Estrogen Receptor Activation Factors (E-RAF) of the Goat and Rat Uteri: Structural and Functional Studies

Thesis Submitted to the University of Hyderabad
for the Degree of Doctor of Philosophy

M. PREM KUMAR

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 134, India.

August 1994

To

MY PARENTS

DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD

Dated: 10 August 1994

DECLARATION

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

Dr. Raghava Varman Thampan
Supervisor.

M. Prem Kumar
Candidate.

DEPARTMENT OF BIOCHEMISTRY

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

HYDERABAD

Dated:10 August 1994

CERTIFICATE

This is to certify that Mr. Prem Kumar 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 RECEPTOR ACTIVATION FACTORS (E-RAF) OF THE GOAT AND RAT UTERI: STRUCTURAL AND FUNCTIONAL STUDIES", for submission for the Degree of Doctor of Philosophy of this University.

Prof. T. Suryanarayana
Head, Department of Biochemistry

Dr. Raghava Varman Thampan
Supervisor.

16.8.94

Prof. N. C. Subrahmanyam
Dean, School of Life Sciences.

ACKNOWLEDGEMENTS

At the outset, it gives me immense pleasure to thank Dr. R. V. Thampan, for his excellent guidance and constant encouragement, without which this endeavour would not have been possible. Apart from offering valuable insights and meticulous corrections, he has also inspired me in acquiring knowledge in this fascinating field through his lively discussions.

I also extend my sincere thanks to the following:

Head, Department of Biochemistry and the Dean, School of Life Sciences for providing me necessary facilities to carry out this work.

University Grants Commission for the award of Junior and Senior Research Fellowships.

My labmates for their timely help and co-operation.

All my friends for their camaraderie and making my stay memorable at this university.

- My parents whose neverfailing affection and encouragement made possible this whole enterprise.

Last but not the least, words fail to express my heartfelt appreciation for Usha for her spirit of co-operation and being beside throughout this adventurous pursuit of knowledge.

PREM

ABBREVATIONS USED

ATP	:	Adenosine 5' triphophate
pABA	:	p-Amino benazamidine agarose
BSA	:	Bovine serum albumin
CANP	:	calcium activated neutral protease
DDW	:	Double distilled water
DEAE	:	Diethylaminoethyl
EDTA	:	Ethylenediamine tetra acetic acid
ELISA	:	Enzyme linked immuno sorbent assay
ER	:	Estrogen receptor
E-RAF	:	Estrogen receptor activation factor
FITC	:	Fluoroscein iso thio cyanate
HAP	:	Hydroxylapatite
HRPO	:	Horse radish peroxidase
PAGE	:	Polyacrylamide gel electrophoresis
PMSF	:	Phenylmethyl sulphonyl fluoride
PPO	:	Diphenyl oxazole
POPOP	:	1,4 Bis (2,5 phenyl oxazyl) benzene
SDS	:	Sodium dodecyl sulphate
TCA	:	Trichloroacetic acid
TBS	:	Tris buffered saline

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Chapter I

Introduction

Receptor dimers in hormone action: **A general survey.**

The nuclear estrogen receptors belong to a super family of receptors whose function is dependent on the binding of small hydrophobic ligands such as steroids, thyroid hormone, vitamin D, and retinoic acid. Functional analysis have shown that these receptors act as transcription enhancing factors by interacting with the corresponding responsive elements, which triggers a cascade of biological responses through modulations of transcription of specific genes (Yamamoto, 1985; Green and Chambon, 1986; Evans and Hollenberg, 1988; Evans, 1988).

Functional domains on the estrogen Receptor:

In the case of estrogen receptor (ER) it has been shown that the hormone binding domain (HBD) and the putative DNA binding domain (DBD) which are responsible for the nuclear binding (Kumar et al., 1986) and specific recognition of responsive element of target genes (Greene and Chambon, 1987; Kumar et al., 1987) correspond to separate discrete elements that can function independently (Webster et al., 1988a; 1988b). The hormone binding domain appears to contain estradiol inducible transcription activation function (Kumar et al., 1987; Webster et al., 1988b). In addition estrogen is required for tight nuclear binding of the receptor.

Estrogen receptor as a homodimer:

It was proposed by Yamamoto and Alberts (1972) that the estrogen receptor binds to DNA as a dimer. The cross linking and

density shift experiments of 5S receptor complex showed that it consisted of two similarly sized units, which turned over with similar half-lives providing an evidence that 5S nuclear receptor complex is a dimer of two 4S monomers (Miller et al., 1985). **With the** use **of** monoclonal antiestrophilins Lindstedt et al., (1986) showed that the receptor dimers are formed in the cytosol following heat-transformation of monomeric cytosolic receptors. It appeared that the major fraction of estrogen receptor extracted from nuclei under these conditions existed as dimers. Computer modelling studies of Gordon and Notides (1986) were also consistent with this model. The studies of Kumar and Chambon, (1988) provided the direct evidence for the formation of homodimers in human estrogen receptors for its binding to responsive elements. The binding of glucocorticoid receptor to DNA as a homodimer was reported by Tsai et al., (1988) Wrange et al., (1989) .

The efficient binding of estrogen receptor to DNA is dependent on hormone in vitro (Kumar and chambon, 1988) . They showed that the action of the hormone does not involve **the** modification of structure of the DNA binding domain. The isolated DBD segment can also bind specifically to estrogen responsive element suggesting that no post translational modification is required for its binding. The receptor dimer binding to estrogen responsive element required the hormone binding domain for stable complex formation. Heterodimers of estrogen receptor were noticed when the receptor truncated for HBD were mixed with mutants

without A/B region (Kumar and Chambon 1988). This was not observed with an intact HBD, and the complex formed was more stable.

Estrogen receptor contains both estrogen induced and costitutive dimerization domains. The binding of hormone favours the stable homodimer and therefore the HBD contains an efficient estrogen inducible dimerization domain. Whereas DNA binding domain does not have such strong dimerization domain, though dimers might also be formed in solutions in the absence of the hormone binding domain. Since the dimer-DNA complexes were formed irrespective of the presence of both 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 et al., 1988; Kumar and Chambon, 1988). Fawell et al., (1990a) have identified a region within the steroid binding domain of mouse estrogen receptor that is required for both receptor dimerization and high affinity DNA binding. The mutant that fails to bind estradiol dimerizes and binds to DNA with high affinity in vitro suggesting that the steroid binding is not an absolute requirement for specific DNA binding. This result contrasts with previous studies on human estrogen receptor expressed in HeLa cells, where dimerization was found to be dependent on estradiol (Kumar and chambon, 1988). It was shown that the inhibition of estrogen receptor DNA binding by anti estrogen was mediated by impaired receptor dimerization (Fawell et al., 1990b). It is interesting that hydroxy tamoxifen can mimic the effect of

estradiol although the complex formed in the presence of this anti estrogen may have an altered structure (Geier et al., 1987).

Zinc Fingers:

The DNA binding domain of the ER is 100% conserved between human and chicken estrogen receptors (Krust et al., 1986) and contains a highly conserved 66 amino acid core sequence that is common to all nuclear receptors and may fold into two DNA binding zinc fingers. The DBD is separated from HBD at the c-terminal region of the receptor by a hinge region (Lannigan and Notides, 1990) and it may be involved in dimerization (Kumar and Chambon, 1988).

Zinc fingers were originally recognized in a RNA polymerase III transcription factor (TF III A) of Xenopus laevis as a new DNA binding motif (Miller et al., 1985). The analysis has revealed potential metal binding fingers as common structures in a variety of nucleic acid binding proteins (Berg et al., 1986).

Isolation and characterization of cDNA's encoding potential finger proteins indicate the existence of at least two classes of proteins with specific nucleic acid recognition capabilities. These classes are characterized according to the number and position of cysteine and histidine residues available for zinc co-ordination (Evans and Hollenberg, 1988). The C2-H2 class typical to TF III A is comprised of a sequence motif of 30 amino acids and is repeated consecutively 9 times. This zinc finger motif is defined by 4 metal ligands and conserved

hydrophobic residues (Schwabe and Rhodes, 1991). Extended x-ray absorption fine structure spectroscopy (EXAPS) and 2D-NMR studies have shown that each 30 aminoacid residue motif folds to form an independent domain with a single zinc ion tetrahedrally co-ordinating between an irregular β-sheet and short α-helix (Diankun et al., 1986; Neuhas et al., 1990).

In steroid receptors the metal ligands are represented exclusively by the cysteine residues. The number of cysteine residues in a finger is variable. This class is represented as Cx. Steroid receptors contains two apparently unrelated fingers encoded by separate exons (Huckaby et al., 1987) with four (C_4) and five (C_5) conserved cysteine residues. The C. family is analogous to C2-H2 class and reguires a single zinc ion, whereas C and C may require multiple metal ions for co-ordination shared by cysteines. This type of interaction is present in metallothionein (Furey et al., 1986), where 3 metal ions share nine cysteine ligands. In Cx class a point mutation in the putative finger loop of GAL4 (yeast transcription factor) increases the zinc concentration required for cell viability and DNA binding in vitro (Johnston, 1987).

The DNA binding domain of estrogen receptor has two putative zinc fingers (Green et al., 1988) and it interacts with DNA through the binding of recognition helix in the major groove. The binding of ER to DNA cellulose is lost following exposure to metal chelators and restored upon addition of zinc (Sabbah et al., 1987). Atkinson et al., (1990) showed the interaction of

bovine estrogen receptor with immobilized zinc.

Leucine Zippers:

Leucine rich regions of proteins, which are known as leucine zippers have been proposed as a possible site of protein dimerization (Landshulz et al., 1988). Such structures can be found in steroid receptors in both N and C terminal. The polypeptide segments containing periodic array of leucine residues are proposed to exist in an α-helical confirmation and the leucine side chain extending from one α-helix interdigitate with those displayed from similar α helix of a second poly peptide, facilitating dimerization. This hypothetical structure is referred to as the leucine zipper and it may represent a characteristic property of a new category of DNA binding proteins (Landschulz et al., 1988). It was proposed that the leucine extend from an unusually long α helix and the leucine side chains of one helix interdigitate with those of a matching helix from a second poly peptide to form a stable non covalent linkage. The periodic array of 4 leucines was noted in the sequence of fos and jun proteins and also in yeast gene regulatory protein GCN4 (Landschulz, 1988). Mutagenesis of fos protein supports the hypothesis that a haptad repeat of leucine residues stabilize the interaction between the fos and jun proteins. The complex between fos and jun can bind DNA more tightly than either protein alone and the basic residues adjacent to the leucine repeat of fos contribute to the DNA binding potential of the complex (Kouzarides and Ziff, 1988). Schuermann et al., (1989) and

Landshulz et al., (1989) have shown evidence that the leucine zipper regions are important for homo and heterodimerization of some proteins.

The circular dichroism spectroscopy of the isolated leucine zipper of GCN 4, fos and jun showed that these proteins will form a stable dimer of α helices oriented in a parallel manner (O'shea et al., 1989). Later the 2D-NMR was used to demonstrate that the helix continues for atleast 32 to 33 residues in the peptide and that the dimer is symmetric. So, it is unlikely that the interdigitation model for the structure of leucine zipper is correct, since it leads to an asymmetric structure in a parallel dimer (Oas et al., 1990). The x-ray structure of GCN 4 leucine zipper revealed that it is a two stranded, parallel coiled coil in which a helices wrap around each other in a shallow left handed super coil. Contacts between the helices include ion pairs and extensive hydrophobic interfaces that contain a distinctive hydrogen bond. The conserved leucines make side-to-side interactions in every other layer of the dimer interface (O'shea, 1991).

Analysis of sequences in the steroid binding domain of mouse estrogen receptor revealed a haptad repeat of hydrophobic residues in this region, which are well conserved in all members of the nuclear receptor super family (Fawell et al., 1990a). The dimerization domain in this receptor has certain features in common with both the leucine zippers (Landschulz et al., 1988) and helix-loop-helix motif (Murre et al., 1989). The dimerization

domain characterized by Fawell et al., (1990a), lies at a distance of more than 250 aminoacids from DNA binding region, in contrast to the leucine zippers and helix-loop-helix motifs, which are adjacent to the basic DNA binding region. The proposed structure is involved in the dimerization of uteroglobin, a dimeric protein, expressed predominantly in the endometrium, that binds progesterone with high affinity (Beato and Bairer, 1975).

POTENTIAL FOR ESTROGEN RECEPTOR HETERODIMER FORMATION;

Jensen and De Sombre (1973) model classifies steroid receptor as an extra nuclear protein. Their model formed the basis for the once widely acclaimed two-step mechanism hypothesis that the estrogen receptor is mainly cytoplasmic in location and that it has to be activated or transformed by the steroid before it could enter the nucleus and bring about gene activation. Receptor activation is the molecular reaction which alters the receptor to an entity with increased binding capacity for the nuclear acceptor systems. A variety of manipulations such as increase in the temperature of the medium, salt treatment or alkaline pH have all been shown to activate the steroid receptors (O' Malley et al., 1974). Transformation has been defined by Muller et al., (1983) as a change in the biochemical form of the receptor which results in increased affinity of the receptor hormone complex for nuclear components including DNA or chromatin.

