was studied. There are reports to show that DTT and monothioglycerol abolishes, reversibly, the type-II receptor binding to estradiol, while the type-I remained ineffective to this treatment (Markaverich and Gregory 1991). Results of a similar nature have been obtained in our experiments with the R-I and R-II. However, it may be pointed out that monothioglycerol even at a concentration of 12mM did not introduce any inhibition in the R-II activity. It is not possible to comment at this stage why monothioglycerol failed in this reaction in which DTT was successful. It is also too early to comment upon the inhibitory effect of quercetin on the E2-R-II interaction.

Bioflavonoid derivatives like methyl p-hydroxyphenyllactate (MeHPLA) have inhibitory effects on the type-II receptor for estradiol binding (Markaveich and Gregory,

1991). I used quercetin, a bioflavonoid, and found that it inhibits the binding of estradiol to the R-II receptors. Markaverich and his associates have used quercetin-Sepharose as a tool for the isolation of rat uterine nuclear type II sites (Markaverich and Gregory, 1991).

Based on the above mentioned data, I am inclined to believe that the R-I is the type I ER and the R-II is the type II. During the purification of these receptors we have been on an alert to identify any nuclear receptor activity that would have escaped our notice. We have observed none other than these two. However qualitative differences are noticed between the estradiol binding properties of the R-II and those of the Type II in the following.

1). The kd of the R-II is lower than the value calculated for the Type II by Markaverich and associates.

2). DTT brought about a maximum of 35% inhibition in the estradiol binding to R-II while a total inhibition of estradiol binding to Type II was effected by a much less concentration of DTT (Markaverich and Gregory, 1991). CNBr fragmentation analysis reveals that the two ERs are two totally distinct proteins, though carrying common epitopes.

The functional significance of the R-II receptor is not clear. It was reported earlier that the nuclear receptors associated with RNA polymerases in the rat uterus were predominantly of the type-II category (Thampan, 1987). The important observation is that the R-II appears to be a receptor that binds to nuclear proteins but not to the DNA. In this aspect the R-I and R-II draw very close parallels from the

chick oviduct progesterone receptors A and B, originally discovered by Schrader and O'Malley (Schrader and O'Malley, 1972; Buller *et. al.*, 1976). How the R-H-hormone complex will influence the RNA polymerase activity is clearly a topic for future study

Fig. 1. DNA cellulose binding assay for **R-I** receptor:

The 10 mM ATP eluate from the ss-DNA-Sepharose column was chromatographed on a DE-52 column equilibrated with TEM buffer. The receptors bound to DE-52 were eluted with TEM buffer containing 0.2 M NaCl. The eluate was collected as 2 ml fractions and subjected TO DNA cellulose binding assay.

Fig. 2. Isolation of **R-II** following hsp-90 chromatography:

The DNA Sepharose flow through fraction of the nuclear sonicate was subjected to hsp-90 Sepharose chromatography as described in materials and methods. The 2 ml fractions collected from the hsp-90 Sepharose column were subjected to HAP binding assay. The fractions were incubated overnight at 4oC with 40 nM [3H]-estradiol + 100 fold excess of DES. Further processing of the HAP was carried out as described by Peck and Clark (1977). The specific binding data was calculated by deducting the non-specific binding data from the total.

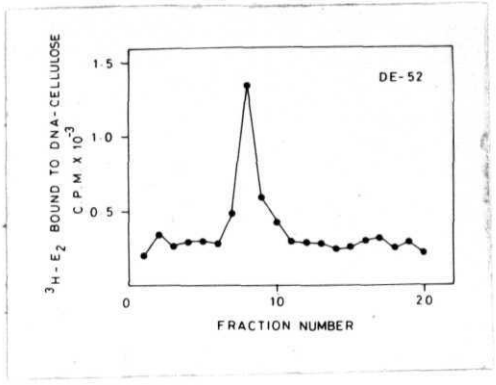

DE-52
$^3H-E_2$ BOUND TO DNA-CELLULOSE C P M X 10^{-3}
1·5
1·0
0·5
0
10
20
FRACTION NUMBER

Fig. 1

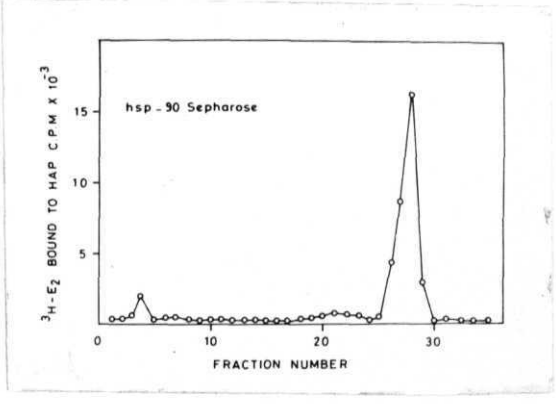

hsp-90 Sepharose
$^3H-E_2$ BOUND TO HAP C P M X 10^{-3}
15
10
5
0
10
20
30
FRACTION NUMBER

Fig. 2

Fig. 3. Electrophoretic analysis of R-I and R-II receptors:

A **and** B: Polyacrylamide gel electrophoresis under non-denaturing conditions of R-I (A) and R-II (B). The proteins were incubated with [3H]-estradiol overnight at 4oC. The incubated proteins were mixed with 2X sample buffer and subjected to electrophoresis in a 6% acrylamide gel,at 100 volts (4oC) for 8 hrs. After electrophoresis the gel was stained with silver nitrate.

C, D **and** E: SDS-PAGE of the receptors: The peak R-I activity fractions from the DE-52 column were pooled and the proteins were precipitated with trichloroacetic acid. The precipitated proteins were subjected to electrophoresis in a 10% polyacrylamide gel and the gel was silver stained (C). The peak R-I activity fractions from the DE-52 column were pooled, concentrated and layered over a Sephadex G-50 column equilibrated with TEM buffer containing 0.3 M NaCl. The void volume was collected and the protein was precipitated and electrophoresed as mentioned earlier (D). (E) shows a SDS-PAGE of the purified R-II, used in the non-denaturing gel (B).

Fig. 4. Non-denaturing gel analysis of the purified R-I and R-II:

The purified proteins were incubated with [3H]-estradiol overnight at 4oC. Polyacrylamide gel electrophoresis was carried out as described in materials and methods. After electrophoresis the gel was sliced into 3 mm pieces and the radioactivity associated with the gel pieces of R-I (A) and R-II (B) analyzed in a liquid scintillation counter.

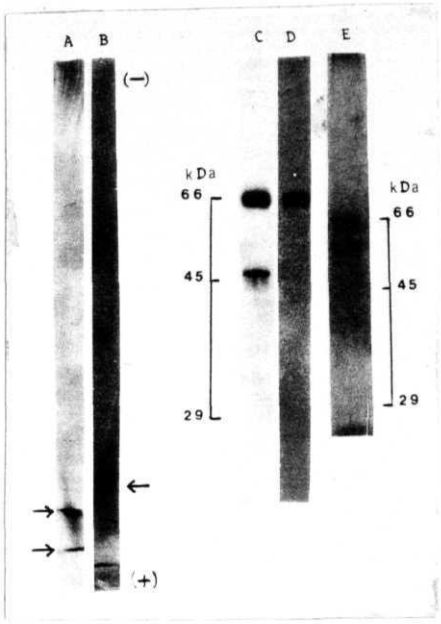

A
B
C
D
E
(−)
(+)
kDa
66
45
29
kDa
66
45
29

Fig. 3

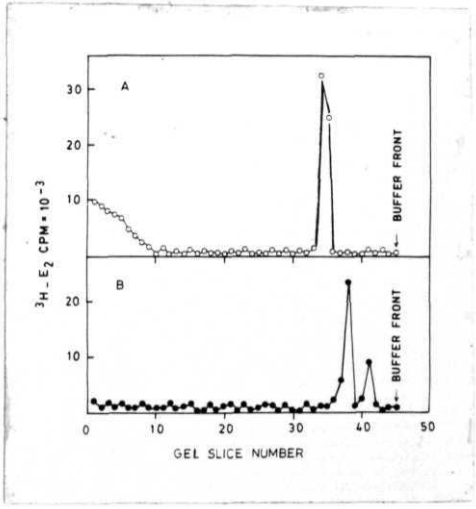

A
B
30
20
10
20
10
BUFFER FRONT
BUFFER FRONT
^3H-E_2 CPM × 10^{-3}
0
10
20
30
40
50
GEL SLICE NUMBER

Fig. 4

Fig. 5. Immunorecognition of R-I and R-II by anti E-RAF IgG:

The purified receptors R-I (A) and R-II (B) were subjected to electrophoresis in a 10% polyacrylamide gel in the presence of SDS. After electrophoresis the proteins in the gel were transferred to nitrocellulose membranes. The membranes were exposed first to anti E-RAF IgG, the primary antibody and later to horse radish peroxidase (HRPO) coupled anti rabbit IgG, the secondary antibody. The membrane was stained with 4-chloro 1-napthol, used as the HRPO substrate

Fig. 6. Sedimentation analysis of the R-I and R-II:

The purified receptors were layered over 5 to 20% linear sucrose density gradients in TEMN buffer containing 0.3 M NaCl(4.5 ml) The tubes were subjected to centrifugation in a Beckman ultracentrifuge at 250,000 x g for 2 hrs using VTi 80 rotor. After centrifugation the fractions were collected by gravity flow (7 drops/150 µl). The R-I receptor fractions were subjected to DNA cellulose binding assay (A), while the R-II receptor fractions (B) were subjected to both DNA cellulose (closed circles) and HAP binding assays (open circles). DNA cellulose binding assay was ca:.ied out for the R-II receptor in order to establish that the R-II receptor is a non-DNA binding protein.

Fig. 7. Determination of Stokes radii of R-I and R-II:

The Stokes radii for the R-I and the R-II receptors were calculated by using a Sephadex G-100 column equilibrated with TEMN buffer containing 0.3 M NaCl. The proteins were concentrated to 200 µl and chromatographed over the column. The column was developed and 2 ml fractions were collected. The fractions of the R-I were subjected to DNA cellulose binding assay (closed circles) while those of the R-II were subjected to HAP binding assay (open circles). The Stokes radii of the proteins were calculated as described by Siegel and Monty (1966).

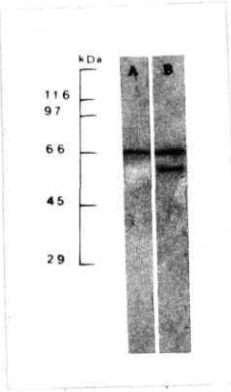


Fig. 5

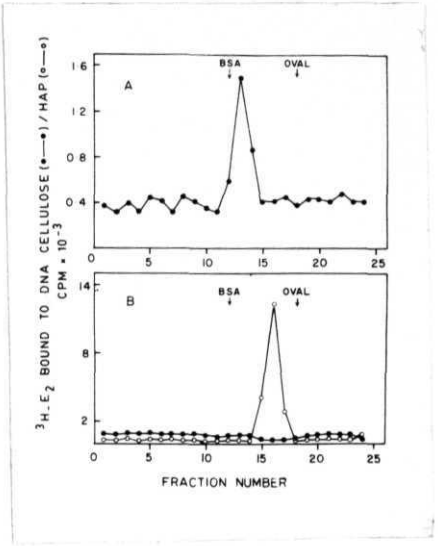


Fig. 6

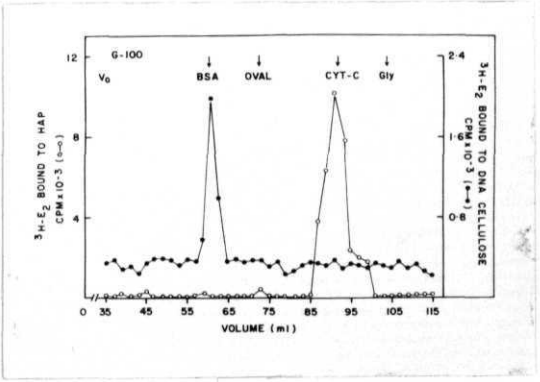


Fig. 7

Fig. 8. Saturation kinetics of estradiol binding to R-I and R-II:

The purified receptors were incubated with different concentrations of [3H]-estradiol (2 to 20 nM for R-I and 4 to 40 nM for R-II) + 100 fold excess of DES. The final volume of the incubation medium was 200 µl. HAP binding assay was carried out in both the cases. The specific binding data was calculated for R-I (closed circles) and for R-II (open circles) by deducting the non-specific binding data from the total. The inset (A) is the Scatchard plot for the receptor R-II and the inset (B) is that for the R-I receptor.

Fig. 9. Cyanogen bromide fragmentation of the purified R-I and R-II:

100 µg of the lyophilyzed protein was dissolved in 10 µl of 70% formic acid. To this suspension 5 mg of solid CNBr was added. Fragmentation was allowed to proceed at room temperature for 1 hr. The reaction was stopped by the addition of 1 ml of distilled water. The lyophilyzed peptides were subjected to electrophoresis in the presence of SDS in a 15% polyacrylamide gel. After silver staining of the gel, the single dimensional peptide profiles were analyzed in an automated laser scanner.

Fig. 10. Effect of quercetin and dithiothreitol (DTT) on estradiol binding to R-I and R-II receptors:

Purified receptors were incubated with [3H]-estradiol (20 nM for R-I and 40 nM for R-II) + 100 fold excess of DES in the presence of varying concentrations of quercetin (50 to 500 nM) (A) or of DTT (0.1 to 1 mM) (B). The final volume of the incubation medium was 200 µl. HAP binding assay was carried out in both the cases The specific binding data was calculated by deducting the non-specific data from the total The receptor samples incubated without quercetin or DTT were used as the controls and the % binding activity was calculated with reference to the respective control values. The closed circles indicate R-I and the open circles indicate R-II.

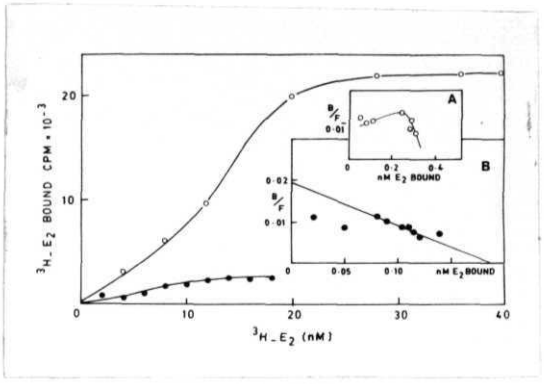


Fig. 8

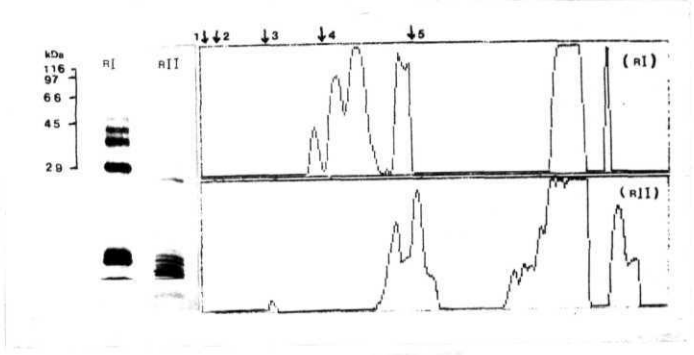


Fig. 9

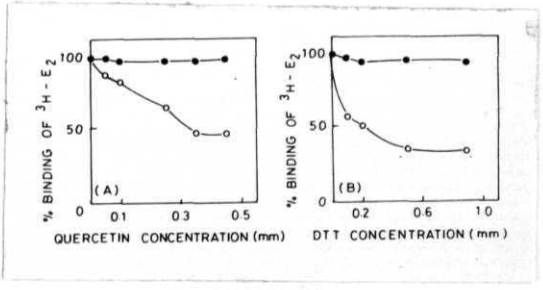


Fig. 10

CHAPTER IV

PLASMA MEMBRANE IS THE PRIMARY SITE OF LOCALIZATION OF THE NON-ACTIVATED ESTROGEN RECEPTOR (naER) IN THE GOAT UTERUS:

HORMONE BINDING CAUSES RECEPTOR INTERNALIZATION

The recent recognition of the involvement of receptor heterodimers in the action of various hormones which include thyroid hormone, vitamin D and retinoic acid (Liao *et.al.*, 1990; Rosen *etal,* 1991) serve to point out the fact that the regular nuclear receptor homodimer is not in a position to account for the initiation of all the gene regulatory events in a given hormone-sensitive system. We, in our laboratory, have been focussing our attention on an estrogen receptor heterodimer in which one monomer binds the hormone while the other binds to the DNA. The receptor has been named as the non-activated estrogen receptor (Anuradha *et.al.*, 1994) since it did not display a DNA binding property under any circumstance. As a result, its partner in the heterodimer, the DNA binding protein, has been named as the estrogen receptor activation factor (E-RAF) in view of the fact that it provides the naER with access to the nuclei. The E-RAF has no capacity to bind estradiol.

The naER was purified to homogeneity and identified to possess a tyrosine kinase activity, capable of effecting both auto and casein phosphorylation (Anuradha *etal.,* 1994). During the course of this study it was noticed that the yield of the purified protein more than doubled when the original medium used in the homogenization contained Triton x-100. This observation, coupled with the assumption that being a tyrosine kinase, the naER could be grouped in a family of proteins that represented growth factors and oncoproteins, prompted us to examine whether naER is a plasma membrane constituent. The results were in the affirmative and the studies also generated some new information with regard to the ultimate biological role of this estrogen receptor.

naER FUNCTIONAL SIGNIFICANCE:

In order to identity a hormone-binding protein as the hormone receptor' it is essential that there should be some information which highlights the fact that the hormone binding to this protein results in some clear change in a given biochemical response. It is true that in this perspective the naER does not qualify at this stage to be identified as an estrogen receptor'. Nevertheless, the available evidences indicate that like any other steroid hormone receptor the site of action of naER is also the cell nucleus, through its postulated conversion to the R-II estrogen receptor. Therefore the naER will qualify to be identified as an estrogen receptor only when the true molecular mechanism of action of the R-II is deciphered. Till then the use of this terminology will remain tentative.

RESULTS

ALTERNATE METHOD FOR THE PURIFICATION OF THE GOAT UTERINE naER:

In the method published earlier from our laboratory (Anuradha *et.ai,* 1994) purification of naER involved three successive chromatographic steps viz. DE-52, phenyl agarose and hsp-90-Sepharose. It was observed that for reasons unknown, a partially proteolysed form of the naER sometimes co-purified with the undegraded naER. In order to avoid this some alterations were introduced into the purification protocol. This involved omission of the phenyl agarose chromatography step and introduction of chromatography on phosphocellulose after the completion of the hsp-90-Sepharose chromatography. The naER, firmly bound to the phosphocellulose column was eluted using a NaCl gradient (Fig. 11). The purified protein appeared as a single band of molecular mass 66 kDa in silver stained SDS-gels (gel shown in Fig. 17).

The yield of naER was further enhanced by the addition of detergents like Triton x-100 or NP-40 (0.2% v/v) to the homogenization buffer (data on recovery not shown). This was an indication to the fact that the naER is a membrane constituent. It was therefore decided to find out first whether the naER is a constituent of the plasma membrane.

ISOLATION OF GOAT UTERINE PLASMA MEMBRANE: DETERMINATION OF THE EXTENT OF PURITY OF THE MEMBRANE PREPARATION:

The method involved in the plasma membrane isolation has been detailed in the experimental section. The inset in figure 12 is a photograph of the sucrose density gradient in the centrifuge tube taken immediately after the termination of centrifugation. The arrow indicates the top layer of the gradient containing purified

Table 1

Analysis of various sub-cellular mailer enzymes in the plasma membrane.

Steps in plasma membrane isolation	ENZYMES			
	SDH[a]	G-6-Pase[b]	5'NUCLEO--TIDASE[b]	CTPase[b]
HOMOGENATE	**41.4**	**0.93**	**0.021**	**0.297**
SUPERNATANT (IOOOOXg)	**14.68**	**0.66**	**0.014**	**0.001**
PELLET (10000Xg)	**56.2**	0.05	**0.072**	**0.332**
PURIFIED PLASMA MEMBRANE	NIL	**0.001**	**2.642**	**0.718**

a) . Expressed as μmoles of dye reduced per mg protein per miniute

b). Expressed as μmoles of Pi released per mg protein per miniute

plasma membrane. The extent of purity of this preparation was determined following the analyses of various marker enzymes in this material. These included succinic dehydrogenase (SDH) and glucose-6-phosphatase (G-6-Pase), known for their localization in the mitochondrial and microsomal compartments respectively and CTPase and 5'nucleotidase, two enzymes known for their association with the plasma membrane. The results showed virtual absence of SDH and G-6-Pase in the membrane preparation and a simultaneous enrichment of CTPase and 5'nucleotidase in this fraction (Table 1).

EXPOSURE OF THE PLASMA MEMBRANE TO ESTRADIOL RESULTS IN DISSOCIATION OF AN ESTROGEN RECEPTOR FROM THE MEMBRANE TO THE MEDIUM:

The plasma membrane fraction suspended in TCMN buffer containing 0.25M sucrose (amounting to 50µg protein per tube) was incubated at 30oC for 30 minutes. Following centrifugation at 10000 X g for 15 minutes, the pellet which represented the washed plasma membrane was collected, resuspended in the same buffer and incubated with 20nM unlabelled estradiol at 30oC for varying time intervals. At the specified interval the tubes were cooled and centrifuged.

The estradiol binding activity associated with the protein components in the membrane pellets and the media were estimated using HAP assay as described in materials and methods'. Control' incubations were carried out using the membrane preparation in the absence of estradiol. It was observed that in the presence of estradiol a gradual release of a hormone binding component from the membrane to the medium took place (Fig. 12) This dissociation did not take place when the incubation was carried out in the absence of estradiol.

THE RELEASE OF ESTROGEN RECEPTOR FROM THE MEMBRANE IS EXCLUSIVELY DEPENDENT ON THE PRESENCE OF ESTROGENS:

Specificity of the hormone dependent release of plasma membrane proteins to the medium was examined using incubation media containing hormonal agents other than estradiol. Following a time dependent incubation of the plasma membrane with the hormone under study, the media were recovered and subjected to SDS gel electrophoresis. Three proteins bands, of molecular mass 66, 55 and 45 kDa were found dissociated from the membrane (Fig. 13 a and b). It was observed that the release of proteins from the membrane took place in the presence of estradiol or of diethylstilbestrol (20nM concentrations) and not in the presence of testosterone, progesterone, dexamethasone or tamoxifen (Fig. 13). Additionally, tamoxifen succeeded in preventing the protein release into the medium influenced by estradiol.

IMMUNOBLOT ANALYSIS OF THE PROTEINS PRESENT IN THE MEDIUM AFTER INCUBATION OF PLASMA MEMBRANE WITH ESTRADIOL:

Immunoblot analysis of the proteins dissociated into the medium following in vitro exposure of plasma membrane to estradiol showed that the anti ER IgG cross reacted with the 66 kDa band alone which showed a time dependent increase in the media and a corresponding decrease in the membrane pellet (Fig. 14). Further, confirmatory evidences were provided to show that ER release into the medium was stimulated only by estrogens.

EVIDENCES TO INDICATE THAT THE PLASMA MEMBRANE ESTROGEN RECEPTOR IS THE naER:

Four different criteria were taken into consideration in order to confirm the naER identity of the plasma membrane estrogen receptor:

a) Convergence in the nature of the purification steps identified for the cytosolic naER isolation.

b) Capacity to dimerize with E-RAF and as a consequence , bind to the DNA.

c) Peptide map comparison of the two proteins.

d) Comparison of the Stokes radii of the two purified proteins.

a). PURIFICATION OF THE PLASMA MEMBRANE RECEPTOR:

The plasma membrane-bound proteins were solubulized in TEM buffer containing 0.3M NaCl and 0.2% (v/v) of NP-40. This concentration of detergent was used because at this concentration the proteins have been shown to retain their activity; increasing the concentration of the detergent has been shown to lead to the loss of the activity as reported by Parikh *et.al.*, (1980). The material containing the solubilized proteins was mixed with TEM buffer in order to reduce the NaCl concentration and was chromatographed on a column of Whatman DE-52. The DE-52 bound receptors were eluted using a salt gradient. The fractions collected were assayed for receptor activity following HAP binding (Fig. 15). The fractions containing the peak receptor activity were pooled and chromatographed on a column of hsp-90-Sepharose.

The fractions containing peak activity from the DE-52 column were pooled and subjected to hsp-90-chromatography as described in the methods'. The fractions eluted from the hsp-90-Sepharose column were subjected to HAP adsorption assay. A single peak of receptor activity was observed (data not shown).

The plasma membrane receptor was purified to homogeneity following chromatography on phosphocellulose. The peak activity fractions obtained from the hsp-90-Sepharose column were pooled and chromatographed on a phosphocellulose column. The receptor bound to the column was eluted with a linear salt gradient of 0-1M NaCl. The fractions collected were subjected to HAP adsorption assay. A single peak of receptor activity was observed, getting eluted from the phosphocellulose column with 0.6-0.7M NaCl concentration (Fig. 15).

b). CAPACITY TO DIMERIZE WITH E-RAF:

The receptor purified from the plasma membrane and also that purified from the cytosol were subjected to DNA cellulose binding assay in the presence of pure E-RAF. Identical patterns of E-RAF dependent DNA binding activity were displayed by the two proteins (Fig. 16). The inset in figure 6 shows the activity of the receptor, dissociated into the medium following exposure of the plasma membrane to estradiol. This receptor was purified to homogeneity subsequently using the procedure described above. DNA binding of this receptor-hormone complex was dependent exclusively on the presence of the E-RAF in the assay medium (Fig. 16 inset).

c). PEPTIDE MAP COMPARISON:

The receptor extracted from the plasma membrane and the cytosolic naER displayed the same molecular mass, 66 kDa and also identical CNBr peptides (Fig. 17).

d). COMPARISON OF THE STOKES RADII:

The plasma membrane receptor displayed the same Stokes radius (36oA) as that of the cytosolic naER (Anuradha *et.al.*, 1994), as observed upon analysis on a column of Sphadex G-100 (Fig. 18). The plasma membrane ER, like its cytosolic counterpart, was found to sediment at 4.8 S (data not shown)

THE naER (EXTRACTED FROM BOTH THE PLASMA MEMBRANE AND CYTOSOL) IS A GLYCOPROTEIN:

Two independent methods were employed in this analysis: a) A concanavalin A linked specific staining of proteins that were transferred to nitrocellulose membrane; b) Chromatography of proteins on a column of concanavalin A Sepharose and elution using a medium containing methyl a-D-glucopyranoside. Both cytosolic naER (Karthikeyan and Thampan, 1994b) and plasma membrane receptor were stained by the dye during the procedure (Fig. 19 inset). Similarly, both the proteins were retained by the

con A-Sepharose column and eluted from the column with the buffer containing 0.3M methyl α-D glucopyranoside (Fig. 19).

RECOGNITION OF naER, PURIFIED FROM THE CYTOSOL AND ALSO THAT **FROM** THE PLASMA MEMBRANE BY ANTIPHOSPHOTYROSINE **IgG:**

Two different approaches were made:

a). Immuno recognition of naER and plasma membrane receptor, transferred to the nitrocellulose, by anti phosphotyrosine antibody and subsequent staining using horseradish peroxidase coupled anti rabbit IgG.

b). Chromatography of naER and plasma membrane receptor on a column of antiphoshotyrosine IgG coupled to Seoharose 4B and the elution of the protein using 0.2 M glycine, pH 2.8.

The antibody cross reacted with both cytosolic and plasma membrane receptors. It cross reacted with phosphotyrosine conjugated BSA but not with unconjugated bovine serum albumin (Fig. 20). Similarly both these proteins, but not the E-RAF, were retained by the immobilized antibody column, to be released following elution with 0.2 M glycine, pH 2.8 (Fig.20).

THE naER IS A TYROSINE KINASE:

A). *Autophosphorylation* by the naER:

Both the plasma membrane and the cytosolic naER showed the capacity for autophosphorylation. The activity increased along with the enhancement in the amount of protein employed (Fig 21 A and B).

B). Inhibitory effect of E-RAF:

Complete inhibition of the protein kinase activity was noticed when equimolar concentrations of E-RAF was present in the naER-protein kinase assay medium (Fig 21C). The activity detected was inversely proportional to the concentration of E-RAF

present in the medium.

C). **Inhibition of** naER ***autophosphorylation*** by Tvrphostin 25:

A graded decrease in the receptor phosphorylation was noticed when the incubation media contained increasing concentrations of the tyrosine kinase inhibitor, Tyrphostin 25 **(Fig.21D)**. Complete inhibition in the activity was noticed when the assay was carried out in the presence of 5|iM concentration of Tyrphostin 25.

D). Inhibition of phosphorylation by estrogens:

Both estradiol and diethylstilbestrol (20nM) inhibited the receptor autophosphorylation while no such inhibition was noticed when the incubation was carried out in the presence of testosterone, progesterone, **dexamethasone** or Tamoxifen **(Fig.21E)**. Further, Tamoxifen reversed the estradiol mediated inhibition of the protein kinase activity.