The transformation of 4S cytosolic estrogen receptor to the 5S form involves an association with a second component or

subunit to 4S (Notides and Nielsson, 1974). The studies carried out by Yamamoto (1974), showed that the transformation of 4S cytosolic ER to the 5S form requires the binding of a second sub-unit, subunit X, to 4S, which is not an estradiol binding protein. Concomitantly, this 5S complex binds to the DNA. These results led to the discovery of a new cytosolic protein, that does not bind estradiol and forms a complex with the 4S estrogen receptor to produce the 5S activated form. This was referred to as an estrogen receptor activation factor (E-RAF) by Thampan and Clark (1981) . This was the first study ever, giving a clue for possible heterodimer involvement in estrogen action. The basis of the discovery was the assumption that ER was primarily localized in the cytosolic compartment of the target cell and it needed to go through a transformation-cum-activation process in order to bind to the DNA.

EXCLUSIVE NUCLEAR LOCALIZATION OF THE ESTROGEN RECEPTOR:

Sheridan (1975) challenged the accepted model of Jensen and De Sombre (1973) based on the conflicting reports of the unoccupied receptors being present in the nucleus and on the evidence for direct nuclear binding of steroid hormones. Sheridan et al., (1979) proposed a model for the intracellular distribution of steroid receptors in which the unbound receptor was in equilibrium between the cytoplasm and the nucleus, partitioned according to their water content. The affinity model proposed by Walters et al., (1981 a, b) suggested the receptor distribution between the cytoplasm and nucleus to be in a dynamic

equilibrium between the concentration of receptors, their affinity for nuclear components and the compartment volumes.

In 1984 there appeared two independent reports which showed that unoccupied estrogen receptors were nuclear in location. King and Greene (1984) used monoclonal antibodies raised against MCF-7 cell ER protein to detect the receptor in various estrogen sensitive tissues. They used cytochalasin to enucleate the cells, thereby reducing to minimum, the possibility of creating homogenization artefacts. They found that receptor resided mainly in the nuclear compartment. The receptor recovered from the cytosol fraction of the homogenate, under these conditions, represented the receptor that was loosely associated with the nucleus. Binding of estradiol to the receptor led to a tighter association with the nucleus, a phenomenon previously interpreted as translocation of receptor from the cytosol to the nucleus.

Simultaneously, Welshons et al., (1984) also showed that the ER was predominantly localized in the nucleus irrespective of whether the hormone was bound to it or not. They proposed that in an intact cell no nuclear translocation of the receptor took place as part of the steroid response, but there occurred an increase in receptor affinity for nuclear elements. In the absence of the steroid, nuclear elements caused the receptor to translocate into the cytosol when the cell was disrupted.

Immuno electron-microscopic studies of Vazquez-Nin et al., (1991) showed that the estrogen receptor was mainly nuclear

in localization. They also showed a smaller but significant section of the receptor to be present in the cytoplasm. The steroid receptors are known to associate themselves with a heat shock protein of molecular mass 90 K (hsp-90) (Jacob et al., 1984, Baulieu 1987, Binart et al., 1989 a, 1989 b; Inao et al., 1990). Hsp-90 is known to be essentially a cytoplasmic protein (Wech and Feramisco, 1982). A small fraction of hsp-90 appears to be located in the nucleus and this may account for the nuclear location of the steroid receptor in their non transformed state in the absence of the hormone (Baulieu, 1987). The demonstration of exclusive nuclear localization of ER (Welshons et al., 1984; King and Greene, 1984) and the reports suggesting cytoplasmic compartmentalization of ER (Walters et al., 1981a, 1981b; King 1986, 1987; Raam et al., 1988) created a shadow of uncertainty with regard to the function of E-RAF. This led to a search for a cytoplasmic estrogen receptor that dimerizes with the E-RAF (Anuradha et al., 1994). This has been identified as the non activated estrogen receptor (naER). Evidences from our laboratory indicate that the goat uterine non activated estrogen receptor (naER) has high affinity to interact with hsp-90. The probability, therefore exists that the cytoplasmic ER identified through immuno electron microscopy was the naER.

ESTROGEN RECEPTOR ACTIVATION FACTOR (E-RAF):

Thampan and Clark (1981) were the first to report the presence of estrogen receptor activation factor (E-RAF) in the mammalian uterus. This protein has no capacity to bind estradiol,

but is a DNA binding protein. The E-RAF, purified from goat uteri (Thampan, 1987), has a molecular mass of about 66 kDa (the 62 kDa reported earlier is an under-estimation) and was found to exist in three molecular forms: E-RAF I, IIa and IIb (Thampan, 1987; 1989). The E-RAF IIa and IIb were shown to destabilize the DNA helix, stimulate DNA strand separation and function as transcription activators under in vitro conditions. E-RAF I displayed the opposite reaction. This protein stabilized native double helical structure of the DNA and inhibited transcription in vitro. E-RAF forms a heterodimer with the non activated estrogen receptor (naER) which is a high affinity estrogen binding protein with no capacity to bind to DNA (Anuradha et al., 1994). This heterodimer exists as a system parallel to the regular estrogen receptor homodimers in the mammalian uterus. E-RAF, naER and the regular ER are immunologically cross reactive (Zafar and Thampan, 1993).

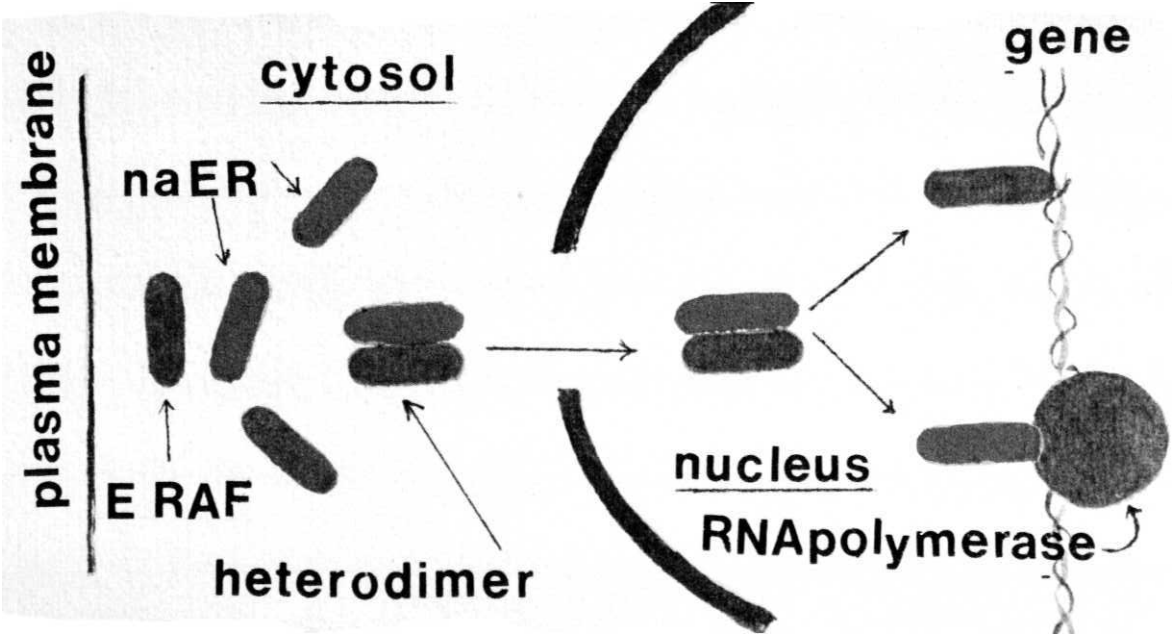
NON ACTIVATED ESTROGEN RECEPTOR (naER):

naER is an estrogen binding protein without any capacity to bind to DNA. The identity of naER as an estrogen receptor has been well illustrated by the observation that (i) it has high affinity to bind estradiol and (ii) the hormone binding site is specific for estrogens. This protein has a molecular mass of 66 kDa, sediments at 4.2S, and has a Stokes radius of 36°A. CNBr peptide analysis reveals that it has a primary structure distinctly different from that of the regular estrogen receptor (Anuradha et al., 1994). These studies further showed that naER

gains access to the DNA only upon dimerization with the E-RAF. This is the first report of heterodimer formation in estrogen action. As the molecular mass of naER is identical to that of the regular estrogen receptor, theoretically in terms of molecular size and weight the regular ER homodimer is very similar to the heterodimer constituted by naER and E-RAF.

Recent studies on naER carried out in our laboratory has shown that the naER is a tyrosine kinase and a glycoprotein localized in the plasma membrane (Karthikeyan, 1994). Keeping this in view, a model (Fig. 1) has been presented in order to identify the probable mode of action of naER-E-RAF heterodimer in the target tissue. Following hormone binding the membrane bound naER enters the cytoplasm and dimerizes with the estrogen receptor activation factor. This hetrodimer enters the nucleus where, upon dissociation, naER (now known as R II estrogen receptor) binds to the RNA-polymerase while E-RAF binds to the DNA.

Figure 1: Model showing the mode of action of naER-E-RAF heterodimer in target tissue. naER= non activated estrogen receptor, E-RAF= estrogen receptor activation factor.


plasma membrane
cytosol
naER
E RAF
heterodimer
gene
nucleus
RNApolymerase

RECEPTOR HETERODIMERS IN THE ACTION OF OTHER HORMONES:

Receptor heterodimers have been identified in the actions of thyroid hormone, retinoic acid and vitamin D. 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 receptor isoforms, TRα and TRβ, encoded on separate genes (Evans, 1988; Lazar et al., 1990). These isoforms bind to specific DNA sequences in target genes called thyroid responsive elements (TRE) as monomers and dimers (Forman et al., 1992; Baniahmad et al, 1990; Holloway et al, 1990; Lazar et al, 1991). TR binding to TRE can be enhanced by heterodimerization with thyroid receptor auxiliary proteins (TRAP) (Yen et al., 1992a; Murray et al., 1989; Darling et al., 1991; O' Donnel et al, 1991; Zhang 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 et al., 1991; Zhang et al., 1992; Yen et al., 1992b; Marks et al., 1992). Physiological concentrations of triiodo thyronine (T) decrease the binding of TRa and TRβ homodimers to TRE, but does not affect the TR-TRAP heterodimer binding (Sugawar et al., 1992; Yen, 1992).

The three genes which encode the high affinity retinoic acid receptors (RAR) have been identified and termed as a, β, γ

(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 receptor a (RXRα). The binding of retinoic acid receptor to its responsive element requires a co-regulator termed as RXRβ, that increases the DNA binding capacity and the transcriptional activity of RAR. RXRβ also heterodimerizes with thyroid receptor and vitamin D receptor increasing both DNA binding and transcriptional function (Yu et al., 1991). RXRa and RXRβ can also heterodimerize with other class of hormone receptors such as thyroid and vitamin D, thereby enhancing their DNA binding capcity and transcriptional activity (Yu et al., 1991; Bugge et al., 1992).

Vitamin D 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 D (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.

Nuclear Localization Sequences (NLS):

The nuclear proteins must contain in their primary structure

a signal that enables them to accumulate selectively in the nucleus (De Robertis et al., 1973). Regions important for nuclear localization have been defined for several proteins and are refered to as the nuclear localization signals/sequences (NLSs). In spite of the availabilty of a lot of data regarding the NLSs, no consensus NLS sequence has so far emerged; the only information available is that it is a sequence rich in basic amino acids lysine and/or arginine.

The NLSs for several proteins,such as Polyoma T, N1/N2, lamin A, c-myc, c-rel, adenovirus E1A, p53, histones, etc. have been identified, but only a few of them, that have been studied extensively, are described here.

In the Simian virus (SV) 40 large T antigen NLS, a sequence of eight amino acids pro.lys.lys(128).lys.arg.lys.val.gly is necessary for its nuclear localization. A point mutation resulting in the substitution of threonine for lysine at the codon 128 position abolishes its ability to localize to the nucleus (Kalderon et al., 1984a). The nucleoplasmin NLS is bipartite and both the basic clusters are interdependent. A nucleoplasmin like motif is seen in several proteins such as p53, N1/N2, No38 and the steroid receptors (Robbins et al, 1991).

Steroid hormone receptor NLSs:

The NLSs of the members of the steroid/thyroid hormone receptor superfamily are located in the hinge region between the DNA and the hormone binding regions. A hormone dependent NLS has been reported for the rat GR (Picard and Yamamoto, 1987), but no

such signal was found in hER (Picard et al., 1990). Ylikomi et al. (1992) preferred to analyze SR NLSs in their natural amino acid sequence context as the possible cooperation between several NLS. The effect of the other domains of the protein on the activity can be assessed only in their natural environment.

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

There exists a hormone inducible p-NLS in the hER hormone binding domain, which on its own is not sufficient for ensuring efficient nuclear accumulation, but can cooperate with constitutive hER p-NLSs or a heterologous NLS. This inducible p-NLS apparently contributes to the nuclear accumulation of the wild type receptor in the presence of the hormone.

Nuclear localization sequence binding proteins (NLSBPs);

NLS containing proteins have to be recognized by proteins termed as the NLSBPs in order to transport them to the nucleus (Goldfarb et al., 1986). The first attempt to identify NLSBPs was made by Yoneda et al. (1988) using a novel approach. They assumed that since the SV 40 large T antigen NLS consists of positively charged amino acids 'lys lys lys arg lys' probably the receptor that interacts with the signal sequence has an electrostatically complementary negatively charged sequence containing either 'asp asp asp glu asp' (DDDED) or 'glu glu glu asp glu'. The anti DDDED

antibody blocked transport of nuclear proteins into the nucleus and cross-reacted with the 69 and 59 kDa proteins of rat liver nuclei (Yoneda et al., 1988).