E). Phosphoaminoacid identification:

The phosphorylated naER (both of plasma membrane and of cytosol) was acid hydrolyzed and the hydrolysate was subjected to paper **chromatography** as described in ' methods', in the presence of authentic phosphoaminoacid standards. The results showed that the only phosphoaminoacid produced was phosphotyrosine **(Fig.21F)**.

DISCUSSION

Based on the molecular characteristics displayed by the non-activated estrogen receptor (naER), purified form the goat uterine cytosol (Anuradha *et.al.*, 1994) it is possible to state that this protein is different from the regular estrogen receptor (ER) in some features while remaining similar in others. The intracellular site of localization of the regular ER is known to be the nucleus (King and Greene, 1984; Welshons *et.al.*, 1984). In this context it was of interest to know the site of intracellular localization of this alternative form of estrogen receptor, the naER

As stated earlier it was the tyrosine kinase activity found associated with the naER (Anuradha *et.al.*, 1994) and the observation that a 100% increase in the yield of the naER was achieved following the introduction of detergents in the homogenization buffer that prompted us to examine whether the naER is a plasma membrane constituent. For this it was essential to have a clean preparation of the uterine plasma membrane, free of other membranous contaminants. From the data on the marker enzyme analysis presented here it is being shown that the preparation is highly pure as the recovery of mitochondrial and microsomal enzymes in the plasma membrane preparation was almost negligible. That the plasma membrane receptor and the cytosolic naER represent one and the same protein is highlighted by the following observations.

1). The protocols involved in the purification of the two proteins are identical.

2). Both the proteins are 66 kDa molecules with identical peptide maps and identical pI values (pI: 5.2).

3). Both cross react with the anti ER IgG.

4). Both dimerize with estrogen receptor activation factor (E-RAF).

5). They are both glycoproteins and easily bind to Con A Sepharose, to be eluted subsequently with methyl α-D-glucopyranoside

6). They have the same Stokes radii, identical patterns of saturation binding with estradiol and the same sedimentation values

7). Both the proteins are tyrosine kinases, capable of autophosphorylation. The two proteins are recognised by anti phosphotyrosine IgG the protein kinase activities were inhibited by their exposure to estradiol, DES, E-RAF or to the inhibitor Tyrphostin.

The precise molecular mechanisms that promote or underlie receptor internalization are not known. There are evidences to indicate that components of the cytoskeleton are related to the entry process for a wide variety of ligand-receptor complexes. There are indications that these activities are likely to be associated with fluxes of ca^{2+} or cyclic nucleotides. These are in turn associated with the phosphorylation/ dephosphorylation of integral plasma membrane proteins and cytoskeletal elements.

Estrogen receptor association with tubulin and actin have been subjected to study in our laboratory (Zafar, 1992; Zafar and Thampan, 1995). The association of ER with the cytoskeletal proteins and the subsequent influence that the receptor hormone complex exerts on their phosphorylation/dephosphorylation point towards the assumption that the receptor involved in the process was the naER. The naER association with the cytoskeletal proteins may be a process that immediately follows receptor internalization and also a mechanism directed toward the intracellular movement of the naER-E-RAF heterodimer. The membrane perturbation generated by

the receptor internalization may lead to a simultaneous entry of heterologous substances (Szego and Pietras, 1984) into the cytoplasm. The calcium entry into uterine cell (Pietras and Szego, 1975; Morley, *et. al,* 1992) that follows the cellular exposure to estradiol might well be a mechanism that is closely linked to this receptor internalization.

The subject of estrogen receptor localization in the uterine plasma membrane has been examined earlier by other research groups (Szego and Pietras, 1981; Muller *et.al.*, 1979) While studies carried out by Szego and Pietras (1981) provided positive identification for the presence of ER in the plasma membrane, Muller *et al.* (1979) concluded that what was detected in the plasma membrane was an artifact, due to cytoplasmic receptor contamination. There is enough evidence to argue against this artifactual association of naER with the plasma membrane. The plasma membrane preparation, in the present experiments is incubated initially for a period of 15 minutes at 30oC, which results in the removal of all the previously dissociated and loosely bound proteins from the membrane. Subsequent exposure of the membranes either to estradiol or to DES alone results in the receptor dissociation along with the 55 and the 45 kDa proteins. I am unable to comment upon the dissociation of the 55 and 45 kDa protein from the plasma membrane in the presence of estradiol. Even after 30 minutes of exposure of the membrane preparation to any of the other hormones tested, including estradiol in combination with Tamoxifen, no membrane protein entry into the medium could be observed. Any non-specifically associated cytosolic protein should have entered the medium from the membrane preparation under these conditions.

Estradiol entry into the uterine cell has been a controversial subject. While Muller *et al.* (1979) identified the hormone entry process as a passive diffusion

mechanism, Milgrom and co-workers (1973) favoured a protein mediated, facilitated transport of estradiol. Muller and co-workers carried out their studies using isolated cell suspensions They observed a specific estrogen binding mechanism in association with the rat uterine plasma membrane but not with the rat diaphragm membranes. Since this represented only 2 to 3 % of the total estrogen binding activity in the uterus, they assumed that what was identified in the plasma membrane was a contaminant from the cytoplasm. In order to test this hypothesis they exposed the uteri to high concentrations of unlabelled estradiol and mixed it with the cytosolic receptor preparation before the tissue was processed and the membranes isolated. They continued to find a small but significant level of estrogen binding in association with the plasma membrane, which they concluded as being a cytosolic contaminant.

Exposure of the uteri to $1X10^{-6}M$ estradiol as in the experiments carried out by Muller *et.al.*, (1979) should have caused the internalization of a significant quantity of the non-activated estrogen receptor (naER) from the plasma membrane. However, under the present experimental conditions It was not possible to bring out a total dissociation of the receptor from the membrane preparation. A residual amount of the activity continued to remain within the membrane as evidenced by the immunoblot analysis involving anti ER IgG. The reason behind this phenomenon remains unknown.

Milgrom and co-workers (1973) proposed that the entry of estrogens into the uterine cells was a protein mediated process. They observed that the hormone entry was a saturable process which could not be the case had it been the result of passive diffusion across the lipophilic framework of the plasma membrane. The only difference they observed was the reduced effectiveness of DES in comparison with estradiol in inhibiting 3H estradiol entry into the uterine cells. Nevertheless, non-estrogenic

steroids failed to compete with 3H-estradiol for the cellular entry. Further, estradiol entry into the uterine cells was found to be sensitive to SH blocking agents.

The assumption that the tyrosine kinase activity of the naER may not manifest within the cytosol is supported by the present experimental data Within the cytosol the naER remains as a glycoprotein and dimerizes with the E-RAF, a process that completely inhibits the protein kinase activities associated with the two proteins (Anuradha *et.al.*, 1994). Similarly, it enters the cytosol, from the plasma membrane, as a hormone receptor complex. So long as its estradiol binding sites are saturated, the tyrosine kinase activity of the protein remains totally repressed. This suggests that the conversion of the naER to R-II takes place within the nucleus possibly with the help of a nuclear glycopeptidase. The deglycosylated naER (R-II receptor) fails to dimerize with the E-RAF and has reduced affinity in contrast to the regular naER for binding estradiol. These changes assure that the tyrosine kinase activity of the R-II (modified naER) within the nucleus will not be influenced by these factors. A major protein phosphorylated by the R-II estrogen receptor appears to be the RNA polymerase II itself. Therefore, naER dependent phosphorylation of cytoplasmic proteins can take" place only under abnormal conditions when, due to mutations or other aberrations, the naER loses its property for estradiol binding and also for dimerization with E-RAF. Under such circumstances it is likely that the naER will fail to reach the nucleus.

Castoria *et.al.*, (1993) purified from the calf uterus a 67 kDa tyrosine kinase from the calf uterus that phosphorylated the estrogen receptor. I am, at present, not in a position to state whether this tyrosine kinase is the naER or whether it is a totally distinct protein

Figure 22 is a model in which I propose to summarize the mechanisms detected in this study. The naER is shown here as a plasma membrane constituent with a conformation that is dependent upon (he integrity of its carbohydrate residues. Estradiol, present in the extracellular medium, binds to the naER following which the receptor-hormone complex gets internalized. The internalized naER dimerizes with the estrogen receptor activation factor (E-RAF). The cross recognition of each other's nuclear localization sequences (Prem Kumar, 1994; Prem Kumar and Thampan, 1994) helps in the transport of the heterodimer into the nucleus.

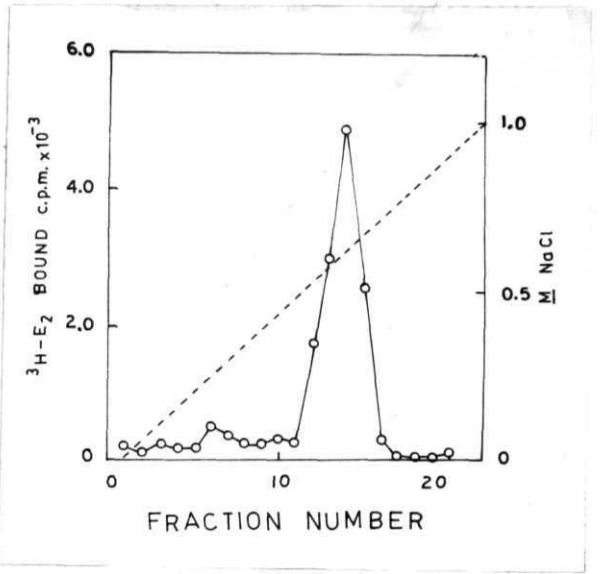
Eventhough Szego and co-workers made extensive studies in the past regarding what they beleived as estrogen receptor localized in the plasma membrane, no additional information on this topic have been generated by Szego's research group in the recent past. Therefore, I wish to exert caution in making comparisons between the naER and the receptor reported by Szego at this stage.

Fig. 11: Final step in the purification of naER through **chromatography** on phosphocellulose.

The peak receptor activity fractions eluted from the hsp 90-Sephnrose column using zero moylbdate buffer were chromatographed over a phosphocellulose column equilibrated with TEM buffer. Following washing, the proteins bound to phosphocellulose column were eluted using a 0-1M linear NaCl gardient in TEM buffer The factions collected were subjected to HAP binding assay as described in the `materials and methods'.

Fig. 12: Dissociation of estrogen receptor from the plasma membrane.

The plasma membrane preparation (50μg protein/assay tube) was subjectd to pre-incubation at 30°C for 15 minutes in order to remove all loosely bound proteins from the membrane preparation. The membranes, recovered following centrifugation were resuspended in TCMN-sucrose buffer and were exposed to 20nM estradiol at 30°C for varying time intervals. After incubation, the membranes were processed as described in `materials and methods'. The reaction volume was 150μl in total. The pellets were suspended in the buffer (same volume as that of the supernatant). Following this both the supernatant and the pellet recovered were subjected to HAP binding assay. The closed and open circles indicate the receptor activities in the supernatants and pellets respectively. The soild lines refer to the incubations in the presence of estradiol while the broken lines indicates the incubations carried out in the absence of estradiol. The inset shows the final separation of plasma membrane upon a sucrose density gradient, subjected to centrifugation as described in the materials and methods'. The arrow indicates the location of the purified plasma membrane preparation that was used in the current study.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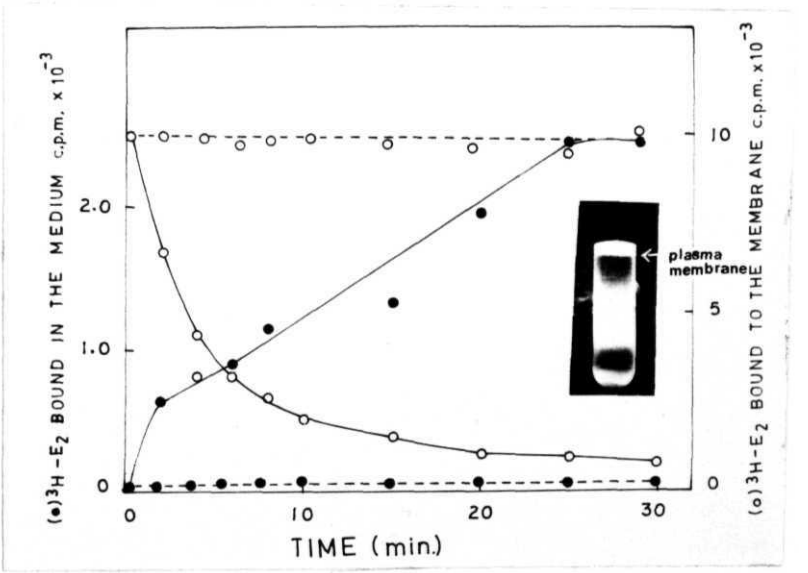
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plasma membrane
0
10
20
30
TIME (min.)

Fig. 12

Fig. 13: Hormone specificity in the release of receptor from the plasma membrane.

The incubation media containing palsma membrane were exposed to 20nM concentrations of (a) estradiol, (b) diethyl stilbestrol, (c) Tamoxifen, (d) Tamoxifen + estradiol, (e) progesterone, (0 testosterone or (h) dexamethasone; for different time intervals. V is the control samples containing no hormone. Following incubation the supernatants collected were lyophylized and later subjected to SDS-PAGE. The numbers on the gels indicate the time in minutes. `M' represents the lanes with marker proteins. The gels were stained with silver nitrate.

Fig. 14: Immunoblot analysis of the medium after incubation of plasma membrane with estradiol.

The plasma membrane fractions were incubated at 30oC with 20nM estradiol for different time intervals. The control' (C) contained no hormone. After incubation the membrane and the media were separated following centrifugation. The media were lyophylized. Both the media (B) and the membrane (A) were subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes. The nitrocellulose sheets were exposed first to anti ER IgG, and subsequently to HRPO coupled anti rabbit IgG. The blot was stained with 4-chloro-1-naphthol. The numbers on the blot indicate the time in minutes. c' refers to the lane having control samples.

(C) Immunoblots of the media collected upon incubating the membranes with 20nM concentration of estradiol (E2), Tamoxifen (T_x), progesterone (P), testosterone (T) dexamethasone (D_x) or diethyl stibestrol (DE) for 30 minutes at 30oC. The media collected were subjected to SDS-PAGE, followed by western transfer. The blot was exposed first to anti ER IgG and subsequently to HRPO-coupled anti rabbit IgG. The membrane was developed using 4-chloro-1-naphthol..