Adam and Gerace (1991) were the first group to demonstrate the identification of NLSBPs by a functional assay. La Casse et al. (1993) identified two NLSBPs for GRs and TRs by cross linking cytosolic proteins with GR/TR NLS peptides. The molecular mass of these proteins were 74 and 58 kDa. Of these, the 58 kDa protein bound the NLS peptide with high affinity. They are yet to be purified and a functional assay needs to be designed to confirm these proteins as the NLSBPs.

The studies carried out in our laboratory has demonstrated the existence of the NLSBP for ER in the goat uterus, which is a 55 kDa protein and transports ER into the nucleus (Bala Nirmala, 1994).

II. GENERAL SURVEY OF CALCIUM ACTIVATED NEUTRAL PROTEASES WITH SPECIAL EMPHASIS ON THE ENZYMES THAT HAVE BEEN SHOWN TO MODIFY STEROID RECEPTORS:

The calcium activated neutral proteases (CANPs) or calpains (E.C.3.4.22.17) are one of the few well characterized non lysosomal proteolytic systems of mammalian cells. The protease activity was thought originally to be accounted for by a single enzyme that requires very high concentrations of calcium (>1mM) for activity in vitro. Later, a second form of CANP was identified and isolated from soluble extracts (Demartino, 1981: Demartino and Croall, 1982; Kishimoto et al., 1981; Mellgren, 1980) indicating the presence of multiple forms of this enzyme. The two isolated CANP activities shared many biochemical and catalytical properties with the exception of their calcium requirements. One enzyme required millimolar concentration of calcium for its activity referred to as type II, CANP-II, or mCANP. The second enzyme type I, CANP-I or μCANP required only micro molar concentration of calcium for its full activity. Most of the mammalian cells contain both the enzymes μCANP and mCANP. The enzyme is a heterodimer consisting of two subunits with molecular weights of 80k and 30k (Croall and Demartino, 1984; Demartino and Croall, 1983; Hatanaka et al., 1985; Inomata et al., 1985; Kitahara et al., 1984). There are several reports showing that the enzyme has been purified as a monomer consisting of the large subunit of 80kDa mass (Croall and Demartino, 1983;

Kubota et al., 1981; Malik et al., 1983; Melloni et al., 1985; Melloni et al., 1986). The large subunit can function as calcium dependent protease in vitro. The reason for the absence of the small subunit has been suggested to be due to various post homogenization modifications adopted in the purification protocols. Commonly reported method of purification of CANP involves calcium dependent affinity chromatography using immobilized substrate like casein-Sepharose (Kubota et al., 1981; Malik et al., 1983). The proteases mCANP and μCANP are distinct enzymes though they have a number of similarities and share a common subunit (30kDa subunit is common in both the forms). Biochemical studies of the isolated protein revealed that the catalytic or large subunit of μCANP and mCANP are distinct (Demartino and Croall, 1985; Inomata et al., 1985; Kitahara et al., 1984; Otsuka et al., 1983). The 80kDa molecular weight subunit of each enzyme comprised of 4 domains of which domain II is involved in catalysis (Metrione, 1986; Ohno et al., 1984) and domain IV in calcium binding (Emori et al., 1986; Krestsinger and Nockolds, 1983).

The catalytic properties of the two enzymes were similar except for their calcium requirement. These enzymes have the pH optimum at 7.5 and are cysteine proteases (cysteine is present at the active site). They are irreversibly inhibited by alkylating reagents such as iodoacetamide, iodoactate and N-ethylmaleimide (Azamga et al., 1979, Kitaharra et al., 1984). Other inhibitors of protease activity are leupeptin, antipain ..etc (inhibitors of

sulfhydral proteases). Casein has been used as a test substrate for the routine assays because it is extensively hydrolyzed to acid soluble peptides and thereby provides a convenient and sensitive basis for spectrometric and radiometric measurements (Demartino 1981; Murakami et al., 1988).

A large number of proteins and peptides have been reported to be proteolyzed by the CANPs in cell free extracts; that includes steroid hormone receptors also. It is being suggested from the folowing that CANPs may play a regulatory rather than a degradative role in cellular metabolism; a) calcium is well established as an important regulator of many key cellular functions like cell growth, secretion and cellular response to hormones (Exton, 1988, Rasmussen and Barrett, 1984; Rasmussen et al., 1987); b) these enzymes appear to modify (and not degrade) numerous cytoskeletal proteins, protein kinases and receptor proteins. Therefore it has been suggested that the enzymes carry out their function through specific limited proteolysis, to regulate the activity, structure or function of a particular protein.

Limited proteolysis is an important regulatory mechanism in the biochemical processes and it is a general phenomenon with steroid receptors. Vedeckis et al., (1980) succeeded in partial purification and characterization of a calcium activated protease, from hen oviduct that hydrolyzes progesterone receptor. They used this enzyme as a tool for analyzing hen oviduct progesterone receptor structure (Vedeckis et al., 1980b). The

optimal calcium concentration required for its activity was 1mM and high levels of calcium do not inhibit enzyme activity. The enzyme is a sulphhydryl protease and it cleaves the progesterone receptor subunits A (79kDa mass) and B (117kDa mass) to two hormone hormone binding fragments (43kDa) and meroreceptor (23kDa). The meroreceptor obtained from A and B proteins are similar in their size and charge which suggests the structural similarity between the two proteins. The proteolytic fragments do not bind to DNA-Cellulose, implying that the hormone and DNA binding regions of A and B exist in separate domains.

The protease purified by Vedeckis et al., (1980) exhibits similarities to the receptor transformation factor (RTF) a calcium activated protease characterized by Puca et al., (1977) which performs a limited proteolysis on the calf uterine estrogen receptor. RTF converts the native estrogen receptor of 120kDa to 60kDa by way of limited proteolysis. It was suggested that this modification is needed for the diffusion of receptor into the nucleus.

A calcium activated neurtral protease purified from goat uterine cytosol has been used in the structural and functional characterization of estrogen receptor activation factor (E-RAF), an estrogen receptor associated protein found in the cytosol. The data is being presented in this thesis. However the role played by these proteases in the E-RAF dependent molecular mechanisms is far from being understood.

SCOPE OF THE THESIS:

Estrogen receptor activation factor (E-RAF) is a DNA binding protein found in the mammalian uterus, that dimerizes with an alternate form of estrogen receptor, the non-activated estrogen receptor (naER). The true functional role of this heterodimer in estrogen action in only beginning to be understood. The fact that the naER is the precursor form for the type II (R-II) estrogen receptor in the goat uterine nucleus and also that the transport of naER to the nucleus is exclusively dependent upon its dimerization with E-RAF point towards the high functional significance of the protein, E-RAF. Added to these is the knowledge that the naER is a tyrosine kinase, while E-RAF functions as a serine kinase.That these protein kinase activities do not manifest so long as the two proteins remain together in the heterodimer highlights the importance ascribed to the process of dissociation of the heterodimer in the nucleus. All these features of the E-RAF point towards a position of paramount importance to the E-RAF in the hierarchy of proteins associated with estrogen action.

The experimental work presented in this thesis serves to highlight some of the structural and functional features of the goat uterine E-RAF. The structural studies carried out on the protein using an endogenous calcium activated neutral protease provided valuable information on the existence of different functional domains on the molecule. The highlights of these

studies were mainly two: (1) the discovery of a specific dimerization domain in the E-RAF involved in dimerization with the naER, apparently through leucine zippers; (2) the indirect evidence indicating that the nuclear entry of the two monomers is dependent upon recognition of the nuclear localization sequence in one monomer by the other protein in the heterodimer.

In vivo regulation of E-RAF biosynthesis in the rat uterus has been analyzed. The method involved exposure of the uteri to subcutaneous implants of various hormones to determine the hormone specificity associated with the protein synthesis. Another method was the E-RAF-ELISA developed in our laboratory while the studies on E-RAF synthesis were in progress. This method was successfully used in estimating ng quantities of E-RAF in the rat uterus during a variety of physiological conditions.

It is expected that these studies would serve to enhance our knowledge on the biology of the E-RAF and provide an ELISA method for the quantitation of E-RAF in biopsies of human mammary and uterine cancer.

Chapter II

Materials and Methods

Adenosine 5' triphosphate, casein, diphenyl oxazole (PPO), 1,4-bis (2,5-phenyloxazol) benzene (POPOP), 17β-estradiol, fluorescein iso thio cyanate (FITC) isomer I, phenyl methyl sulphonyl fluoride (PMSF), progesterone, testosterone, diethylstilbestrol, dexamethasone, Tamoxifen, 4-chloro-1-naphthol, benzamidine-HCl and poly L-aspartic acid were purchased from Sigma Chemical Co.,St. Louis, USA. DEAE Cellulose (DE-52), GF/C glass micro fiber filters, Whatman filter papers (3mm), cellulose cc-31 and phosphocellulose were purchased from Whatman Biosys. Ltd., England. Sepharose 4B, and Sephadex series G-100, G-50, and G-25 were obtained from Pharmacia Fine Chemicals, Sweden. 2,4,6,7- [H] estradiol-17β (specific activity 101 Ci/ m mol) and high specific activity tritiated amino acid mixture (Sp. act. 93-130 Ci/ m mol) were purchased from Amersham. ^{35}S Methionine (Sp. act. lOOOCi/ m mol) was obtained from Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC), Bombay. Nitro cellulose membrane sheets were purchased from Kodak. Horse radish peroxidase-coupled anti rabbit IgG and tetra methyl benzidine (TMB) was purchased from Genei, Bangalore. Goat anti rabbit IgG and antibodies against estradiol coupled to BSA, raised in goat, were purchased from Lupin laboratories, Bombay. Dulbecco's modified Eagles medium was prepared in the laboratory. The medium was deficient in phe, leu, lys, tyr, pro and met. MALA-N (Indian Drugs and Pharmaceuticals Limited, Hyderabad), an oral contraceptive that contains Norgestrel and Ethinylestradiol, was obtained from the University of Hyderabad Health Centre. All other reagents used

were of analytical grade, purchased from local commercial establishments.

Water used in the preparation of buffer was processed as follows. Raw water was first subjected to pressure filtration in order to remove suspended elements. This was deionised using a Pure water DM-75 deionizer. The deionized water was subjected to sigle distillation using either a glass or a stainlees steel unit. The single distilled water was redistilled using a glass/quartz unit. This is termed as double distilled water (DDW). Goat uteri were obtained from local slaughter, house transported on ice to the laboratory and stored at -75 C until further use.

BUFFERS USED IN THE EXPERIMENTS:

Buffers used in the purification of ER. E-RAF and naER:

TEM: 10mM Tris-HCl, pH 7.6, 1mM EDTA and 12mM monothioglycerol, containing 0.2mM PMSF.

TEMN: TEM with 50mM NaCl. pH 7.6.

TCKM-Sucrose: 50mM Tris-HCl, pH 7.5, 4mM $MgCl_2$, 20mM KCl, lmM CaCl and 250mM sucrose. This buffer was used in the isolation of nuclei from the uterus.

Buffers used in the purification of CANP:

Homogenization buffer: 2 0mM $NaHCo_3$, lmM EDTA pH 7.5.

pH 7.5 buffer: 20mM Tris-HCl pH 7.5, 100mM NaCl, 5mM EDTA and lOmM β-mercapto ethanol.

Substrate for CANP: This contained 0.21% casein, 5mM CaCl , 25mM β- mercapto ethanol and 0.1M sodium glycerophosphate, pH 7.5.

SDS-PAGE buffers:

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

Lower Tris (4X): 1.5M Tris-HCl pH 8.8 containing 0.4% SDS.

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

APS solution: 2% APS solution (prepared freshly).

Reservoir Buffer: 0.1M Tris, pH 8.2 containing 0.8M Glycine.

Sample buffer: 1ml glycerol, 50μl β-mercapto ethanol, 3ml 10% SDS and 1.25ml upper tris solution (4X). Final volume was made upto 10ml with DDW.

Buffers for Western transfer:

Towbin buffer: 25mM Tris-HCl, pH 8.3, 190mM Glycine in 20% methanol.

Tris Buffered Saline (TBS): 10mM Tris and 0.9% NaCl pH 7.4.

Coating buffer: 10mM Sodium carbonate and 40mM Sodium bicarbonate pH 9.6, with 0.02% sodium azide.

PBS-Tween: 0.1M phoshate buffered saline pH 7.5 containing 0.05% Tween-20.

Bradford's reagent for protein estimation: 10mg of Coomassie Brilliant Blue G-250 was dissolved in 5ml of ethanol. To this 10ml of 85% (w/v) phosphoric acid was added. The resulting solution was made upto 100ml with DDW and was finally filtered through Whatman # 1 filter paper.

Scintillation cocktails for radioactivity measurement:

Toluene base: 5g PPO, 500mg POPOP per liter that contained Triton

X-100 and toluene in a ratio 33:67.

Brays Mixture: 60g naphthalene, 20ml ethylene glycol, 100ml methanol, 4g PPO, 200mg POPOP made upto 1 lit with dioxane.