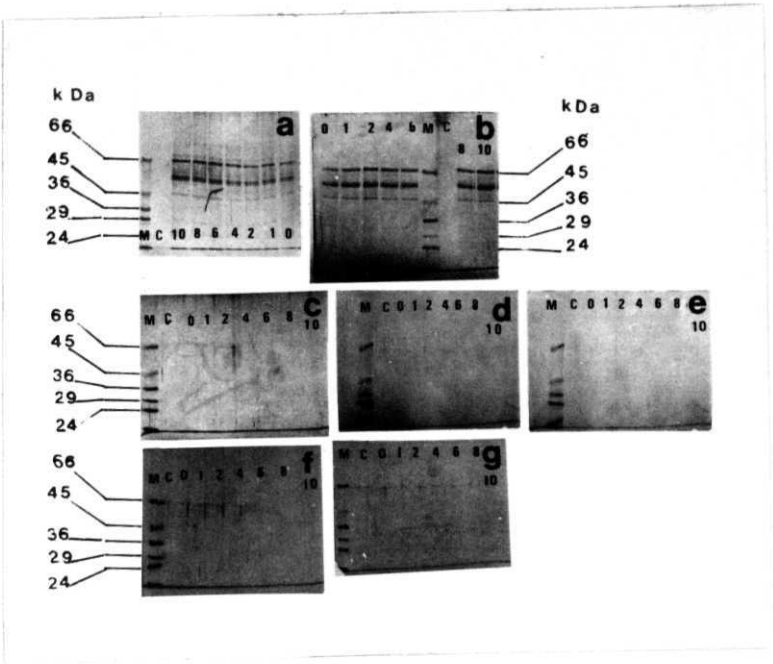
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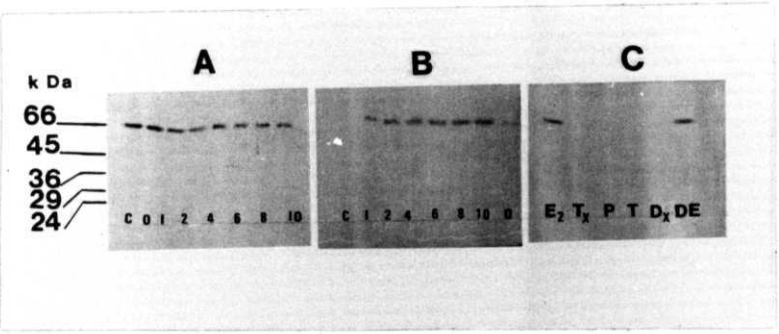
Fig. 13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E2 Tx P T Dx DE

Fig. 14

Fig. 15: Chromatographic purification of plasma membrane estrogen receptor.

(A). DE-52 chromatography: The purified plasma membrane preparation was suspended in TEM buffer containing 0.3M NaCl and 0 2% NP-40. Solubilized membrane proteins, separated from the membranes following centrifugation, were chromatographed on a Whatman DE-52 column equilibrated with TEM buffer. The column was washed with TEM buffer and the DE-52 bound proteins were eluted using 0-1M linear NaCl gradient in TEM buffer. Fractions collected were subjected to HAP binding assay as described in 'materials and methods'.

(B). Phosphocellulose chromatography; The DE-52 fractions containing peak receptor activity were pooled. The NaCl concentration was reduced to 0.05M by dilution. This material was chromatographed over a column of hsp 90-Sepharose equilibrated with TEM buffer containing 10mM NaMo: The column was washed with the same buffer and the proteins bound to hsp 90 were eluted using molybdate-free TEM buffer. The fractions collected were subjected to HAP binding assay. The fractions containing receptor activity were pooled and chromatographed over a column of phosphocellulose equilibrated with TEM buffer. The column was washed with TEM buffer and the proteins bound to phosphocellulose were eluted using a 0-1M linear NaCl gradient in TEM buffer. The eluate collected was subjected to HAP binding assay.

Fig. 16: DNA cellulose binding assay to detect the naER- E-RAF dimerization.

The ER purified from the plasma membrane (open cicles) and the naER purified from the cytosol (closed circles) were incubated overnight with 3H-estradiol at 4°C. The ER-3H-estradiol complex was recovered following treatment with dextran coated charcoal and was incubated with DNA cellulose and varying concentrations of E-RAF at 30°C for 30 minutes. The 3H-estradiol bound to the DNA cellulose pellet was measured. The inset shows the corresponding activity associated with the ER, dissociated from the plasma membrane following exposure to estradiol at 30°C for 30 minutes.

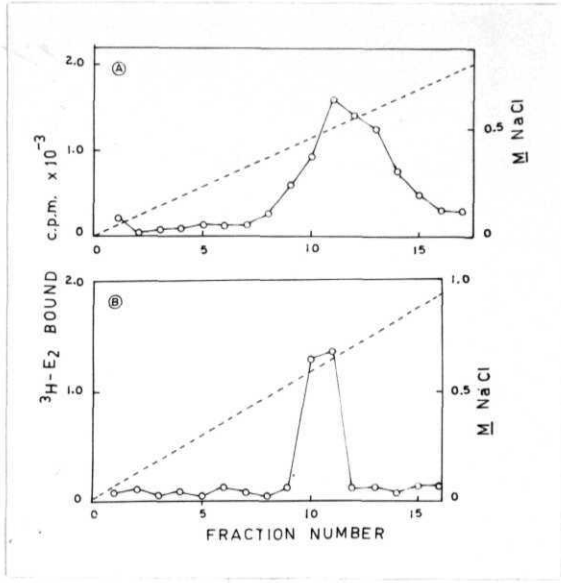
Ⓐ
Ⓑ
c.p.m. x10^{-3}
^{3}H-E$_2$ BOUND
M NaCl
FRACTION NUMBER

Fig. 15

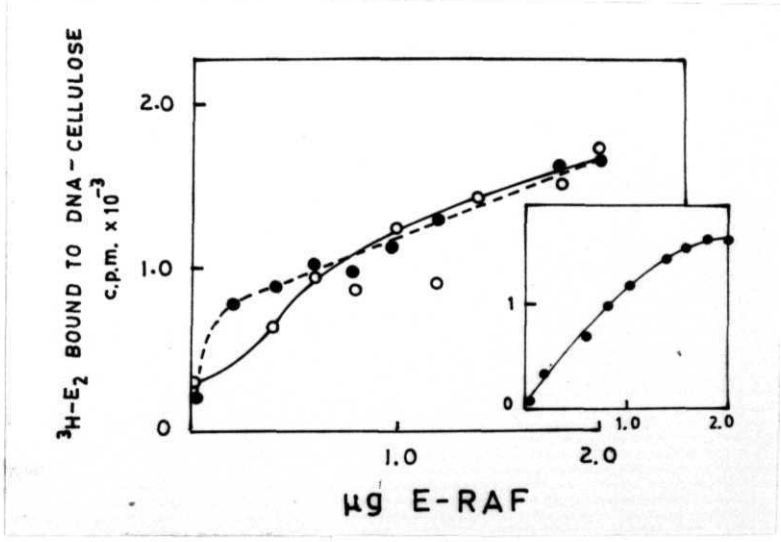
^{3}H-E$_2$ BOUND TO DNA-CELLULOSE
c.p.m. x10^{-3}
µg E-RAF

Fig. 16

Fig. 17: SDS-gel and CNBr peptides of the naER purified from the cytosol and of the ER purified from the plasma membrane.

`A'** and **`B' represent the SDS gels of the naER purified from the cytosol and the ER purified from the plasma membrane respectively. X' and **`D'** show the CNBr peptides of the plasma membrane ER and the cytosolic naER respectively.

Fig. 18: Gel filtration on Sephadex G-100 of the ER isolated from the plasma membrane.

The receptor purified from the plasma membrane was concentrated to 200µl and subjected to chromatography on a column of Sephadex G-100 equilibrated with TEMN buffer containing 0.3M NaCl. The column was developed with the same buffer and the fractions collected were subjected to HAP binding assay. The column was precalibrated with bovine serum albumin (1), ovalbumin (2) and cytochrome c (3). The Stokes radius calculated for the plasm membrane ER was the same as that identified for the cytosolic naER (Anuradha *et.al.*, 1994)

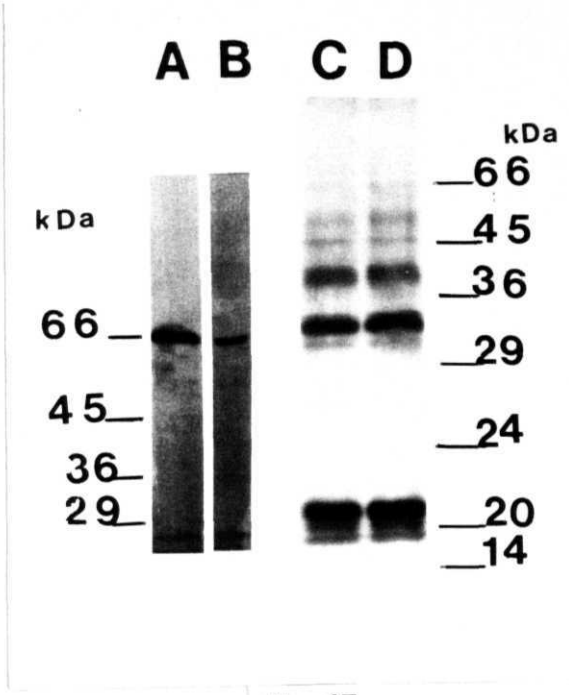
A B C D
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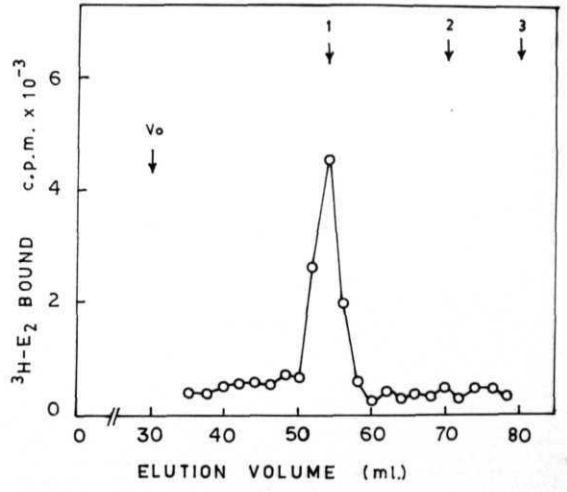
Fig. 17


1
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6
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40
50
60
70
80
ELUTION VOLUME (ml.)

Fig. 18

Fig. **19:** Detection of glycoprotein nature of the naER.

The purified receptor preparations were chromatographed on a column of concanavalin A Sepharose equilibrated with TCMMN buffer. The column was washed with the same buffer. The proteins bound to con A were eluted using 0.3M methyl a-D-glucopyranoside in TCMMN buffer. Fractions collected were measured for absorbance at 280nm. The open circles and the closed circles indicate the respective elution profiles of the plasma membrane ER and the cytosolic naER.

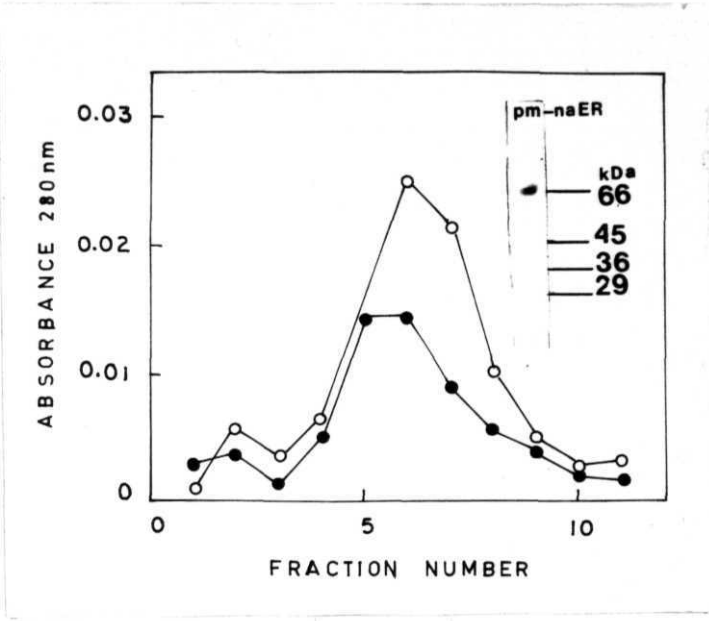
The inset shows a western blot of the plasma membrane receptor showing the presence of carbohydrate moiety in the receptor. The proteins were transferred to a nitrocellulose membrane. The membrane was first blocked with BSA and was later exposed to concanavalin A. The membrane was further exposed to HRPO and developed using 4-chloro-1-naphthol.

Fig. 20: Immunorecognition of naER and plasma membrnae receptors by anti-phosphotyrosine IgG.

The purified receptors were allowed to phosphorylate in the presence of ATP as described in materials and methods'. The phosphorylated proteins were chromatogrphed over a column of anti phosphotyrosine IgG Sepharose equilibrated with 10mM Tris HC1 pH 7.6 containing 12mM monothioglycerol, 6mM $MgCl_2$, 20mM NaCl and 0.2mM PMSF. The column was washed with the buffer and the proteins bound to the column were eluted using 0.2M glycine pH 2.8. Fractions collected were measured for their absorbance at 280nm. The open and the closed circles indicate the elution profile of plasma membrane receptor and that of the naER respectively. The triangles indicate the non-binding nature of the E-RAF, which is a serine kinase.

The inset displays the blots. `N' and `P' refer to the naER and plasma membrane receptor respectively recognized by anti phosphotyrosine IgG. The proteins were subjected to electrophoresis and subsequently transferred to a nitrocellulose membrane. The membrane was exposed to anti phosphotyrosine IgG and subsequently developed using 4-chloro-1-naphthol.

`A' and `B' are presented to show the specificity of the antibody. `A' shows the cross reactivity of the antibody with phosphotyrosine-BSA conjugate and `B' shows the absence of cross reactivity with unconjugated BSA. The proteins were western transferred to a nitrocellulose membrane following which the membrane was processed as described earlier.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FRACTION NUMBER
pm-naER
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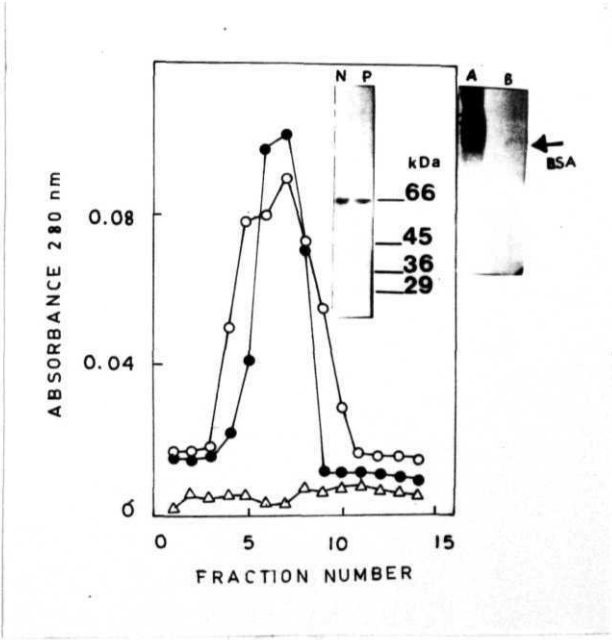
Fig. 19


ABSORBANCE 280 nm
0.08
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15
FRACTION NUMBER
N
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A
B
BSA
kDa
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36
29

Fig. 20

Fig. 21: **Autophosphorylation** of the receptors and the identity of phospho aminoacid formed.

'A' shows the autophosphorylation of the plasma membrane receptor and **`B'** displays the autophosphorylation of the cytosolic naER. The numbers on the **autoradiogram** indicates the concentration of the receptor used in the incubation medium. **`C'** shows the effect of E-RAF on the autophosphorylation of the plasma membrane receptor. The plasma membrane receptor (10μg) was incubated in the presence of different concentrations (μg) of E-RAF (represented by the numbers on the autoradiogram).

`D' shows the effect of the tyrosine kinase inhibitor (Tyrphostin 25) on the autophosphorylation of the plasma membrane receptor **(right)** and the cytosolic naER **(left).** 10μg of the receptor was incubated with different concentrations (μM) of Tyrphostin 25 (indicated by the numbers on the autoradiogram).

`E' shows the effect of estradiol, Tamoxifen or **diethylstibestrol** on the autophosphrylation of the plasma membrane receptor (right) and of the cytosolic naER **(left).** The receptors were incubated with 20nM concentrations of estradiol **(E_2),** Tamoxifen **(Tx),** Tamoxifen + estradiol (ET) or diethylstibestrol **(DE)**.

`F'** represents the identification of the phosphorylated aminoacids associated with the autophosphorylated naER and the plasma membrane receptor. The phosphorylated proteins were subjected to acid hydrolysis and the hydrolysates were subjected to paper **chromatography** as described in `materilas and methods'. Phospho tyrosine **(P-Tyr), phospho serine **(P-Ser)** and phospho threonine **(P-Thr)** were used as standards. The chromatograms were subjected to **autoradiography**.

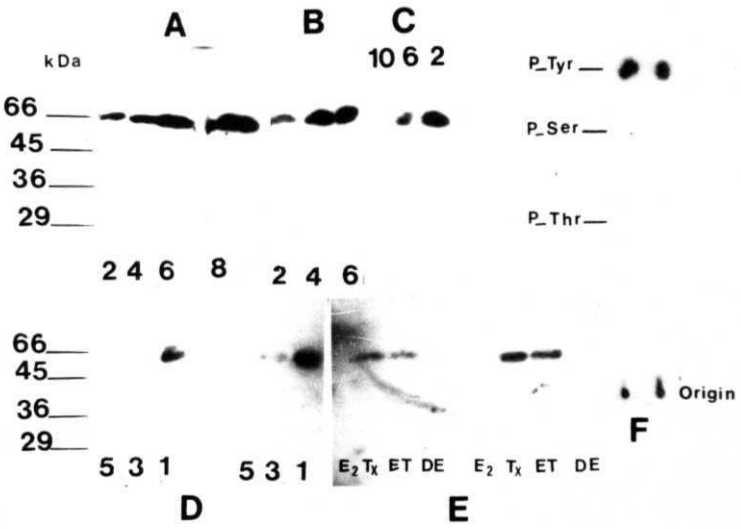

A
B
C
kDa
10 6 2
P_Tyr
P_Ser
P_Thr
66
45
36
29
2 4 6 8
2 4 6
66
45
36
29
Origin
F
5 3 1
5 3 1
E2 Tx ET DE
E2 Tx ET DE
D
E

Fig. 21

Fig. 22: A model summarizing the observations presented in this chapter.

Stage (1): The naER is shown here as a constituent of the plasma membrane. The sugar residue is shown in such a way as to emphasize its role in the maintenance of the structural integrity of the naER. Stage (2): Estradiol binds to the naER. Stage (3): The internalization of the hormone-receptor complex takes place. Stage (4): The internalized naER dimerizes with the estrogen receptor activation factor (E-RAF). The heterodimer thus formed enters the nucleus.

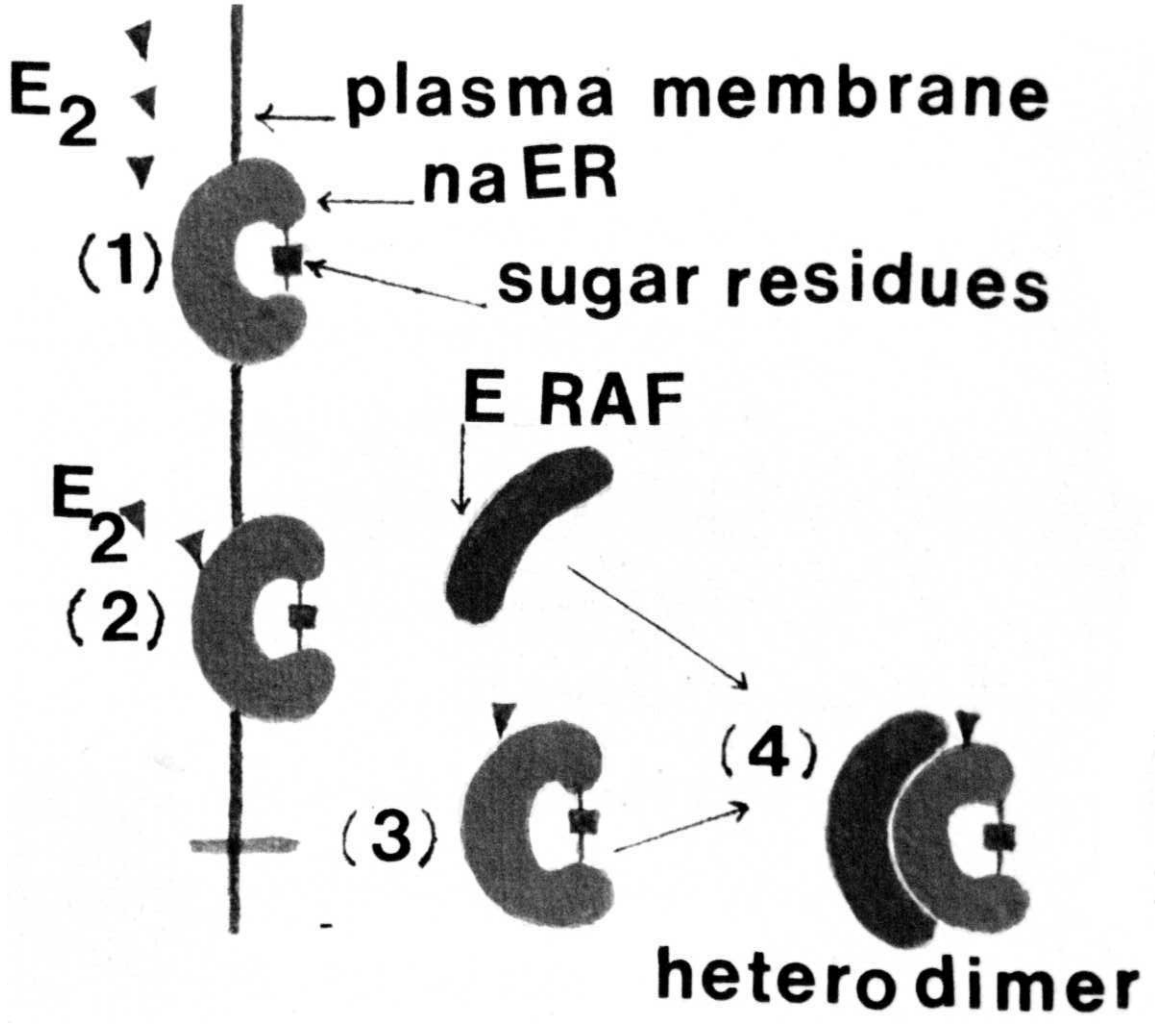

E2
plasma membrane
naER
(1)
sugar residues
E RAF
E2
(2)
(4)
(3)
hetero dimer

Fig. 22

CHAPTER V

THE NUCLEAR ESTROGEN RECEPTOR R-II OF THE GOAT UTERUS:

DISTINCT POSSIBILITY THAT THE R-II IS THE DEGLYCOSYLATED FORM OF THE NON-A CTIVATED ESTROGEN RECEPTOR (naER).

The type II nuclear estrogen receptor of the mammalian uterus was first identified by Eriksson *et.ai,* (1978) several years ago Subsequent studies carried out by Clark and his associates revealed that the presence of this receptor was closely linked to active transcription and cellular proliferation in the rat uterus (Clark *et.al.*, 1979; Markaverich *et.ai,* 1989; Markaverich *et.ai,* 1990). In comparison to the regular nuclear estrogen receptor homodimer, this receptor possessed greater capacity for estradiol binding, with a concomitant reduction in its affinity. Purification and characterization of the type II estrogen receptor posed a challenging task to investigators for several years.

During his studies on the rat uterine estrogen receptor activation factor (E-RAF), Thampan observed that the nuclear type II estrogen receptor was predominantly associated with the nuclear RNA polymearses and also that it was incapable of binding to the DNA (Thampan, 1983; 1989). It was this observation that prompted us to design a method for the purification of the estrogen receptor, type-II. we have succeeded in our attempt to purify and characterize this nuclear receptor (Karthikeyan and Thampan 1994a; chapter III of this thesis). However, in order to avoid controversies we decided to name it as R-II.

Purification and characterization of the goat uterine non-activated estrogen receptor (naER) has been accomplished (Anuradha *et.ai,* 1994; Karthikeyan and Thampan 1994b; chapter IV of this thesis). It was the absolute identity observed in the procedures employed in the purification of the cytosolic naER and those of the nuclear R-II that projected the question as to whether the two proteins were one and the same. Nevertheless, the noticeable differences between the hormone binding characteristics and the structural features of the naER and those of the R-II served to abandon, temporarily, experiments aimed to test this view point. The discovery of the glycoprotein nature of the naER (Karthikeyan and Thampan 1994b; chapter IV of this thesis) and of the absence of carbohydrate residues in the R-II served to re-initiate this line of thought. With the results presented in this chapter I feel that I have nearly succeeded in identifying the precursor-product relationship that exists between the

cytosolic naER and the nuclear R-II, respectively.

As mentioned earlier, in the previous chapter, it is imperative that a hormone binding protein comes to be identified as a hormone receptor only when the hormone-protein interaction is shown to lead to a cascade of biological responses. The present evidences show that the R-II binds to the nucleaar RNA porymearse n. Whether this interaction will result in activation of the RNA porymearse and the consequent enhancement in the expression of a given gene can not be addressed at this stage, since I do not have the neccessary experimental evidence now. It requires additional experimentation to understand the molecular biology of the R-II. Till then the use of the terminology receptor' for this high affinity estrogen-binding protein may not seem realistic.

RESULTS

PURIFICATION OF NUCLEAR ESTROGEN RECEPTOR **R-II:**

Using the method published earlier (Karthikeyan and Thampan, 1994a; chapter III of theis thesis) it was not possible to isolate a highly pure preparation of nuclear R-II receptor since a 55 kDa protein, presumably a proteolytic product of the **R-II,** co-purified with the 66 kDa protein. In the modified procedure presented here a phosphocellulose chromatography step has been introduced. This was the only change that was introduced when the original naER isolation procedure (Anuradha *et.al.*, 1994) was modified. As a result I have succeeded in isolating a highly purified preparation of R-II (Fig.23; SDS gel given in Fig.28).

COMPARATIVE STUDIES USING **R-II** AND naER:

A). SATURATION BINDING OF **ESTRADIOL:**

The saturation binding data for the naER showed a parabolic curve while that for the R-II had a sigmoidal shape (Fig.24). While saturation in hormone binding to naER was achieved in the presence of 15-16nM estradiol, saturation binding to R-II required double this concentration of estradiol, 30-40nM.

B). STOKES RADIUS AND SEDIMENTATION COEFFICIENT:

Stokes radii of R-II and naER were calculated following chromatography of the proteins on a column of Sephadex G-100 equilibrated with the TEMN buffer containing 0.3M NaCl. The column was precalibrated. The Stokes radii of the proteins were calculated as described by Siegel and Monty (1966). While the naER displayed a Stokes radius of 36oA, the R-II showed a value of 22oA for its Stokes radius (Fig.25). While naER showed a sedimentation value of 4.6S, the R-II was seen to sediment at 3.8S (Fig.25 inset).

C). E-RAF DEPENDENT BINDING TO DNA:

Binding of the receptor-hormone complex to DNA, in the presence of varying concentrations of E-RAF was examined. The naER displayed a proportional increase in its **DNA** binding capacity in response to the increase in the concentration of E-RAF **used** in the medium until the two proteins arrived at an **equimolar** concentration (Fig.26). On the other hand, the R-II failed to bind to the DNA in the presence of E-RAF (Fig.26).

D). EXAMINATION OF THE GLYCOPROTEIN IDENTITY:

Two different experimental approaches were made: 1) concanavalin A mediated staining of glycoproteins; 2) chromatography on a column of concanavalin A-Sepharose and elution with a buffer containing methyl a-D-glucopyranoside. As observed earlier (Karthikeyan and Thampan, 1994b) the naER clearly showed its identity as a glycoprotein. It appeared as a stained protein in the blot and showed excellent capacity to bind to con A-Sepharose, to be eluted with 0.3M methyl a-D-glucopyranoside in TCMMN buffer. On the other hand, there was no glycoprotein identity associated with the nuclear receptors R-II and R-I (Fig.27 and inset).

E). PEPTIDE MAPS:

The purified R-II appeared as a single homogeneous band of 66 kDa in silver stained SDS gel (Fig.28). CNBr peptides of the naER and the R-II were subjected to comparison. The peptide maps displayed by the two proteins were identical (Fig.28). The peptide map of the nuclear receptor R-I which is totally different from those of **R-II** and naER has been shown here for comparison.

EXPERIMENTS TO EXAMINE WHETHER naER COULD BE CONVERTED TO R-II;

The identical methods of purification, the same molecular mass, identical nature of CNBr peptides and the recognition by anti ER IgG of both naER and ER, all pointed towards the fact that the R-II may be a modified naER. Since the major structural difference between the two proteins pertained to the absence of carbohydrate residues associated with the R-II, it was decided to find out whether the naER to R-II conversion could be accomplished by deglycosylating the naER.