Maintenance and surgery of animals:

3 months old female rats from an inbred colony of Wistar strain were used in the experimental studies. The animals were maintained under natural dark and light cycles (12 hours of light and 12 hours of darkness). Food (standard pellet food supplied by Hidustan Lever Ltd., India) and water were available ad libitum to the animals. Ovariectomy, adrenalectomy and the subcutaneous implantations of hormone-bees-wax pellets were done in rats under light ether anaesthesia. Adrenalectomized rats were given normal saline (0.9% sodium chloride) instead of water for drinking.

Preparation of hormone-wax pellets:

Estradiol-17β, testosterone, Tamoxifen or Dexamethasone - bees wax pellets had a hormone to bees wax ratio of 1:1000. Progesterone: bees wax was in 1: 2 00 ratio. On an average the pellets to be implanted weighed between 50-70mg. Mala-N bees wax pellets were made in 1:1000 ratio with reference to its ethinylestradiol concentration. The steroid was extracted into alcohol before mixing with molten bees wax.

Detection of the stages of estrus cycle and pregnancy in rats:

The vaginal smears were observed under a light microscope to determine the stage of the estrus cycle of the rat (Zarrow et al., 1964). The vaginal smear taken during the proestrus contained leukocytes and numerous small, rounded nucleated epithelial cells.The vaginal smear of the estrus showed cornified

cells and those of metestrus had cornified cells along with large nucleated cells and a limited number of leukocytes.Diestrus vaginal smear contained large number of leukocytes, mucus and debris. The females in proestrus stage were caged with healthy males and the day one of pregnancy was determined following the detection of vaginal plugs.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was performed as described by Laemmli (1970). The electrophoresis was performed by casting 10% gel to check the protein homogeneity and also for peptide mapping.

Silver staining of SDS-gel:

Silver staining of polyacrylamide gels was carried out according to the procedure described by Blum et al., (1987). The gels were fixed in a solution of 50% methanol and 12 % glacial acetic acid and 0.5ml of formaldehyde/ liter for 1 hour. After fixation of the proteins the gels were washed with 50% ethanol for 3 x 20 minutes. After pre-treatment with 0.2g/liter sodium thiosulphate (1min) the gels were washed 3 times with water and then impregnated for 20 minutes with 2g/ liter silver nitrate. Following this the gels were rinsed in water and developed with 60g/ liter sodium carbonate. The proteins appeared as silver stained bands.

Radioimmuno assay of estradiol:

The procedure described by Niswender et al., (1975) was followed.

Sample preparation: The hormone from ratr serum was extracted with diethyl ether solvent. 500µl of serum was mixed with equal

volume of ether and the tubes were left in a freezer at -75°C. The top organic layer that contained the hormone was collected by decanting it into an assay tube.

Estradiol Standards: Standards were prepared in the 20-200ng range by first making a stock of 200µg of estradiol/ml methanol and diluting it to make the corresponding working solution with 0.01M phosphate buffered saline containing 0.1% gelatin.

Labeled estradiol: H-estradiol of 40nm concentration was diluted 100 times and an aliquot of 100µl was added to each tube during the assay. The assay was carried out as follows.

All the standards were run in triplicates. To the standard tubes varying concentrations of unlabelled estradiol was added, in the 20-200pg range. This was followed by the addition of PBS to a final volume of 1ml. 100µl of labeled estradiol was added to the above mixture, mixed and to this 10µl of anti-estradiol-BSA raised in goats was added. The tubes were incubated at $4^{o}C$ for 12 hours. At the end of the incubation, 500µl of dextran coated charcoal (0.1% dextran and 1% charcoal in PBS) was added to the tubes which were subsequently kept in ice for 30 minutes. The tubes were subjected to centrifugation at 5000xg for 5 minutes at $4^{o}C$. The supernatant was collected and the radioactivity associated with the samples measured using Bray's mixture (scintillation cocktail). The experimental samples were also treated in a similar fashion. Blanks were the tubes that contained labelled hormone alone. Total counts were calculated from the tubes that contained labelled hormone and estradiol antisera. Non specific counts were calculated from the tubes that

contained labeled hormone and control sera (goat IgG).

$$\%\text{binding} = \frac{(\text{Standard counts} / \text{Sample counts} - \text{Blank} + \text{NSB}}{\text{Total counts}} \times 100$$

The graph was plotted with the % of binding on Y-axis and concentration of estradiol on X-axis.

Preparation and liqand coupling to cyanogen bromide activated Sepharose:

Cyanogen bromide (CNBr) activation of Sepharose 4B was performed as described by March et al.,(1974). One volume of Sepharose 4B along with equal volume of 2M sodium bicarbonate was taken in a beaker and was left to stir slowly. The rate of stirring was increased and 0.05 volumes of 2mg of CNBr/ ml of acetonitrile solution was added all at once. After stirring vigorously for 15 minutes, the slurry was transferred into a coarse sintered glass funnel and was washed with 10 volumes of 0.1M sodium bicarbonate pH 9.5.

The ligand was dissolved in one volume of 0.2M sodium bicarbonate, pH 9.5. Coupling of the activated Sepharose 4B was done for 20 hours in the absence and for an additional 4 hours in the presence of 1M glycine at 4°C. This gel 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 suspended in TEM buffer containing 0.02% sodium azide at 4 C.

Isolation of heat shock protein 90 (hsp-90):

The hsp-90 was isolated from the the goat liver by the

procedure of Sullivan et al., (1985) with minor modifications. Goat liver was homogenized in +molybdate (+Mo) buffer (10mM potassium phosphate, pH 7.0 containing 10mM sodium molybdate and 10mM monothioglycerol). The homogenate was centrifuged at 12,000xg at 4°C for 20 minutes. The resultant supernatant was centrifuged at 100,000xg for 90 minutes in a Beckman L8-M Ultracentrifuge using a SW-28 rotor.

The liver cytosol thus obtained was stirred with 0.1 volume of phosphocellulose which was then filtered under vacuum. The filtrate was stirred with heparin agarose (40ml, also preequilibrated with +Mo buffer) for 20 minutes. The heparin agarose was washed with 20 volumes of +Mo buffer aided by vacuum filtration. The gel was resuspended in +Mo buffer.The heparin-eluate was mixed with DE-52 (30ml equilibrated with -Mo buffer) and stirred for 20 minutes.

The DE-52 was pelleted following centrifugation and the gel was washed with 20 volumes of -Mo buffer (phosphate buffer without molybdate) containing 0.3M potassium chloride; 2ml fractions were collected. The absorbance at 280nm was determined for each fraction. The peak fractions containing hsp-90 were dialyzed overnight against -Mo buffer and lyophilized.

Immobilization of hsp-90 and E-RAF II on Sepharose 4B:

Covalent coupling of E-RAF II and hsp-90 to CNBr activated Sepharose 4B was carried out following the method described by March et al., 1974.

Isolation of uterine nuclei:

The procedure described by Thampan (1985) was followed. The

goat uterus was homogenized in 20 volumes of TCKM-Sucrose buffer pH 7.5, using a Polytron homogenizer PT 45-80 and the homogenate was filtered through a cheese cloth. Following filtration the suspension was subjected to centrifugation at lOOOxg for 10 minutes. The pellet was treated with Triton X-100, washed thrice with detergent free buffer and resuspended in 10ml of TCKM-Sucrose buffer. This was layered over 30ml of TCKM buffer containing 340mM sucrose and centrifuged at lOOOxg for 10 minutes. The purified nuclear pellet was resuspended in TCKM-Sucrose buffer.

Isolation of DNA:

DNA was isolated following the method of Marmur (1961). The nuclei were isolated from goat liver following the method of Thampan (1985) and suspended in saline EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0). To this a 25% SDS solution was added. The mixture was incubated for 10 minutes in a water bath at 60 C and was subsequently cooled to room temperature. Sodium perchlorate was added to the viscous suspension to a final concentration of lM.This suspension was mixed thoroughly with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) in a ground glass stoppered flask for 30 minutes. The emulsion was centrifuged at 5,000xg for 5 minutes. The upper aqueous phase was carefully withdrawn. Two volumes of ethanol were gently layered on the aqueous phase to precipitate the nucleic acid. The precipitate was dispersed in saline citrate and was subjected to re-extraction with chloroform- isoamyl alcohol mixture until very little protein remained at the interphase. The DNA was reprecipitated with

ethanol.

Preparation of single stranded (ss) DNA-Sepharose:

DNA from the goat liver was extracted as described by Mannur (1961). The DNA was denatured by dissolving in 0.5M NaOH followed by heating in a boiling water bath for 15 minutes. Equal volume of 0.4M sodium bicarbonate solution of pH 9.5 was added. The denatured DNA was coupled to one volume of cyanogen bromide activated Sepharose 4B, prepared as described by March et al., 1974. The gel was suspended in TEMN buffer containing 0.04% sodium azide.

Preparation of DNA-Cellulose:

Rat liver DNA was extracted following the procedure of Marmur (1961). The DNA was adsorbed to cellulose (Whatman cc-31) as described by Alberts and Herrick (1971), with the modifications introduced by Thampan (1987). 60mg of goat liver DNA was denatured by dissolving it in 0.1N NaOH and then heating in a boiling water bath for 15 minutes. The solution was then cooled in an ice bath and the pH was adjusted to 7.0 with HC1. It was then mixed with a solution of native DNA (60mg DNA in 10mM Tris pH 7.5 and 1mM EDTA). 12g of cellulose was slowly added. The slurry was dried overnight at room temperature, lyophilized and suspended in 120ml of TEMN buffer. The suspension was stored in 3ml aliquots at -75 C.

Purification of E-RAF II:

The method developed by Thampan (1987) was followed. The experimental steps were all carried out at 0-4 C. Goat uterine cytosol was first chromatographed over a column of

p-aminobenzamidine-agarose equilibrated with TEMN (10mM Tris-HCl, pH 7.6, containing 1M disodium EDTA, 12mM monothioglycerol, 50mM NaCl and 0.2mM phenylmethylsulphonyl fluoride) buffer. The flow-through fraction from this column was collected. This step was chosen in order to reduce the protease contamination of the cytosol during the purification of the protein. The flow-through fraction was chromatographed over a 25ml column of Whatman DE-52, equilibrated with TEMN buffer. The flow-through fraction from the DE-52 column was chromatographed over a 10ml column of single stranded DNA Sepharose. The column was washed extensively with the buffer. Elution of E-RAF from the column was achieved using TEMN buffer containing lOmM ATP. Ammonium sulphate was added to this ATP-eluted fraction to give a final 70% saturation of the salt. The precipitated proteins were dissolved in a small volume of TEMN buffer and dialyzed extensively against the same buffer. The fraction was chromatographed over a 10ml column of hydroxylapatite equilibrated with lOmM sodium phosphate, pH 6.8. The column was washed with 200mM sodium phosphate and elution was carried out with 250mM sodium phosphate. The 250mM sodium phosphate-eluted fraction contained E-RAF II which appeared as a single band of 66kDa in silver stained poly acrylamide gels.

Purification of regular estrogen receptor (ER):

The procedure developed by Zafar and Thampan (1993) was followed. The goat uterine cytosol was chromatographed over pABA. The pABA flow-through fraction of the cytosol was mixed with a suspension of Whatman DE-52 in TEMN buffer, in a beaker kept in an ice bath. The protein fraction eluted from the matrix using

TEMN buffer containing 0.2M NaCl was collected. This was mixed with a suspension of phospho cellulose in TEMN buffer containing 0.2M NaCl. The gel was washed with this buffer and the receptor bound to the matrix was eluted with the buffer containing 0.3M NaCl. The NaCl concentration of this eluate was diluted to 50mM following which the material was mixed with a suspension of ss-DNA-Sepharose in TEMN buffer. The gel was washed with the buffer and the receptor bound to the DNA was eluted with the buffer containing 10mM ATP. The ATP-eluted fraction contained purified estrogen receptor, appearing as a single band Of 66kDa in silver stained gels.

Purification of non activated estrogen receptor (naER):

The method developed by Anuradha et al., (1994) was followed. Minor modifications were introduced into this procedure in order to increase the yield of the protein. Goat uterus was homogenized (20%) in TEMN buffer using a Polytron PT 45-80 homogenizer. The cytosol, prepared following the method described by Van der Hoeven (1981) was chromatographed on a column of Whatman DE-52. After extensive washing, once each with TEM buffer and TEM buffer containing 0.3M NaCl, the proteins bound to the matrix were eluted using TEM buffer containing 0.5M NaCl. The NaCl concentration in the eluate was diluted to 0.05M and solid sodium molybdate was added to this preparation giving a final concentration of 10mM. This material was chromatographed over a column of hsp-90-Sepharose. The column was washed extensively and the proteins bound to hsp-90 -Sepharose were eluted using zero molybdate TEM buffer. Fractions collected were analyzed for their

absorbance at 280nm. The peak fractions were chromatographed on a column of phosphocellulose equilibrated with TEM buffer to achieve final purification of the receptor. The naER was eluted from the phosphocellulose using a NaCl concentration between 0.6M and 0.7M. The purified protein appeared as 66kDa band in silver-stained SDS gels.