The details regarding the reaction media used in the deglycosylation of naER by the glycopeptidase have been given in the 'methods'. Three criteria were examined for the naER to R-II conversion: a) change in the saturation binding data for estradiol binding; b) loss in the capacity of naER to dimerize with E-RAF and the consequent binding to the DNA ;c) alteration in the Stokes radius of the naER, as shown by the chromatographic elution profile on a Sephadex G-100 column. The results are presented in Figure 29.

The glycopeptidase treatment of the naER caused a distinct change in the estradiol binding properties of the naER. While the control naER showed the typical saturation curve, arriving at saturation binding with 15-16nM estradiol, the glycopeptidase treated protein achieved saturation hormone binding in the presence of 30-32nM estradiol (Fig 29A). Treatment of the naER with the glycopeptidase virtually destroyed its capacity to dimerize with E-RAF and bind to the DNA (Fig.29B). Also, the enzyme treatment made a significant change in the Stokes radius of the naER, from 36oA to 23 oA (Fig.29C). The sedimentation values calculated for the treated and untreated naER were 3.9S and 4.6S respectively (data not shown).

RECOGNITION OF **R-II,** BUT NOT THE **R-I** BY **ANTIPHOSPHOTYROSINE IgG:**

As described earlier (Karthikeyan and Thampan, 1994b; chapter IV of this thesis) for the analysis of the naER using anti phosphotyrosine IgG, two types of experiments were performed: 1) immunorecognition of the receptor immobilized on nitrocellulose membrane by anti phosphotyrosine-BSA IgG; 2) chromatography of the receptor protein on a column of anti phosphotyrosine IgG-Sepharose and the subsequent elution using 0.2M glycine, pH 2.8. The anti phosphotyrosine antibody crossreacted with the R-II, but not with the R-I nuclear estrogen receptor (Fig.30). Similarly, the R-II but not the R-I was retained by the immunoaffinity column (Fig.30).

ASSOCIATION OF TYROSINE KINASE ACTIVITY **WITH** THE **R-II,** BUT NOT WITH THE **R-I:**

A). **AUTOPHOSPHORYLATION:**

An increase in the autophosphorylation of R-II was observed in relation to the increase in the R-II protein concentration employed (Fig.31 A). No such activity was demonstrated by the R-I (Fig.31B).

B). ANTAGONISM BY ESTROGENS:

The autophosphorylation of R-II was not inhibited by the presence of estradiol or of diethylstilbestrol, a feature in which the R-II differed from the naER (data not shown).

C). EFFECT OF E-RAF:

E-RAF failed to inhibit the R-II autophosphorylation (Fig.31C). While equimolar concentration of E-RAF totally inhibited naER autophosphorylation (Fig.31C and D).

D). EFFECT OF TYRPHOSTIN 25:

A graded decrease in the R-II autophosphorylation was observed in response to an increase in the dose of Tyrphostin 25 employed (Fig.31E).

E). IDENTITY OF THE AMINO ACID PHOSPHORYLATED:

The phosphoaminoacid present in the autophosphorylated R-II was identified as phosphotyrosine (Fig.31F).

R-II BINDS TO NUCLEAR RNA POLYMERASE II:

Cytosolic naER and the R-II nuclear estrogen receptors were labeled with fluorescein isothiocyanate and the binding of these proteins to RNA polymerase II was studied as given in the experimental section A very clear binding of R-II to RNA polymerase was observed as demonstrated by increasing transfer of fluroscence from R-II to the RNA polymerase (Fig.32). The activity was not so pronounced with the naER as it showed only 1/4 the activity that was associated with the R-II.

PHOSPHORYLATION OF RNA POLYMERASE II BY R-II:

The phosphorylation of RNA polymerase was studied both in the presence and in the absence of estrogen receptors. In the absence of any added protein two subunits of the RNA polymerase (molecular mass 105 kDa and 30 kDa) were found phosphorylated. Two additional protiens were found phosphorylatd (molecular mass 66 kDa and 40 kDa) in the presence of naER and two others (molecular mass 91 kDa and 20 kDa in addition to the 40 kDa) in the presence of R-II (Fig.33). The 66 kDa protein that was observed phosphorylated in the naER-protein kinase system was not observed in the R-II containing media.

TABLE 2:

COMPARISON OF STRUCTURAL AND FUNCTIONAL FEAUTURES OF naER WITH THOSE OF DEGLYCOSYLATED naER AND OF R-II:

A).

	Mol. mass	CNBr peptides	Cross reactivity with anti ER IgG	Tyrosine kinase activity
naER	66 kDa	IDENTICAL	+	**+**
Deglycosy--lated naER	66 kDa	IDENTICAL	**+**	**+**
R-II Receptor	66 kDa	IDENTICAL	**+**	**+**

B).

	Sediment--ation values (S)	Stokes radius **(°A)**	Dimerizat--ion with E-RAF	Saturation binding **of** estradiol	Glycoprot--**ein nature**
naER	4.6	36	YES	20nM	YES
Deglycosy--lated **naER**	3.9	23	NO	**30nM**	NO
R-II Receptor	3.8	23	NO	30nM	NO

DISCUSSION

Purification and characterization of the Type II (R-II) nuclear estrogen receptor of the goat uterus was reported by us earlier (Karthikeyan and Thampan, 1994a). The R-II receptor thus purified showed the presence of two bands: one at 66 kDa and the other at 55 kDa. A similar feature was often encountered during the purification of the cytosolic naER as well. This problem was circumvented following the introduction of a phosphocellulose chromatography step in the purification protocol whereby the 66 kDa protein was purified to absolute homogeniety (Karthikeyan and Thampan, 1994b; chapter IV of this thesis). The same modification has been introduced into the protocol for the purification of nuclear R-II estrogen receptor. The naER from the cytosol and that from the plasma membrane and the nuclear R-II were all purified following an identical procedure. The two proteins share the same molecular weight, display identical peptide maps and are both recognized by anti ER IgG. Like the naER the nuclear R-II is a tyrosine kinase; the two proteins crossreact with anti phosphotyrosine IgG. Further, the tyrosine kinase activities of the two proteins are blocked by the inhibitor, Tyrphostin 25. They display the same pI values (5.2). These identical features of the two proteins indicated the possibility that the two receptor forms may represent one and the same protein with altered structural and functional features. Table 2 presents a comparative data on these proteins. As can be observed, the deglycosylation of naER produced drastic changes in the structural and functional characteristics of the protein. The change in Stokes radius from 36oA to 23oA and sedimentation values from 4.6S to 3.9S brought these physical features of the altered naER very close to the corresponding features of the R-II. The deglycosylation did not change the molecular mass of the naER; it remained at 66 kDa. We have, therefore generated all possible evidences except those regarding the N-terminal / C-terminal residues in order to confirm that R-II is the deglycosylated naER.

The features in which the nuclear R-II differs from the naER are as follows:

1). R-II has no capacity to dimerize with E-RAF. E-RAF forms a heterodimer with naER

2). R-II is not a glycoprotein while naER is one as evidenced by specific staining in the concanavalin A horse radish peroxidase method and also its retention on a con A Sepharose column, to be eluted by methyl α-D-glucopyranoside.

3). R-II has lower Stokes radius in comparison to the naER and a lower sedimentation value too.

4). Saturation binding of estradiol to naER is a parabola, reaching saturation limits with approximately 15 nM estradiol. On the contrary R-II displays a sigmoidal relationship in hormone binding, reaching saturation binding with approximately 30nM estradiol. The much higher capacity of the R-II to bind estradiol in comparison with naER is associated with a drastic reduction in the former's affinity for the hormone.

5). Tyrosine phosphorylation by naER is blocked by its exposure either to estradiol or to E-RAF while these have no influence on the tyrosine phosphorylation effected by R-II

Since the distinct structural feature in which naER differed from the R-II was the glycoprotein nature of the former, it was decided to examine whether deglycosylaion of naER using a glycopeptidase introduced the necessary structural changes in the protein facilitating its conversion to R-II. The results of the experiments provided positive indications in this direction. Exposure to the glycopeptidase destroyed the capacity of naER to dimerize with E-RAF. The enzyme treatment reduced the Stokes radius of the naER to the R-II level. The enzyme treated naER showed a much higher capacity to bind estradiol than the untreated control. Possibly, the loss of the carbohydrate associated with naER as a result of the glycopeptidase action brought about irreversible structural changes in the protein. Consequently a distinct alteration in its shape could have taken place as evidenced by the reduced Stokes radius value The structural changes also may have resulted in the

exposure of additional estradiol binding sites characteristic of the R-II receptor. The ultimate result of these changes was the apparent loss of the property of the naER to dimerize with the E-RAF

It is possible that the naER to R-II conversion is brought about by a nuclear glycopeptidase. Had this enzyme been present in the cytosol the naER-E-RAF dimerization would not have materialized There is enough experimental evidence to indicate that the two proteins are totally dependent on each other for their nuclear movement. Absence of either one in the medium abolishes the nuclear entry of **the** surviving protein (Premkumar, 1994).

The decreased affinity of the naER brought about by its deglycosylation in the nucleus would indicate that the hormone originally bound to the naER in the heterodimer is liable for rapid dissociation. This view is in perfect agreement with the data reported by Clark and colleagues regarding the type II estrogen binding sites of the rat uterus (Clark *et.al*., 1979). In their studies Clark *et. ai,* (1979) found it **not** possible to pre label the type-II following its exposure to labelled estradiol as **the** hormone bound to the receptor dissociated rapidly. It was always necessary to identify its presence in nuclei through nuclear exchange assay or hydroxylamatite binding assay. The presence or absence of estradiol did not make any difference in the capacity of R-II either to autophosphorylate or phosphorylate RNA polymearse II subunits. This would mean that dissociation of the hormone bound to the naER that occurs as a result of its conversion to R-II is a necessary molecular event, making estradiol available for binding to the type-I estrogen receptor.

There is a distinct difference between the characteristics of the R-II in binding to and phosphorylating the RNA polymerase and those of the unmodified naER. In **the** experiment in which the binding of FITC-labelled naER or **R-II** to the RNA polymerase II was examined the two proteins were seen to follow a ~ 4:1 ratio in the respective capacities of R-II and naER to bind to the enzyme. The RNA polymerase II

is known to have intrinsic protein kinase activity (Edwards, et.al., 1990; Kolodziez et.al., 1990). This apparently results in the phosphorylation of 105 kDa and 30 kDa subunits of the enzyme. In the presence of naER two additional proteins were seen phosphorylated, of which the 66 kDa protein is most likely the naER itself. The other is the 40 kDa subunit. In addition to the 40 kDa subunits, two others were phosphorylated by the R-II; the 91 kDa and the 20 kDa subunits. No 66 kDa phosphorylated protein was found associated with the RNA polymerase phosphorylated by R-II receptor.

Evidences have been provided (Karthikeyan and Thampan, 1994; chapter IV of this thesis) for the plasma membrane origin of the naER To describe this plasma membrane estrogen receptor mediated events as `non-genomic action of estradiol' is incorrect. The site of action of the heterodimer is the nucleus. This nuclear movement of the heterodimer is influenced by the binding of a monomer to the nuclear localization sequences in the other monomer (Premkumar, 1994). The remaining sequences of reactions are presented in the model given in figure 34. Once within the nucleus the possible glycopeptidase action may dissociate the heterodimer into its monomeric units. The modified naER, now known as R-II, binds to nuclear RNA polymerase II while E-RAF, an activator of transcription (Thampan, 1989), binds to the gene. The exact gene location to which E-RAF binds remains unknown. R-II phosphorylates the RNA polymerase II; how this phosphorylation of three subunits of RNA polymerase II by the R-II influences transcription cannot be explained at this stage.

Fig. 23: Phosphocellulose chromatography during the final purification of the R-II nuclear estrogen receptor.

The fractions containing the peak receptor activity eluted from the hsp 90-Sepharose column (carried out as described in 'methods'), were chromatographed on a phosphocellulose column equilibrated with TEM buffer. The column was washed with TEM buffer. The receptor protein bound to phosphocellulose was eluted using a 0-1M linear NaCl gradient in TEM buffer. The fractions collected were subjected to HAP binding assay.

Fig. 24: Saturation binding of estradiol to naER and R-II receptor.

The purified receptor preparations were incubated overnight at 4oC with different concentrations of [3H]-estradiol (4-40nM) + 100 fold molar excess of DES. HAP binding assay was carried out at the end of the incubation. The specific binding activity was calculated for naER (open circles) and R-II (closed circles) by deducting the respective non-specific binding data from the total.

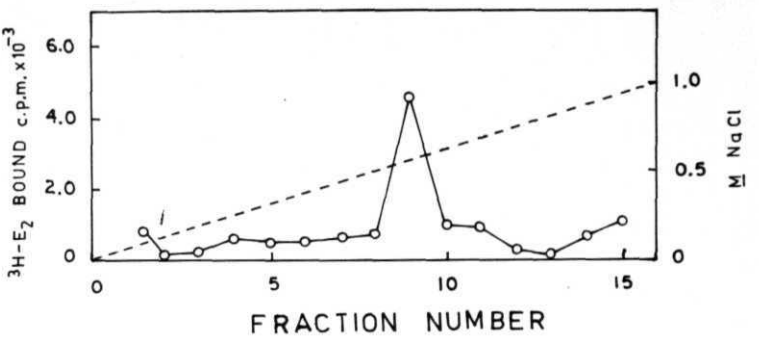

^3H-E_2 BOUND c.p.m. x 10^{-3}
6.0
4.0
2.0
0
0
5
10
15
FRACTION NUMBER
1.0
0.5
0
M NaCl

Fig. 23

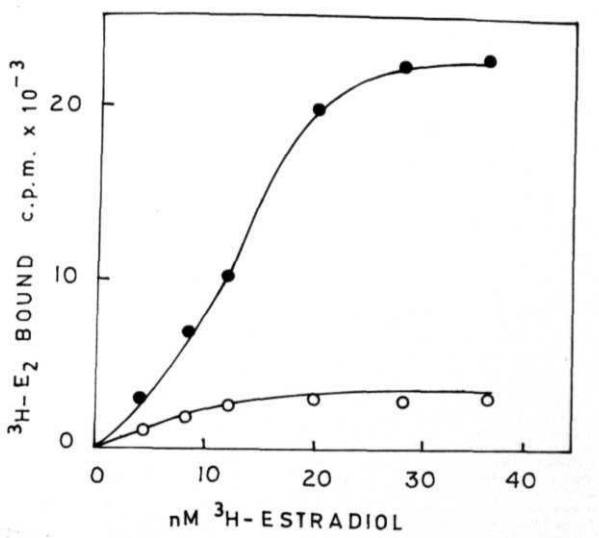

^3H-E_2 BOUND c.p.m. x 10^{-3}
20
10
0
0
10
20
30
40
nM ^3H-ESTRADIOL

Fig. 24

Fig. 25: Comparison of the Stokes radius and sedimentation coefficient of the naR with the corresponding values of R-II.