FITC-Labeling of naER and E-RAF:

naER and E-RAF were labeled with fluorescein isothio cyanate isomer I following the method of Nargessi and Landon (1981). The purified protein was dissolved in 0.1M NaHCO buffer pH 9.0 (1mg of protein was dissolved in 100µl of buffer) . To this 100µl of 1mM solution of FITC in buffer was added and incubation was carried out for 12 hours at 30°C. The free FITC was removed by gel filtration on a Sephadex G-25 column. The labeled protein that was eluted in the void volume was collected and concentrated for further use.

Antibodies;

Equal volume of the antigen (lmg/ ml) and complete Freund's adjuvent were mixed. The mixture was injected into the foot pad and four subcutaneous locations of a New Zealand white rabbit. Three booster injections with two weeks intervals between two boosters were given mixing the antigen with incomplete Freund's adjuvent. Bleeding of the rabbit from the ear vein was carried out a week after the third booster injection. and the serum was collected.

The serum collected was chromatographed over a DE-52 column, equilibrated with 10mM Tris-HCl buffer pH 7.6. The flow-through

fraction which contained Ig G was collected. Affinity columns were prepared by coupling the immunogen covalently to CNBr activated Sepharose 4B. The crude IgG fraction was chromatographed over the affinity column equilibrated with 10mM Tris-HCl pH 7.6. The column was washed with three column volumes of the buffer and the IgG bound to the column was eluted using 4M $MgCl_2$. The eluate was collected in an equal volume of 100mM Tris. The resulting mixture had a pH of 7.2. The eluted protein was concentrated by ultrafiltration and was chromatographed over a column of Sephadex G-25 equilibrated with salt free, 10mM Tris-HCl buffer, pH 7.6. The void volume was collected and used as a monospecific polyclonal antibody preparation.

Western blotting:

Western transfer of proteins from SDS gels to nitrocellulose membranes using a Bio-Rad transblot equipment was done as described by Towbin et al., (1979). The pure proteins were subjected to SDS-PAGE. Following electrophoresis the gel was equilibrated with Towbin transfer buffer for 30 minutes. Two pieces of Whatman No. 3 filter papers were cut to the size of the gel and the nitrocellulose membranes were saturated by soaking in transfer buffer for 30 minutes. A pre wetted fiber pad and the soaked filter paper sandwiched the gel and nitrocellulose sheet. Air bubbles were removed at each step of the assembly of the sandwich by 'roll-pin' exclusion using a glass pipette. The transfer was carried out for 5 hours at 60V (limit 0.21). The proteins transferred to the nitrocellulose membrane were exposed to the primary antibody (1:500 dilution) at room temperature for

12 hours, after pretreatment of the membrane with 3% BSA for one hour. The membrane was then coupled to HRPO-coupled anti rabbit IgG for 2 hours at room temperature. The membrane was washed with Tris buffered saline and stained with 4-chloro-1-naphthol.

Ouantitation of proteins by Bradford's assay:

To 100µl of test sample 1ml of Bradford's reagent was added, mixed and the colour developed was read at 595nm after 5 minutes. BSA was used as the protein standard.

DNA estimation in rat uteri:

Rat uteri were homogenized (10%) in 10mM Tris-HCl ph 7.5. Equal volume of 10% trichloro acetic acid (TCA) was added to the homogenate following which the acidified homogenate was incubated in a boiling water bath for 15 minutes. Following centrifugation at 5,000xg, 0.1ml of the hot TCA extract was mixed with 5% TCA in order to make up the final volume to lml. 4ml of freshly prepared diphenylamine solution (3.0g of recrystallized diphenylamine in 300ml of glacial acetic acid and 8.25ml of concentrated sulphuric acid) was added to the 10 fold diluted TCA extract. The mixture was incubated in a boiling water bath for 10 minutes. The tubes were immersed in an ice water bath to facilitate rapid cooling and the absorbance was measured at 600nm. Calf thymus DNA was used as the DNA standard.

Purification of type I, III, and V collagens from goat uteri:

Different types of collagens were purified according to the procedure described by Miller et al., (1982) using neutral salt solvent.

Goat uteri were homogenized in TEMN buffer (0.05M Tris-HCl

pH 7.5, 1mM EDTA, 4.5M NaCl with 0.02M PMSF). Following centrifugation at 10,000xg for 10 minutes the pellet was collected. The pellet was subjected to washing with DDW to reduce the concentration of NaCl and was suspended in 20 volumes of solvent containing 0.05M Tris pH 7.5 and 1.0M NaCl. Extraction was carried out into the neutral salt solvent for 24 hours and the extraction mixture was filtered through a cheese cloth. The collagen solution was subjected to centrifugation at 100,000xg for 2 hours to get a clear supernatant that contained different types of collagens.

Collagens differential were separated by differential salt precipitation. Type III collagen was precipitated from the extract by the addition of solid NaCl to 1.8M. This was followed by the precipitation of collagen type I by increasing the salt concentration of the solution to 3.5M. Finally type V collagen was precipitated with 4M NaCl. Precipitates were recovered by centrifugation at 35, OOOxg for 1 hour. All the 3 collagens were dissolved in 0.5M acetic acid and re-precipitated by the addition of 2M NaCl. The different collagens thus obtained were used in collagenase assay.

Purification of collagenase:

Crude collagenase was prepared from rat uteri as described by Weeks et al., (1976). The uteri were homogenized in 10 volumes of ice cold 0.01M calcium chloride solution containing 0.25% Triton X-100. The homogenate was centrifuged at 4 C for 20 minutes to obtain the 6,000xg pellet. The pellet was resuspended in 0.1M Tris-HCl buffer, pH 7.6 and incubated at 60 C for 4

minutes. After cooling in ice it was subjected to centrifugation at 10,000x g for 10 minutes and the supernatant collected was used for the assay of collagenase activity.

Collaaenase assay using type I, III and V as substrate:

100µl of crude collagenase was incubated with 150µg of collagen at 37 C for one hour. Undigested collagen was removed by centrifugation at 5,000xg for 10 minutes. To the supernatant equal volume of 12N HC1 was added at 100°C in order to hydrolyze the peptides released by the action of collagenases. The hydrolysate was evaporated at 130°C. The residue left behind was assayed for hydroxyproline.

Measurement of hydroxyproline:

Hydroxyproline (OHP) was assayed as described by Woessner et al., (1961). Chloramine-T solution was made by dissolving 1.41g of chloramine-T in 20ml of DDW to which 30ml of methyl cellosolve and 50ml of 0.26M citrate buffer pH 6.0 containing 0.21M acetic acid and 1.45M sodium acetate trihydrate were added. 1ml of chloramine-T solution was added to each tube containing the hydrolyzed peptide residues of collagen. Hydroxyproline was allowed to be oxidized for 20 minutes following which the reaction was terminated by the addition of1ml of 3.15M perchloric acid. The contents of the tubes were mixed and allowed to stand for 5 minutes.Finally lml of 20% of p-dimethyl amino benzaldehyde dissolved in methyl cellosolve was added to the mixture and the tubes were placed in a water bath at 60°C for 20 minutes. The absorbance at 557nm was determined spectro photometrically.

Purification of calcium activated neutral protease (CANP) from goat uterus:

Goat uteri were obtained from a local slaughter house, transported in ice to the laboratory, and kept stored at -75°C until used. A 20% homogenate of the tissue in the homogenization buffer (20mM $NaHCO_3$, 1mM EDTA, pH 7.5) was made using a blendor. The homogenate was filtered through glass wool and centrifuged at 12,000x g for 20 minutes. The supernatant was recentrifuged at 105,000x g for 2 hours using a sw-28 rotor in a Beckman ultracentrifuge, to obtain the uterine cytosol.

The cytosol thus obtained was chromatographed over a column of p-aminobenzamidine agarose (pABA) equilibrated with 20mM Tris-HCl pH 7.5 containing 100mM NaCl, 5mM EDTA and 10mM β-mercaptoethanol. The proteins bound to the pABA column were eluted with 2mM benzmaidine-HCl in the same buffer. The eluate was chromatographed over a column of hydroxylapatite equilibrated with 10mM sodium phosphate buffer following which the column was washed with the buffer. The enzyme bound to the column was eluted with 250mM sodium phosphate buffer, pH 7.5. The sodium phosphate eluate was chromatographed over a column of DE-52, equilibrated with TN buffer (lOmM Tris, lOOmM NaCl pH 7.5). The protease bound to the column was eluted using a linear gradient of NaCl (lOOmM to 1M). Fractions (2ml each) collected were subjected to CANP assay. All the isolation steps were carried out at 4°C, unless otherwise mentioned.

CANP assay:

Casein was used as the substrate and it was prepared as

described by Ishura et al., (1978). The preparation contained 0.24% casein, 5mM $CaCl_2$, 25mM β-mercaptoethanol and 0.1M sodium glycerophosphate, pH 7.5. To 500μl of the substrate, 250μl aliquots of the DE-52 fractions were added and the mixture was incubated at 30°C for 1 hour. The reaction was stopped by the addition of 750μl of 10% trichloroacetic acid (ice cold) following which the tubes were kept in ice for an additional 30 minutes. The supernatant was collected following centrifugation in a J2-21 Beckman refrigerated centrifuge using a JA 18.1 rotor. The absorbance at *X* 280 nm of the TCA supernatants was measured in a Shimadzu spectrophotometer. The result of CANP action was examined using Bradford's assay (Bradford, 1976). One unit of the enzyme activity is expressed as equivalent to that activity which enhances digestion of casein by 0.1 absorption unit at 595nm in Bradford's assay.

E-RAF fragmentation by CANP and separation of the fragments on ss-DNA Sepharose:

To 100μg of E-RAF in the substrate buffer 200μl of enzyme fraction (10 units) was added. The mixture was incubated at 37°C for one hour. Reaction was stopped by the addition of 100μl of DE-52 slurry equilibrated with TEM buffer. Following centrifugation at 4,000x g for 5 minutes the supernatant was collected and chromatographed over a column of ss-DNA Sepharose. Flow-through fractions from this column were collected and the peptide bound to the DNA was eluted using TEM buffer containing 10mM ATP. Both the fractions were concentrated and subjected to SDS-PAGE analysis.

Dissociation of naER and E-RAF heterodimer:

The purified naER (100μg in TEM) was incubated overnight with 20nm H-estradiol at 4 C. Following this an equal volume of dextran coated charcoal suspension (1% charcoal and 0.1% dextran in TEM buffer) was added to the incubation medium and the mixture was left in ice for 30 minutes. The supernatant was collected following centrifugation and was chromatographed over an E-RAF Sepharose column, E-RAF immobilized on CNBr activated Sepharose 4B. The column was washed with TEM and elution was carried out using varying concentrations of salt (sodium chloride 0.2M to 2M) and also with 10mM concentrations of various amino acids belonging to different chemical categories (leu, gly, cys, tyr, lys, asp, val, phe, met, and ile) . The radioactivity associated with the eluates was measured using Brays mixture in a LS 1801 Beckman liquid scintillation counter.

Experiments to identify the E-RAF fragment involved in the dimerization:

To 0.5 ml of FITC labeled naER (100 μg) 100μg of E-RAF in 0.5ml of TEM was added. The mixture was incubated at 30°C for one hour. Both naER and E-RAF were dissociated by the addition of leucine to a final 10mM concentration. Separation of naER and E-RAF was carried out by the addition of 250μl of DE-52 slurry equilibrated with TEM (E-RAF appears in the flow-through fraction while the naER remains bound to DE-52). The mixture was subjected to centrifugation and the supernatant containing the E-RAF was collected. This E-RAF was treated with CANP as mentioned earlier and the fluorescence associated with the two fragments measured

in a fluorescence spectrophotometer (Shimadzu RF-5000) at excitation wavelength of 470nm and emission wavelength of 520nm.

Assay for dimerization between naER and one of the two E-RAF fragments:

To 100µg of FITC labeled naER in TEM an equal amount either of a or of β fragment of the E-RAF was added and the reaction mixture was incubated at 3 0°C for one hour. The complex was then subjected to gel filtration on a column of Sephadex G-100 equilibrated with the TEM containing 0.3M NaCl. The column was calibrated previously with blue dextran and the marker proteins and the fractions (4ml/ fraction) were collected. An elution volume that was three times the void volume (bed size of 90ml and void volume of 28ml) was collected. Fluorescence associated with each fraction was measured as mentioned before.

Effect of estradiol on E-RAF-naER dimerization:

FITC-naER (100µg) was incubated with varying concentrations of unlabeled estradiol (0-20nM) overnight at 4 C which was followed by the addition of 100µg of E-RAF in TEM buffer to the incubation mixture. The incubation was continued for one hour at 30°C. Dissociation of both the proteins was carried out by the addition of leucine to a final 10mM concentration. Separation of E-RAF from naER was achieved following the addition of 100µl DE-52 slurry into the reaction mixture. The fluorescence associated with the DE-52 flow-through fraction (E-RAF) was measured in order to determine the extent of dimerization in the presence of varying concentrations of estradiol.

Cross reactivity of naER. E-RAF. and the a and β fragments of E-RAF with anti polvaspartate antibodies:

Following SDS-PAGE, naER, E-RAF, and the a and β fragments of the E-RAF were transferred to nitrocellulose membranes as described by Towbin et al., (1971). The immuno blots were exposed to anti polyaspartate IgG for 12 hours at room temperature. Subsequently the membranes were exposed to HRPO coupled anti rabbit IgG for 2 hours. Blots were washed with Tris buffered saline and stained with 4-chloro-1-naphthol, a HRPO substrate.

Assay for interdependence of E-RAF and naER for the nuclear migration of the heterodimer:

This experiment was designed in order to look into the conditions that influence the transport of naER and E-RAF to the nucleus. Goat uterine nuclei in sucrose buffer (50mM Tris-HCl pH 7.6 containing 2mM MgCl , 20mM KCl, 1mM CaCl , 250mM sucrose and 0.2mM PMSF) used in this procedure was prepared as mentioned earlier. To show the dependence of E-RAF on the transport of the heterodimer into the nucleus, 100μg of naER in 100μl of TEM was incubated with 20nM of ^{3}H-estradiol *11(3,* overnight at 4°C. To this equal volume of dextran coated charcoal was added. Following centrifugation, after an incubation in ice for 30 minutes, the supernatant was collected. This was mixed with varying concentrations of E-RAF (0-100μg in TEM) and incubated for another 30 minutes at 30°C. The nuclear suspension (200 μl/ tube corresponding to 1mg of DNA in sucrose buffer) was added to the reaction mixture mentioned above and the incubation at 30 C was continued for one hour. The pellet was collected after

centrifugation at 1000g for 10 minutes and washed twice with the sucrose buffer. Radioactivity associated with the nuclei was measured following extraction with 1ml ethanol in a Beckman LS 1801 liquid scintillation counter using a toluene based scintillation cocktail containing 5g PPO, 500mg POPOP/ lit of toluene: Triton x 100 (67:33 v/v) mixture.

To show the dependence of naER on the nuclear transport of the heterodimer, 100μg of FITC labeled E-RAF was incubated with varying concentrations of naER-estradiol 17β complex (0-100μg) for 30 minutes at 30°C. To this 200μl of goat uterine nuclear suspension in sucrose buffer corresponding approximately to 1mg DNA was added. The mixture was incubated for one hour at 30°C, centrifuged and the 1000X g pellet was collected. The pellet was washed twice with the sucrose buffer. The FITC-labeled E-RAF, bound to the nuclei, was dissociated by the addition of lml of TEM containing 0.5M NaCl. The fluorescence in the supernatant was measured at excitation wavelength of 470nm and emission wavelength of 52 0nm.

Cross reactivity of a and β fragments with anti-ER and anti-E-RAF IgGs:

Both the fragments were transferred to nitrocellulose as mentioned above and the cross reactivity of the fragments with anti-ER or anti E-RAF IgG was studied following exposure of the blots first to the primary antibody and subsequently to HRPO-coupled anti rabbit IgG. The blots were stained with 4-chloro-1-naphthol.

In Vitro incorporation of labeled amino acids into E-RAF:

The uteri from rats belonging to different experimental groups were removed, cleared free of fat and connective tissue, slit longitudinally and immediately transferred to Dulbecco's modified Eagles medium (1 uterus/ml) deficient in phe, leu, lys, tyr, pro and met. The uteri were incubated in the medium with a H-labeled amino acid mixture (leu, lys, phe, pro, tyr) and [^{35}S] met (5μCi each) at 37°C for 5 hours. Each incubated uterus was homogenized in 3.0 ml of TEMN buffer (10mM Tris-HCl, pH 7.6 containing 1mM EDTA, 12mM monothioglycerol, 50mM NaCl and 0.2mM phenyl methylsulphonyl fluoride). The homogenate was centrifuged at 10,000x g and the supernatant was collected. To 1ml of the supernatant, 50μl of a DE-52 slurry, equilibrated with TEMN, was added and mixed gently. The tubes were incubated in ice for 30 minutes, centrifuged and the supernatant was collected. This was mixed with anti goat uterine E-RAF IgG (final dilution of 1:200 with monospecific polyclonal antibody) overnight at 4°C. Afterwards goat anti rabbit Ig G was added to the mixture (1:1000 final dilution) and the incubation was continued for another 2 hours. The tubes were centrifuged at 10,000 xg for 10 minutes and the precipitates collected. All the centrifugation protocols mentioned above were carried out using a JA 18.1 rotor in a Beckman refrigerated centrifuge. The precipitates were redissolved in 500μl of TEMN buffer and filtered through Whatman GF/C glass fiber filters using a Millipore (12 placed) filtration unit connected to a vacuum pump. The filters were washed twice with 10% TCA in order to remove the unincorporated, labeled amino

acids and twice with ethanol to dry them. The radioactivity associated with the dry filters was measured using a scintillation cocktail (5g PPO and 500mg POPOP/ liter of toluene: Triton X-100 67:33 (v/v) in a Beckman LS 1801 liquid scintillation counter.

CNBr fragmentation of E-RAF II and analysis of the cross reactivity of the fragments with anti-ER IgG:

E-RAF II was subjected to cyanogen bromide fragmentation following the method of Kasper (1970). The fragments were chromatographed over a column of anti ER IgG-Sepharose equilibrated with 10mM Tris-Hcl, 12mM mono thioglycerol, pH 7.2. The column was washed with this buffer. The protein fragments bound to the column were eluted with 4M MgCl . Both the flow through fraction and the 4M MgCl eluate of the column were collected. The eluate was collected in a beaker containing equal volume of 100mM Tris. The resulting mixture displayed a pH of 7.2. Estrogen receptor was also treated with CNBr in a similar fashion and the resulting fragments were chromatographed on a column of anti ER IgG-Sepharose. The flow through and the 4M MgCl eluate of the column were collected. The peptide fragments of ER and E-RAF were transferred to a nitrocellulose membrane which was in turn exposed first to anti E-RAF IgG for 12 hours and later to the secondary antibody, anti rabbit IgG coupled to HRPO for 2 hours. The proteins were stained using a HRPO substrate, 4-chloro-1-naphthol.

ELISA FOR E-RAF:

Indirect microplate ELISA procedure described by Voller et

al., (1987) was followed with modifications. Varying concentrations of E-RAF (0-200 ng) in 200µl coating buffer (10mM carbonate and 40mM bicarbonate buffer pH 9.6, having 2mM sodium azide) was coated on to a 96 well ELISA plate and the plate was incubated for 18 hours at 4°C. Following this the plate was blocked using 5% BSA in the coating buffer (200µl/well) for 4 hours. The plate was washed twice with phosphate buffered saline pH 7.6, containing 0.05% Tween-20. Following this 200 µl of rabbit anti goat E-RAF IgG was added (1: 500 dilution) to each well and the incubation was continued at 37°C for 6 hours. The plate was subjected to the washing procedure twice again with PBS-Tween, following which the secondary antibody (goat anti rabbit IgG coupled to HRPO) was added to the wells (200µl/ well). Following incubation for 2 hours at 37°C the plate was subjected to washing and the substrate for HRPO, 3, 3',5, 5'-tetramethyl benzidine (TMB) was added to the wells (200µl/ well). The reaction was stopped after 30 minutes by the addition of 50µl of 1N sulphuric acid. The colour developed was read in a Thermomax microplate reader at A 450nm.

The tissue samples, for the measurement of E-RAF concentration, were homogenized (20% homogenate) in the coating buffer. The homogenates were centrifuged at 10,000 g and the supernatants collected. To 1ml of the supernatant 50µl of DE-52 slurry, equilibrated with the coating buffer, was added. The tubes were incubated in ice for 30 minutes and centrifuged following which the supernatant was collected. An aliquot of this supernatant was taken for the estimation of E-RAF through ELISA.

Chapter III

Estrogen Receptor Activation Factor II (E-RAF II) of the Goat Uterus: Use of an Endogenous Calcium Activated Neutral Protease in its Structural Characterization.

Calcium activated neutral proteases (CANP) are the non lysosomal proteolytic enzymes identified in several mammalian cells (Dice, 1987; Hershko et al., 1986). These enzymes are reported to be involved in the proteolysis of a large number of proteins including steroid receptors. The enzyme has previously been used in the structural characterization of progesterone receptor (Vedeckis et al., 1980a, 1980b) and estrogen receptor (Puca et al., 1977). In the present study CANP has been purified and used in the structural characterization of E-RAF II. The protease cleaves the E-RAF into two distinct fragments α and β. The studies carried out on these fragements are presented here.

Purification of CANP:

Chromatography of the uterine cytosol on a column of p-aminobenzamidine agarose facilitated adsorption of proteases to the immobilized p- aminobenzamidine, a protease inhibitor. These enzymes were subsequently eluted from the column using benzamidine-HCl. The enzymes were subsequently adsorbed to the calcium phosphate gel (hydroxylapatite, HAP) where the calcium dependent immobilization of the enzyme took place. Elution of these proteins was achieved with the help of 250 mM sodium phosphate buffer. The eluate from the HAP column was chromatographed over a column of DE-52. Elution of purified CANP from this column was achieved with the help of a linear (0.1M to 1.0M) NaCl gradient. Figure 1 shows (a) the absorbance at 280 nm of the purified protein and (b) the CANP assay data.

Purity of the enzyme and the display of calcium dependent activity:

The protein eluted from the DE-52 column appeared as a 80 kDa band in silver-stained SDS gels (Figure 2 inset). Maximum enzyme activity was observed when the assay was carried out in the presence of 1mM concentration or above of calcium chloride, in the medium (Figure 2).

CANP effect on naER and E-RAF:

E-RAF (100 μg) or naER (100μg) was each incubated with 10 enzyme units of CANP (one enzyme unit is eguivalent to that activity which enhances digestion of casein by 0.1 unit of absorption at 595 nm in the Bradford assay) at 30°C for one hour following which the reaction products were subjected either to chromatography on Sephadex G-50 or to SDS gel electrophoresis. The E-RAF broke up into 2 fragments, of molecular mass 32 and 30 kDa (Figure 3 inset) as a result of CANP action, while the enzyme failed to effect a similar change in the naER (Figure 3). The 32 kDa fragment was designated as the α and the 30 kDa fragment as the β.

E-RAF α retains the DNA binding domain:

Following exposure of E-RAF to CANP in the presence of lmM calcium chloride, the enzyme was removed by adsorption to DE-52; the E-RAF appeared in the DE-52 flow-through fraction while the enzyme remained bound to the matrix. This CANP treated E-RAF was chromatographed on a column of single stranded DNA-Sepharose. The flow-through fraction and the DNA-bound protein, eluted with 10mM ATP, were collected and were subjected to SDS gel analysis. The

30 KDa fragment appeared in the DNA Sepharose flow-through fraction while the 32 KDa protein was detected in the fraction eluted with ATP from the DNA Sepharose column (Figure 4 left panel). Both these fragments cross reacted with anti ER IgG as **well** as with anti E-RAF IgG. It was of interest to see whether these fragments enhanced the DNA binding of the naER following dimerization. In this experiment constant quantity of naER **was** incubated with DNA in the presence of varying quantities of the α fragment, either alone or in the presence of the *(3.* A positive dose response relationship was noticed between the E-RAF a concentration and the quantity of H-naER bound to the DNA cellulose (Figure 4 Right panel) . The presence of E-RAF β in the medium did not make any difference in this pattern.

E-RAF a retains the dimerization domain:

FITC labeled naER (100μg) was incubated either with E-RAF a or with E-RAF β, at 30°C for 30 minutes following which the mixture was chromatographed on a column of Sephadex G-100, equilibrated with the TEMN buffer. The basis of the experiment was that if one of the fragment dimerized with naER the resultant fluorescent complex should appear in the void volume of the G-100 column. It **was** observed that the α fragment of E-RAF possessed the capacity to dimerize with the naER (Fig. 5a) . The high molecular weight complex was not formed between E-RAF β and naER (Fig. 5b).

Leucine dissociates E-RAF-naER heterodimer:

The non-destructive conditions under which E-RAF dissociates from its dimer with naER was analyzed. naER was incubated with

^{3}H-estradiol overnight and the free radioactivity was removed following the addition of dextran-coated charcoal. The H-estradiol-naER complex was chromatographed on an E-RAF Sepharose column. The column was washed with the buffer and the elution with a variety of media was attempted in order to dissociate H-estradiol-naER from the E-RAF. Sodium chloride concentration as high as 2M did not help in this dissociation while elution with buffer containing 10mM leucine completely dissociated the naER from the E-RAF (Fig. 6) . None of the other amino acids tested, belonging to various chemical categories, were effective in bringing about this dissociation.

Additional confirmations for the presence of dimerization domain in the E-RAF a:

FITC-labeled naER was incubated with equimolar concentrations of E-RAF at 30 C for 30 minutes. Leucine was added to this medium to give a final lOmM concentration following which the mixture was exposed to a slurry of DE-52, equilibrated with the TEMN buffer. This step was undertaken in order to obtain the free E-RAF in the DE-52 flow-through fraction. The fluorescence associated with the following fractions was measured: 1) naER dissociated from the heterodimer and bound to the DE-52; 2)total E-RAF; 3) E-RAF a bound to DNA-Sepharose, after E-RAF digestion with the CANP and 4) E-RAF β which appeared in the DNA-Sepharose flow-through fraction. The basic assumption was that a fluorescent transfer should occur from the FITC-naER to the E-RAF site lying close to the naER.