Sedimentation values were calculated folowing sucrose density gradient centrifugation and Stokes radii following gel filtration of the proteins on Sephadex G-100. naER is represented by the open circles and the R-II by the closed circles.

Fig. 26: E-RAF dependent DNA cellulose binding assay for naER and R-II.

The purified receptors were incubated overnight (4oC) with [3H] estradiol (20 nM for naER and 40nM for R-II receptor). The hormone receptor complex, isolated following DCC treatment of the incubated media, was further incubated with DNA cellulose in the presence of varying concentrations of E-RAF. The E-RAF dependent DNA cellulose binding assay was carried out as described in materials and methods'. The closed and open circles indicate the activity associated with the naER and the R-II receptor respectively.

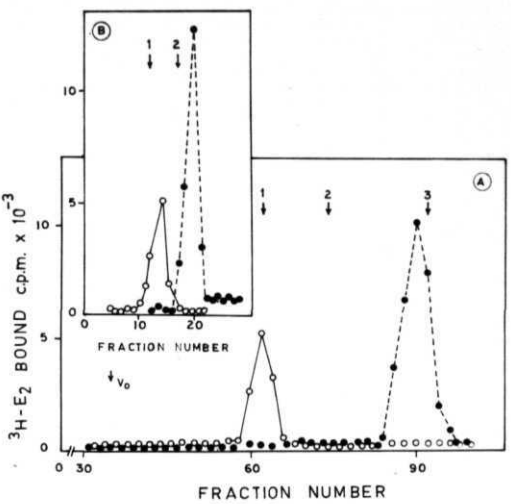

B
A
1
2
3
^{3}H-E$_2$ BOUND c.p.m. x 10^{-3}
FRACTION NUMBER
V$_o$
0
30
60
90

Fig. 25

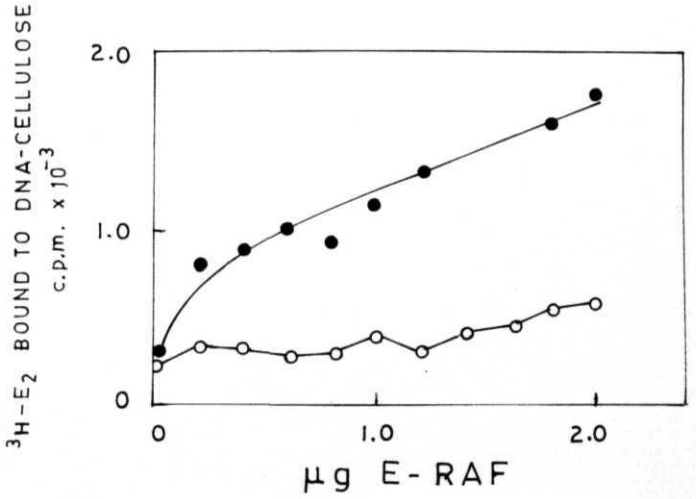

^{3}H-E$_2$ BOUND TO DNA-CELLULOSE
c.p.m. x 10^{-3}
2.0
1.0
0
1.0
2.0
µg E-RAF

Fig. 26

Fig. 27: Detection of carbohydrate moiety in the receptor.

The purified naER and R-II receptors were chromatographed over a concanavalin A Sepharose column (3ml) equilibrated with TCMMN buffer. The column was washed with the same buffer and the con A-bound proteins were eluted using 0.3M methyl α-D-glucopyranoside in TCMMN buffer. The eluate, collected in fractions, was measured for its absorbance at 280nm. The closed and open circles indicate the elution profile of naER and that of R-II receptor respectively. The triangles indicate the activity associated with the R-I nuclear estrogen receptor.

The inset is a blot showing con-A dependent staining for carbohydrate moiety. The proteins were transferred to a nitrocellulose membrane. The membrane was processed as described in materials and methods'. `N' represents the naER, 'R II' the R-II receptor and R I' the R-I receptor.

Fig. 28: Electrophoretic analysis of receptors.

'A' shows the SDS gel of the purified R-II receptor. The fractions containing peak receptor activity eluted from the phosphocellulose column were pooled, concentrated and the proteins precipitated with trichloro acetic acid. The precipitated protein was subjected to electrophoresis on a 10% SDS gel followed by silver staining.

`B', *C and `D' shows the peptides generated following CNBr fragmentation of naER (B), R-II receptor (C) and R-I receptor (D) respectively. The purified protein (100μg) was dissolved in 10μl of 70% formic acid. To this was added 5mg of solid CNBr. The reaction was allowed to proceed for 60 minutes at room temperature following which it was stopped by the addition of 1ml of distilled water. The lyophylized proteins were subjected to electrophoresis on a 15% SDS gel. The gels were stained with silver nitrate.

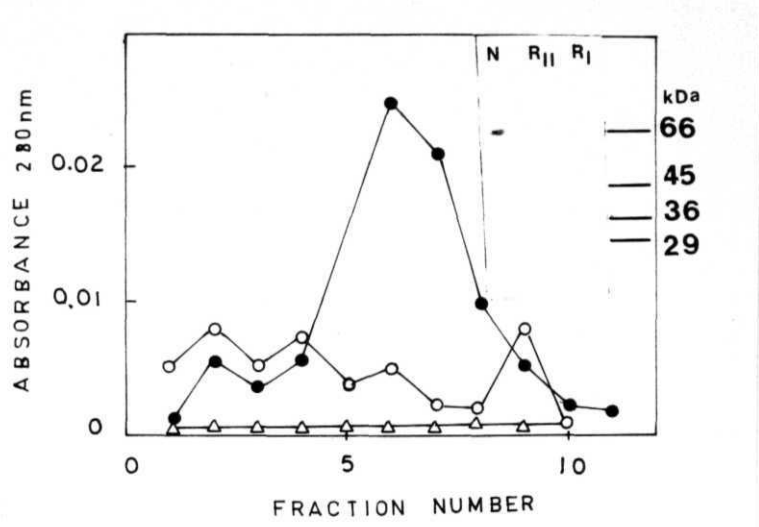

ABSORBANCE 280nm
0.02
0.01
0
0
5
10
FRACTION NUMBER
N
R_{II}
R_I
kDa
66
45
36
29

Fig. 27

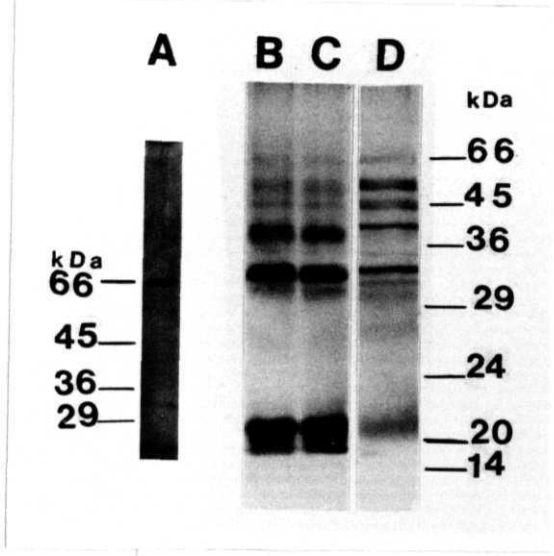

A
B
C
D
kDa
66
45
36
29
kDa
66
45
36
29
24
20
14

Fig. 28

Fig. 29: Conversion of naER to R-II receptor through deglycosylation.

150μg of naER in TEM buffer was incubated with 10 units of glycopeptidase F enzyme as described in `materials and methods'. Upon deglycosylation, NaMo was added to the media to give a concentration of lOmM and the enzyme was separated from the receptor following adsorbtion of the latter to hsp 90-Sepharose equilibrated with TEM buffer containing lOmM NaMo. The matrix was allowed to settle and was washed twice with the NaMo containing buffer. The receptors bound to hsp 90 were eluted with molybdate free TEM buffer. The eluate was used for further analysis. Control samples (not exposed to the enzyme) were also processed in a similar way.

**`A'** shows the saturation binding of estradiol to both the enzyme treated and the control naER. The HAP binding assay was carried out as described in `materials and methods'

**`B'** represents the E-RAF dependent DNA cellulose binding assay for the enzyme treated as well as the control naER. The E-RAF dependent DNA **cellulose** binding assay was carried out as described in `materials and methods'.

`C' shows the chromatographic elution profiles of control and the enzyme treated naER from a Sephadex G-100 column. The samples were concentrated to 200ul prior to chromatography on a Sephadex G-100 column. The column was precalibrated with blue dextran, bovine serum albumin, ovalbumin, cytochrome c and glycine. The fractions collected were subjected to HAP binding assay.

In **`A'** and 'C the enzyme treated naER has been represented by the open circles while the closed circles represent the control naER. In **B'** the closed circles represent the enzyme treated naER while the open circles represent the control naER.

Fig. 30: **Immunorecognition** of receptors by anti phosphotyrosine **IgG.**

The purified R-I and R-II receptors were incubated with lOmM ATP at 30oC for 60 minutes prior to their exposure to anti phosphotyrosine IgG. The incubated proteins were chromatographed on a column of anti phosphotyrosine IgG Sepharose equilibrated with lOmM Tris HC1 pH 7.6 containing 12mM monothioglycerol, 6mM MgCl , 20mM NaCl and 0.2mM PMSF. After washing the column with the buffer the proteins bound to the column were eluted with 0.2M glycine pH 2.8. Fractions collected were measured for absorbance at 280nm. The open and closed circles indicate the elution profiles of R-II and R-I receptors respectively.

The inset is a western blot showing the immunorecognition of R-II, but not R-I, by the anti phosphotyrosine IgG.

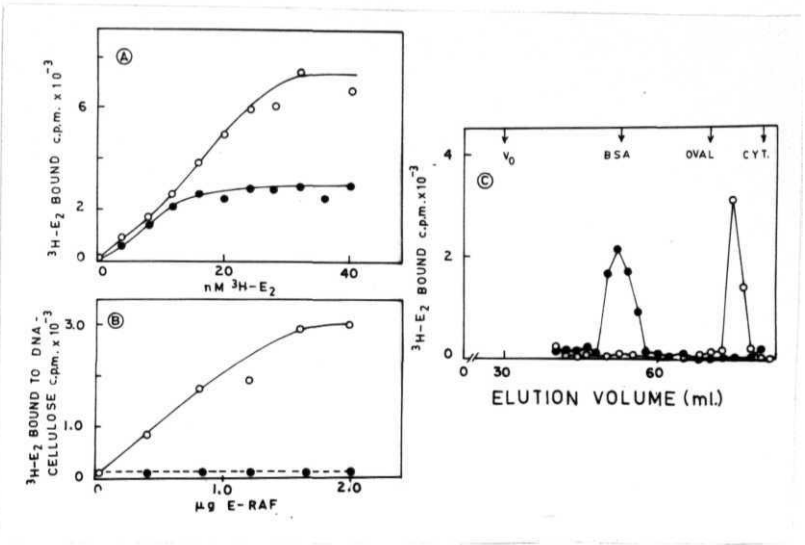

A
^3H-E_2 BOUND c.p.m. x 10^{-3}
nM ^3H-E_2
B
^3H-E_2 BOUND TO DNA-CELLULOSE c.p.m. x 10^{-3}
µg E-RAF
C
V_0
BSA
OVAL
CYT.
^3H-E_2 BOUND c.p.m. x 10^{-3}
ELUTION VOLUME (ml.)

Fig. 29

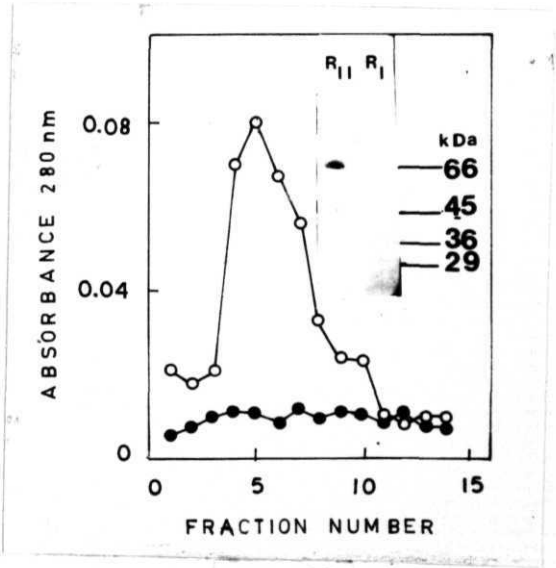

R_{II} R_I
kDa
66
45
36
29
ABSORBANCE 280 nm
FRACTION NUMBER

Fig. 30

Fig. 31: Fluorescence transfer from R-II or naER to RNA polymerase II.

The receptor proteins (naER, R-I and R-II receptors) were labelled with fluorescein isothiocyanate (FITC). The FITC labelled proteins were incubated with purified RNA polymerase II in TEMG buffer for different time intervals at 30oC. The incubation was stopped by cooling the tubes in ice. The complex (receptor-RNA polymerase II) was dissociated following the addition of 0.5M NaCl. The RNA polymerase II was immunoprecipitated by the addition of anti RNA polymerase IgG. The immunoprecipitates, collected following centrifugation, were analysed for fluorescence intensity in a spectrofluorometer.

In the figure the closed circles indicate the % transfer of fluorescence to RNA polymerase from FITC labelled R-II receptor. Open circles represent the transfer from FITC labelled naER and triangles represent that from FITC labelled R-I receptor. The total fluorescence activity associated with the fluorescent labelled protein was taken as 100% activity for this purpose.

Fig. 32: Autophosphorylation by R-II receptor.

'A' and `B' display data on the autophosphorylation of R-II receptor and of R-I respectively. The numbers on the autoradiogram indicate the concentration (in μg) of the protein used in the incubation medium. Estrogens failed to inhibit R-II autophosphorylation (data not shown).

`C' and `D' refers to the effect of E-RAF on the phosphorylation of R-II receptor (C) and naER (D). 10μg of the purified receptors were allowed to phosphorylate in the presence or absence of E-RAF (lOug). The numbers 0' and 10' on the autoradiogram indicate protein concentration (in mg) of E-RAF used in the incubation medium.

`E' shows the effect of the tyrosine kinase inhibitor (Tyrphostin 25) on the autophosphorylation of R-II receptor. 10mg of R-II receptor was exposed to different concentrations (μM) of Tyrphostin 25 (indicated by the numbers on the autoradiogram).

*P is an autoradiogram showing the identification of phosphorylated aminoacid associated with the autophosphorylated R-II receptor. The phosphorylated proteins were subjected to acid hydrolysis and the hydrolysates were subjected to paper chromatography as described in materials and methods'. Phospho tyrosine (P-Tyr), phospho serine (P-Ser) and phospho threonine (P-Thr) were used as standards. The paper chromatogram was subjected to autoradiography.