The data presented in figure 7A shows the distribution of

fluorescence in naER, E-RAF and the E-RAF fragments a & β, under these conditions. It was observed that incubation of FITC-naER with E-RAF caused almost a 50% loss in the naER fluorescence. What was lost from the naER appeared in the E-RAF. 85% of the total fluorescence detected in the intact E-RAF was recovered in the E-RAF a with practically no fluorescence being detected in the β (Fig. 7A).

naER-E-RAF dimerization occurs independent of estradiol;

It was of interest to know whether estradiol influenced naER-E-RAF dimerization. E-RAF and FITC-naER were incubated at 30°C for one hour in the presence of varying concentrations of estradiol following which the dissociation of E-RAF from the complex was achieved upon exposure of the complex to 10mM leucine. The labeled E-RAF was recovered in the DE-52 flow-through fraction as described for the experiments detailed in figure 7A. The fluorescent activity in the E-RAF was measured, taken as an index for having dimerized with the naER. The presence or absence of estradiol did not make any significant difference in the degree of fluorescent labeling of the E-RAF, apparently indicating the fact that the dimerization was independent of the presence of estradiol (Fig. 7B).

Both naER and the E-RAF (a & β) cross react with anti polyaspartate IgG:

Purified naER, E-RAF and the a and *(3* fragments of the E-RAF were subjected to SDS-PAGE. The proteins, western transferred to nitrocellulose, were exposed to anti polyaspartate IgG, to be followed by exposure to HRPO-coupled goat anti rabbit IgG. It was

observed that both the naER and the E-RAF, including the two E-RAF fragments, cross reacted with anti polyaspartate IgG (Fig. 8).

Interdependence of E-RAF and naER for each other's movement into

$H\text{-}E_2$ naER migration into isolated goat uterine nuclei was studied both in the absence and presence of E-RAF; similarly nuclear migration in vitro of FITC-labeled E-RAF was examined both in the presence and absence of naER. Virtually no nuclear movement of naER took place in the absence of E-RAF. The naER binding to nuclei increased along with the increase in the concentration of E-RAF added to the medium (Fig. 9A).

Similarly, no intra nuclear migration of E-RAF took place when the medium was deficient in naER. Enhanced binding of FITC-E-RAF to nuclei took place as the concentration of naER in the medium increased (Fig. 9B) .

3
H-E^-naER migration into uterine nuclei in the presence of one
2
of the two E-RAF fragments:

It was of interest to see whether any of the two E-RAF fragments could stimulate naER entry into the nucleus, independently. Experiments in this direction were carried out as described for those presented in figure 9. Neither of the E-RAF fragments could stimulate the entry of naER into the nucleus (data not shown).

DISCUSSION

Intracellular protein degradation is recognized as a very important regulatory process influencing the levels of individual proteins as well as the development and degeneration of body tissues (Goldberg & Dice, 1974; Goldberg & St. John 1976). Non-lysosomal mechanisms have been shown to be responsible for a major part of the intracellular protein degradation (Dice 1987; Hershko & Ciechanover 1986). CANPs have been recognized as one of the best studied non lysosomal protein degradation systems. Two possibilities exist for the CANPs to influence cellular functions:

a) protein metabolism leading to cellular degeneration and death.
b) a constructive regulatory role in cellular metabolism leading to cell secretion, cellular responses to various hormones and cellular growth in general (Exton, 1988; Rasmussen et al., 1984, 1987). We wish to focus the attention on the goat uterine CANP in both these perspectives.

Regulatory role for calcium in the biology of estrogen action in the uterine cell is a much discussed proposition (Batra and Sjogren, 1982; Szego & Pietras 1984). Szego and Pietras (1984) identified a remarkable increase in the intracellular calcium concentration in uterine cells upon exposure to estradiol. This enhancement in cell calcium may well be an estrogen receptor mediated phenomenon as there is positive evidence available regrading the primary location of the non-activated estrogen receptor (naER) to be the plasma membrane (Karthikeyan, 1994). Hormone binding to the receptor results in

an immediate dissociation of the receptor estrogen complex from the plasma membrane and its entry into the cytosol. The possibility exists that this dissociation of the receptor hormone complex from the plasma membrane can lead to major changes in the plasma membrane organization, resulting in the opening up of a calcium channel. The increased calcium level in the cytosol could immediately influence a variety of calcium dependent metabolic regulators including the CANP. We do not have the data regarding the action of this CANP on proteins other than E-RAF and naER. The conditions under which the CANP cleaves E-RAF into two distinct fragments are incapable of influencing the CANP to act upon the naER. The formation of E-RAF a and *(3* fragments of near identical molecular size as result of the CANP action is indicative of the existence of a highly specified domain in the E-RAF molecule where CANP binds. Since both a and *β* cross react with anti polyaspartate IgG, it may be assumed that both these fragments are capable of binding to nuclear localization sequences in the naER. Theoretically, then, both the fragments should posess the property to help in the naER transport to the nucleus independently which, as it appears, is not the reality.

There **are** conflicting reports regrading the molecular organization of CANPs in mammalian cells. A large number of reports indicate that the enzyme exists as a heterodimer consisting of subunits with molecular mass 80 kDa and 30 kDa (Croall et al., 1984; Demartino et al., 1983; Hatanaka et al., 1985; Inamato et al., 1988; Kitahara et al., 1984). Many others identify the enzyme as a 80 kDa monomer (Croall & Demartino 1983;

Malik et al., 1983; Melloni et al., 1985, 1986; Zimmerman & Schlaepfer 1984). Croall and Demartino in their review (1991) observed that calcium dependent affinity chromatography using immobilized substrate is used in the methods which purify the monomeric form of CANP. The enzyme involved in our study is a 80 kDa monomer. The isolation method involves the enzyme immobilization on a protease inhibitor, elution and the subsequent adsorption on to the calcium phosphate gel. Whether the absence of the 30 kDa protein in our studies is the result of this type of isolation procedure can not be ascertained at this stage.

CANPs have been used earlier in the characterization of steroid receptors (Vedeckis et al., 1980; Puca et al., 1977). E-RAF is not a steroid receptor, but a receptor associated protein. As it is observed from the data presented in this report it is an unavoidable factor in transporting naER to the nucleus just as naER is required in the movement of E-RAF to the nucleus.That shows a true synergistic relationship between the two proteins. While the DNA binding and the dimerization domains are retained by the a fragment of the E-RAF, the NLS-recognition domains are shared between both a and β. Figure 10 is a model representing **this** distribution of the dual recognition motifs between E-RAF and naER. The naER and the E-RAF are represented here as coming close to each other at three sites: (1) the dimerization region in the α-E-RAF and the naER (2) the naER-NLS recognition domain of E-RAF a and the α-E-RAF-NLS recognition domain of the naER; (3) naER NLS recognition of the E-RAF β and β

E-RAF-NLS recognition domain of the naER. If, as discussed earlier, the a and β fragments of E-RAF are capable independently of transporting naER to the nucleus, it will not be difficult to perceive a system of regulation of the gene that is under the control of the E-RAF, the DNA binding protein in the heterodimer.

The controlling element in such a situation will be the cytosolic CANP. Since that is not the case, it is clear that the naER movement into the nucleus is dependent upon the structural integrity of the E-RAF.

I have used E-RAF and naER labeled with FITC to understand the proximity relationship between the two proteins. FITC labels amino groups in proteins. It has been well established that electronic excitation energy can be transferred effectively over a distance as large as 70°A between a fluorescent energy donor and an acceptor (Stryer, 1978). Forster (1948) proposed a theory to explain this dipole-dipole energy transfer. Stryer and Haughland (1967) further suggested that the energy transfer could be used as a spectroscopic ruler in the 10-60°A range to identify proximity relationship among biological macro molecules. One of the two properties of the heterodimer that helped us to examine the fluorescent energy transfer between naER and E-RAF without using a cross linking agent was the stability of the dimer in high ionic strength media. The other was the ease with which the dimer could be dissociated into its monomeric units in the presence of 10mM leucine. What we believe is that by adopting this method which did not introduce any conditions like cross linking that is likely to endanger the delicate biological

function of the proteins we have succeeded in studying this protein-protein interaction in the most non-destructive manner possible. This feature is evident in the experiment that led to the identification that the E-RAF a contains the dimerization domain. Almost 85% of the total fluorescent activity that was lost from the naER was recovered from the E-RAF α, with practically no activity being detected in the β fragment of the E-RAF. We anticipate that this method should prove useful in understanding the proximity relationship between monomeric units of other heterodimeric systems like the Fos and the Jun.

We do not have any direct evidence for the involvement of leucine zipper in the dimer formation between naER and E-RAF. However, the observation that only leucine is capable of dissociating the monomeric units of this heterodimer is apparently indicative of the leucine zipper involvement. In this arrangement polypeptide residues containing an array of leucine residues are proposed to exist in an a helical configuration, with the leucine side chains extending from one a helix interdigitaing with those displayed from a similar a helix of a second polypeptide, facilitating the dimerization (Landschulz, 1988).

Experimental evidences suggest that the naER is a glycoprotein (Karthikeyan, 1994). This glycoprotein dimerizes with the E-RAF and the heterodimer enters the nucleus. A specific change in this organization takes place within the nucleus during which a nuclear glycopeptidase cleaves off the sugar residue on the naER. This de-glycosylated naER is incapable of dimerization

with E-RAF, possibly due to a distinct irreversible structural change that has taken place. It will not be out of context to state here that studies on this heterodimer, its dissociation and subsequent intra-nuclear movement of the monomers will provide vital clues to understand the molecular biology of estrogen action.

Figure 1: Isolation of CANP through DE-52 chromatography. The CANP bound to the DE-52 column was eluted using a linear NaCl gradient. The bed volume of the column was 15ml and elution was carried out using a 50ml gradient of NaCl (100mM to 1M) and 2ml fractions were collected. The absorption profile (280nm) is given in Fig. 1A. Fig. 1B displays the reuslts of the assay of CANP. Caesin was used as the substrate. The peptides released by the action of the enzyme were measured either using Bradford's assay (absorption at 595nm) (O) or by observing the absorbance at 280 nm (O).

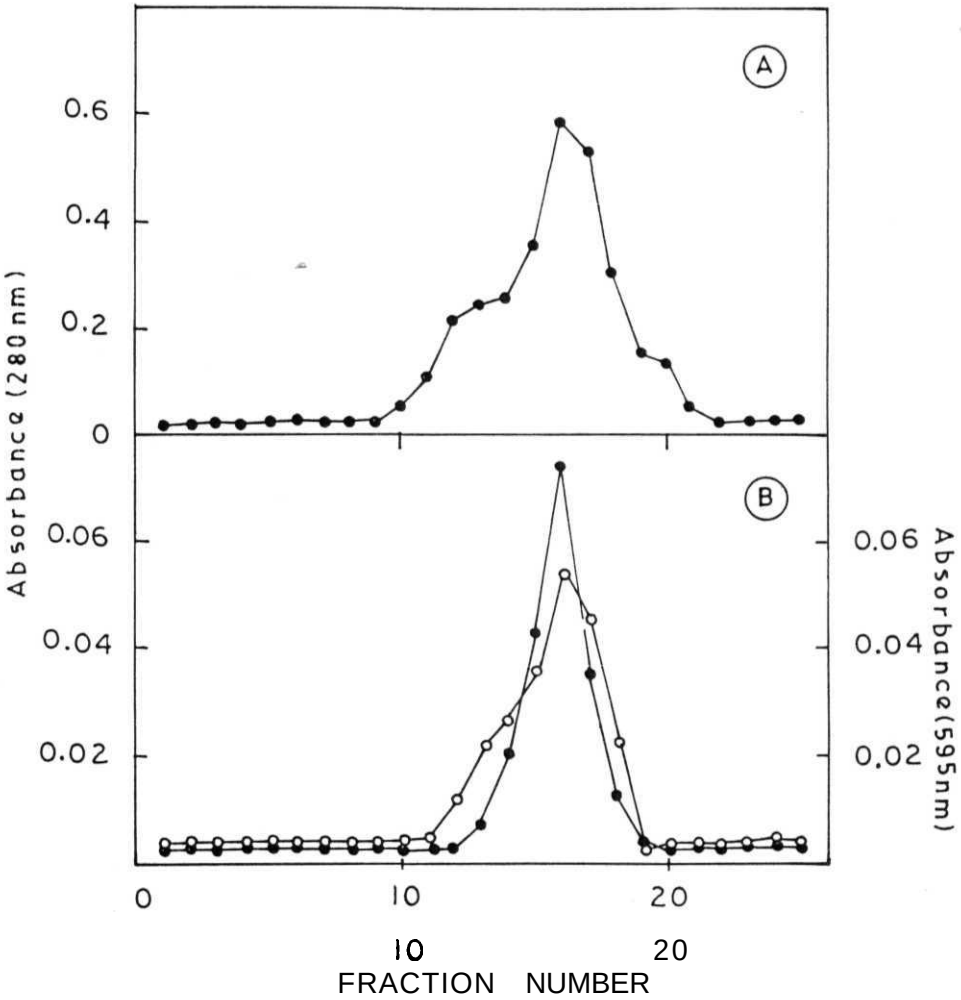

A
B
0.6
0.4
0.2
0
0.06
0.04
0.02
Absorbance (280 nm)
Absorbance(595nm)
0
10
20
FRACTION NUMBER

Figure *2:* Optimal calcium requirement for the CANP activity. Casein was incubated with CANP, in the presence of varying concentrations of calcium chloride (0-1.5mM) and the peptides released were estimated by measuring absorbance at 280nM. The inset is a SDS-PAGE of the purified enzyme existing as a 80kDa monomer.