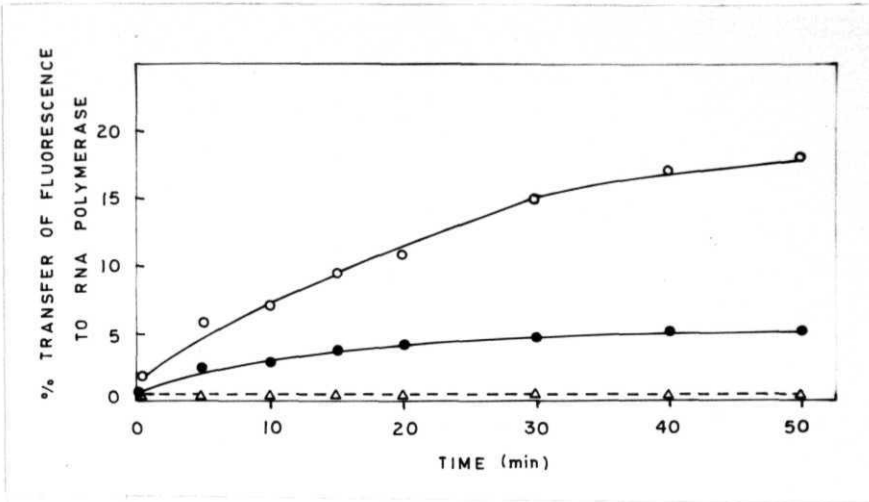

% TRANSFER OF FLUORESCENCE TO RNA POLYMERASE
0
5
10
15
20
0
10
20
30
40
50
TIME (min)

Fig. 31

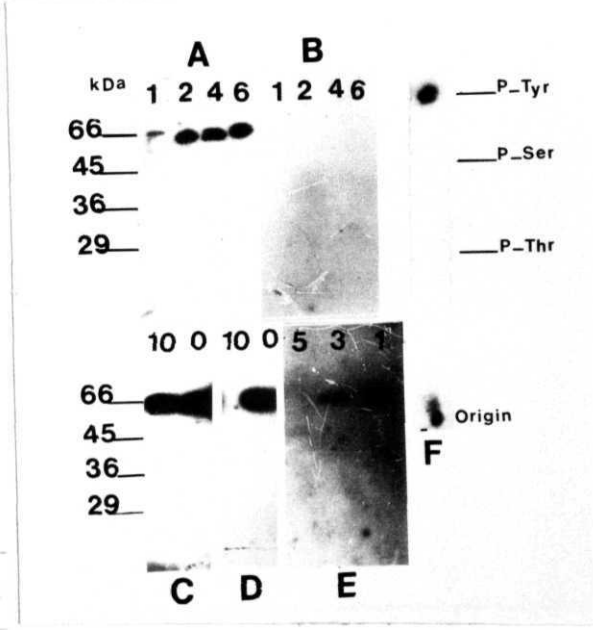

A
B
kDa
1 2 4 6 1 2 4 6
66
45
36
29
P-Tyr
P-Ser
P-Thr
10 0 10 0 5 3 1
66
45
36
29
Origin
F
C
D
E

Fig. 32

Fig. 33: phosphorylation of nuclear RNA polymerase II.

'A' is a non-denaturing gel of the purified enzyme and `B' a 15% SDS gel The RNA polymearse, incubated with the protein kinase assay medium either in the presence or absence of estrogen receptors (R-II or naER) was subjected to SDS-PAGE. The dried gels were subjected to autoradiography (C). 1 and 2 display the subunits phosphorylated in the absence of estradiol. 3 and 4 correspond to those phosphorylated by the R-II in the presence and absence of estradiol respectively. 5 and 6 indicate those phosphorylated by naER in the presence and absence of estradiol. 7 indicate the phosphorylation of subunits in the presence of R-I receptor The arrows (in B) indicat the subunits which were phosphorylated.

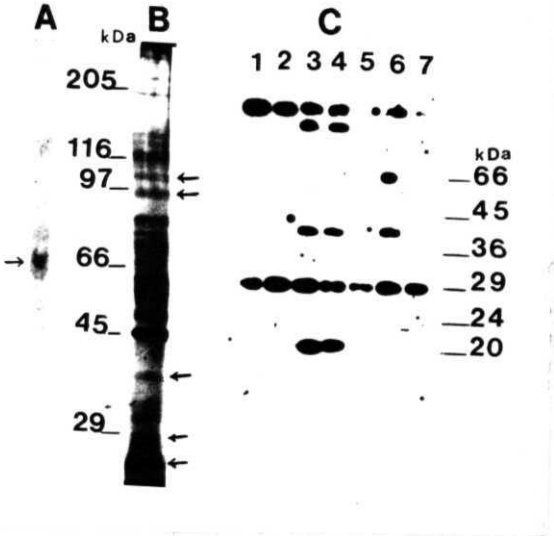
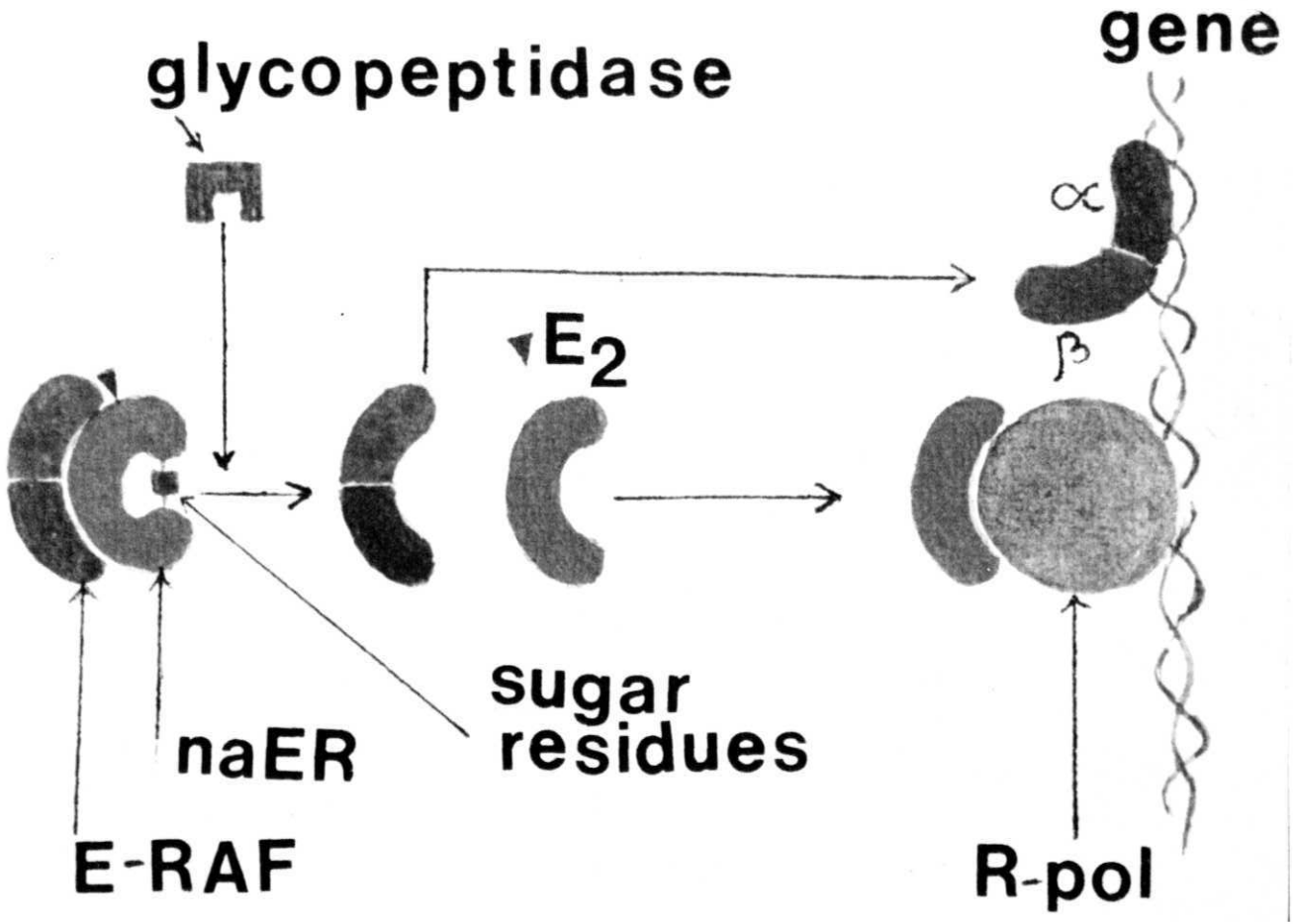


Fig. 33

Fig. 34: A model to summarize the observations presented in this chapter.

E-RAF and naER enter the nucleus as constituents of a heterodimer. A nuclear glycopeptidase acts on the naER and the deglycosylated naER dissociates itself from the E-RAF. The hormone bound to the receptor also dissociates due to the decrease in the hormone binding affinity introduced by the deglycosylation process. The modified naER is now designated as the R-II which binds to the RNA polymerase.


gene
glycopeptidase
α
β
E2
sugar
residues
naER
E-RAF
R-pol

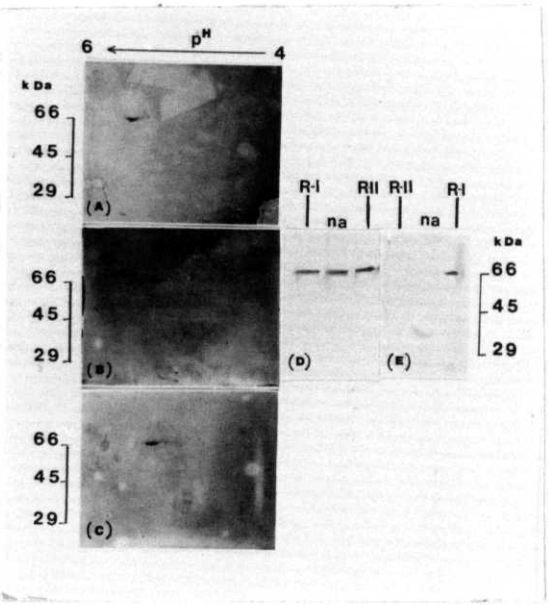

6
pH
4
kDa
66
45
29
(A)
(B)
(C)
R-I
na
RII
R-II
na
R-I
kDa
66
45
29
(D)
(E)

Fig. 35: Two dimensional gel and immuno electrophoretic analysis of R-I, R-II and naER.

A, B and C represent the 2-D gels of R-I, naER and R-II respectively. The R-I has a pI of 5.6 and R-II and naER display a pI of 5.2 each. *D'* is an immunoblot showing the cross-reactivity of R-I, naER and R-II with anti hER (bacterially expressed) polyclonal antibody (SB208). E' is an immunoblot showing the cross-reactivity between R-I and the monoclonal antibody against hER (Mab17).

CONCLUSIONS

1). The following species of estrogen receptors have been purified to absolute homogeneity from the goat uterus. The homogeneity is demonstrated by the silver stained two dimensional gels **(R-I, R-II** and naER).

a). Nuclear estrogen receptor R-I **(Type-I)**

b). Nuclear estrogen receptor R-II **(Type-II)**

c). Non-activated estrogen receptor (naER) from the cytosol

d). Non-activated estrogen receptor (naER) from the plasma membrane

2). All of them are 66 kDa proteins, cross-react with rabbit anti-estrogen receptor (R-I) IgG and the polyclonal antibody against the **bacterially** expressed human estrogen receptor (hER). Monoclonal antibody against hER cross reacts only with the R-I (type I) estrogen receptor.

3). The R-II and the naER displayed identical CNBr peptide maps, distinctly different from the peptide map of the R-I receptor

4). naER forms a **heterodimer** with estrogen receptor activation factor (E-RAF), a DNA binding protein having no capacity to bind estradiol. E-RAF does not **dimerize** with R-I or R-II.

5). Plasma membrane appears to be the primary site of localization of the naER. Binding of estradiol is shown here to result in the dissociation of the naER-E2 complex from the plasma membrane into the medium under *in vitro* conditions. This naER dissociation from the plasma membrane is an estrogen-specific mechanism.

6). Unlike the R-I and the R-II estrogen receptors, the naER is shown here as a glycoprotein.

7). Both naER and R-II are tyrosine kinases. The tyrosine kinase activity is inhibited by Tyrphostin-25.

8). While the tyrosine kinase activity of the naER is totally inhibited by the presence of estradiol/ E-RAF in the assay medium, similar treatments have no effect on the tyrosine kinase activity of the R-II.

9). R-II binds to and phosphorylates specific subunits (91 kDa, 40 kDa and 20 kDa) of the goat uterine RNA polymeraseII.

10). Deglycosylation of naER using a commercially available glycopeptidase resulted in the transformation of the naER to a R-II-like protein. It's affinity for binding estradiol reduced significantly as it's capacity to bind the hormone increased following the deglycosylation. The deglycosylated naER failed to dimerize with E-RAF. The values of Stokes radius and the sedimentation coefficient of the enzyme treated naER came very close to the corresponding values of the R-II. This apparently indicated that the deglycosylation brought about a total change in the shape and the molecular conformation of the naER, transforming it to a protein similar to the R-II.

11). The results are being summarized as follows: Estradiol binding to the naER in the plasma membrane is shown here to result in the dissociation of the naER into the cytosol along with two additional membrane proteins of molecular mass 55 kDa and 45 kDa. The functional significance of the latter two proteins remains unknown. The naER dimerizes with the E-RAF. A possibility is hereby indicated that a nuclear glycopeptidase

deglycosylates naER causing irreversible structural changes in the naER. I wish to speculate that the deglycosylated naER is the R-II estrogen receptor. The R-II binds to nuclear RNA polymerase II and phosphorylates three subunits of the enzyme. Eventhough it is possible to demonstrate the physical association of R-II ER with the nuclear RNA polymerase II, the mechanisms associated with the functional regulation of RNA polymerase II through phosphorylation of these three subunits remain unknown.

The term 'estrogen receptor' has been used with caution in order to identify both naER and the R-II. It has been realized that in a strict sense these two proteins do not have this qualification at this stage since the net result of the hormone binding to these proteins remains shrouded in mystery. This, however, does not dilute my confidence in that the real recognition of the R-II as an authentic nuclear estrogen receptor will be made sooner than later as there are experimental evidences, not presented in this volume, that substantiate this point. Confirmation of the precurssor-product releationship between the naER and the R-II then will automatically qualify the naER to be identified as an estrogen receptor.

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