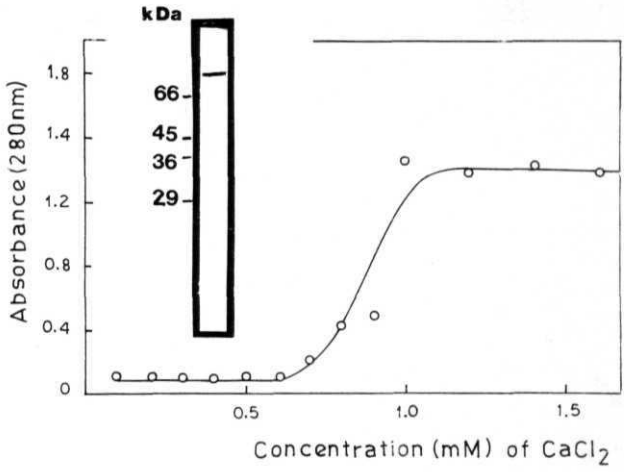

kDa
66
45
36
29
Absorbance (280nm)
1.8
1.4
1.2
0.8
0.4
0
0.5
1.0
1.5
Concentration (mM) of $CaCl_2$

Figure 3: Effect of CANP on naER and E-RAF. naER was labeled with FITC. Following the treatment with the CANP the FITC-naER was subjected to gel filtration on a column of Sephadex G-50 (bed volume of 90ml and void volume V of 33ml). 4ml fractions were collected. Fluorescence associated with each fraction was measured usinq a fluorescence spectrophotometer at excitation wavelength of 470nm and emission wavelength of 520nm. The inset is the SDS-PAGE of E-RAF digested by CANP, showing the 30 and 32kDa fragments of E-RAF. The enzyme was separated from the E-RAF following addition of a DE-52 slurry to the incubation medium. The diffuse band at 66 kDa is the uncleaved E-RAF.

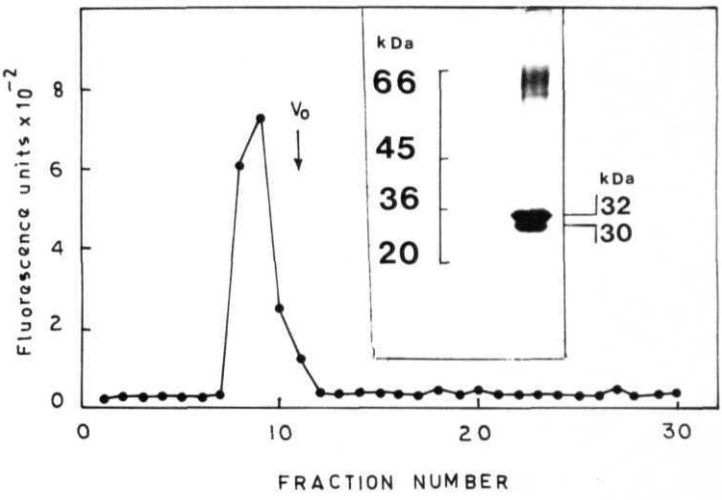

Fluorescence units x10^{-2}
8
6
4
2
0
V_o
kDa
66
45
36
20
kDa
32
30
0
10
20
30
FRACTION NUMBER

Figure 4: Separation of the a and β fragments of the E-RAF resulted following the CANP action. The details regarding separation of the fragmets have been given in 'methods'. a is the fragment that is retained by the ss-DNA-Sepharose column while the β is recovered from the DNA-Spharose flow-through fraction. A and B represent the silver stained SDS gels of a and β respectively. A and B indicate the cross reactivity of the α and β fragments respectively with anti ER IgG while A and B represent the corresponding fragments recognized by anti E-RAF IgG. The panel on the right exhibits the results of a DNA-cellulose binding assay for naER, influenced by varying concentrations of α, either in the presence or absence of β.

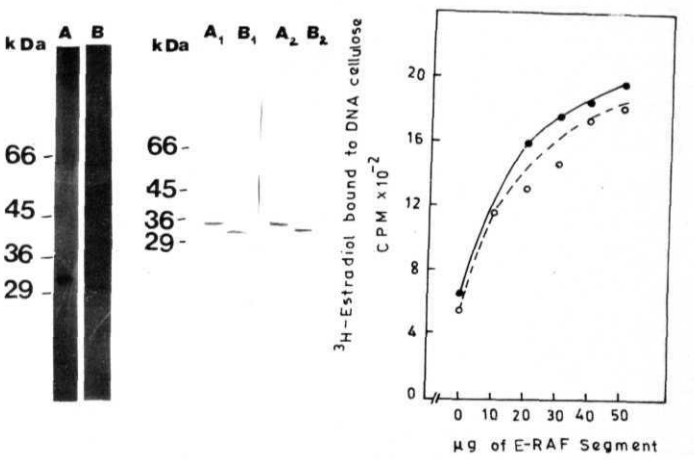

kDa
A
B
66
45
36
29
kDa
A1
B1
A2
B2
66
45
36
29
3H-Estradiol bound to DNA cellulose
CPM x10-2
20
16
12
8
4
0
0
10
20
30
40
50
µg of E-RAF Segment

Figure 5: Dimerization studies between FITC-labeled naER and the E-RAF fragment a or β. The E-RAF fragment α (Fig. 5A) or ft (Fig. 5B) were incubted separately with FITC-labeled naER and the protein mixture was subjected to gel filtration on Sephadex G-100 (void volume V = 28ml, bed volume 50ml) and fractions (4ml/ fraction) were collected. The fluorescence associated with each fraction was measured at excitation λ= 470nm and emission X= 520nm.

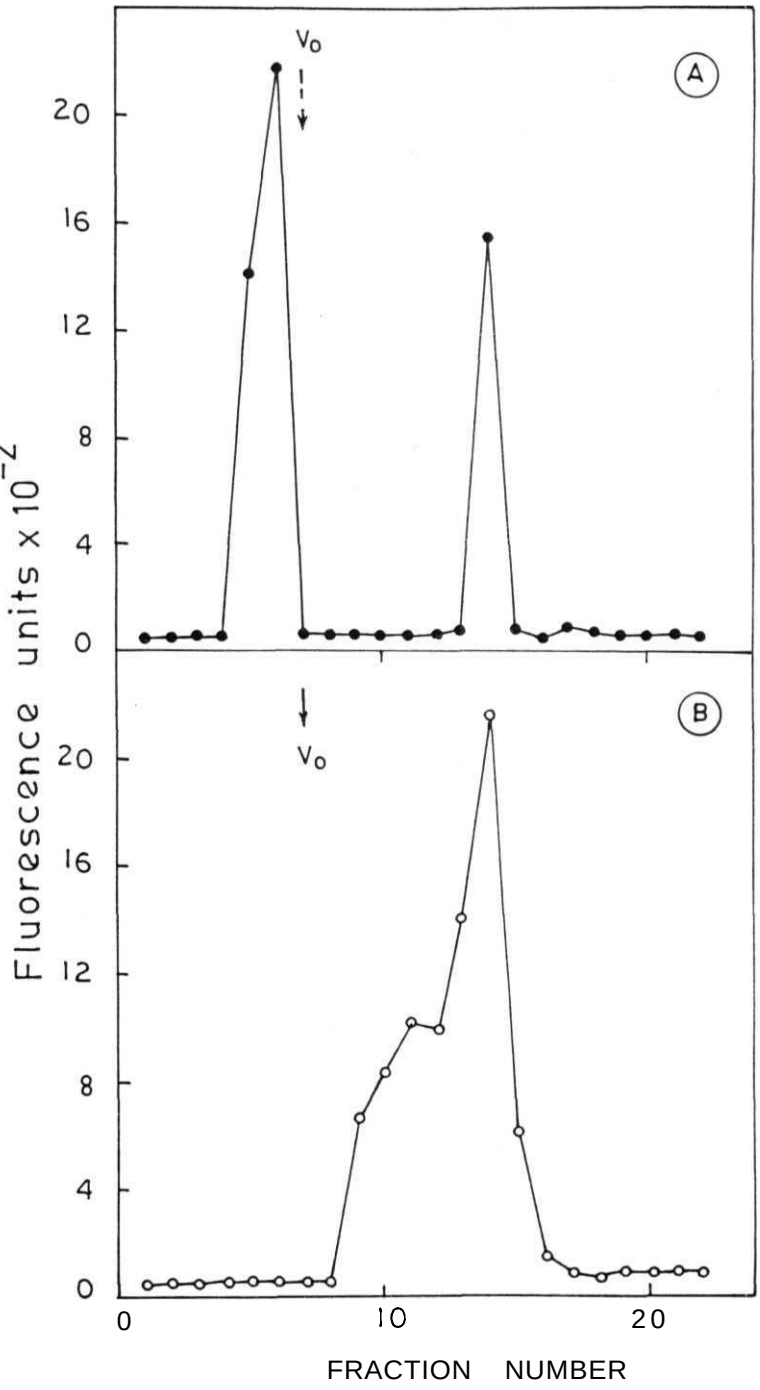

A
B
V_o
V_o
Fluorescence units x 10^{-2}
0
4
8
12
16
20
0
10
20
FRACTION NUMBER

Figure 6: Dissociation of the naER and E-RAF heterodimer under various experimental conditions. The free amino acids (2-10) were used at 10mM concentration. (1) 2M NaCl, (2) leucine, (3) glycine, (4) cysteine, (5) tyrosine, (6) lysine, (7) aspartate, (8) valine, (9) phenylalanine, (10) methionine.

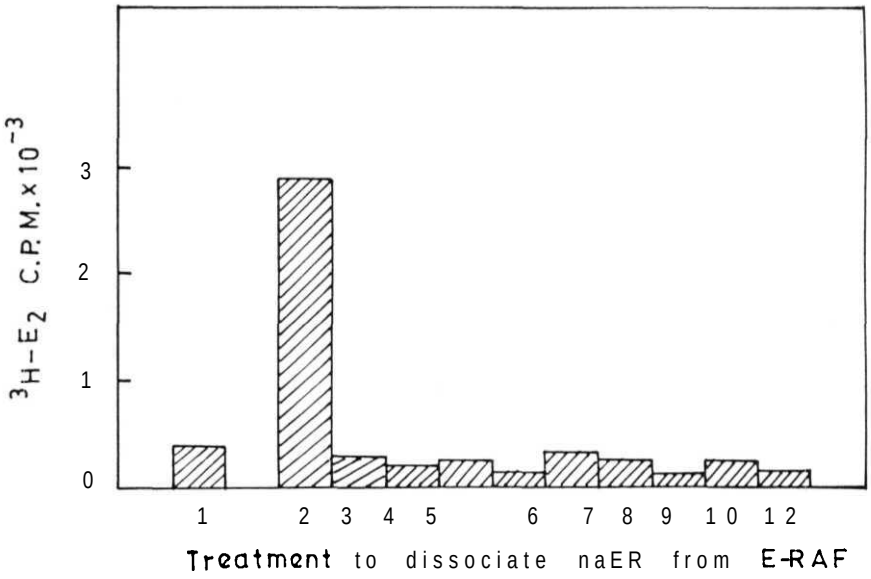
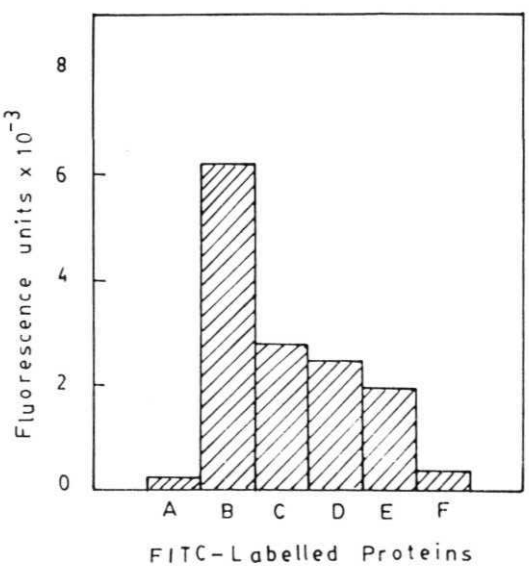

$^3H-E_2$ C.P.M. x 10^{-3}
3
2
1
0
1
2
3
4
5
6
7
8
9
10
12
Treatment to dissociate naER from E-RAF

Figure 7: Identification of the dimerization domain in E-RAF and studies on the effect of estradiol on dimerization between E-RAF and naER. Figure on the left shows the fluorescence transfer from FITC-naER to E-RAF in general and the a and β fragments in particular. The fluorescence associated with A= control (naER not labeled with FITC), B= FITC-naER, C= naER after separation from the dimer, D= E-RAF dissociated from the dimer, E= α fragment of E-RAF, F= β fragment of E-RAF.

The figure on the right displays the effect of estradiol on naER-E-RAF dimerization. FITC-naER was incubated with increasing concentrations of estradiol (0-20 nM) and it was made to dimerize with equimolar concentrations of E-RAF. Dissociation of the complex was brought about following exposure of the complex to 10mM leucine. The fluorescence associated with the E-RAF, dissociated from the heterodimer, was measured. 0T= total fluorescence associated with FITC-naER before dimerization. The values represent the concentration (nM) of estradiol used.


Fluorescence units x 10^{-3}
8
6
4
2
0
A B C D E F
FITC-Labelled Proteins

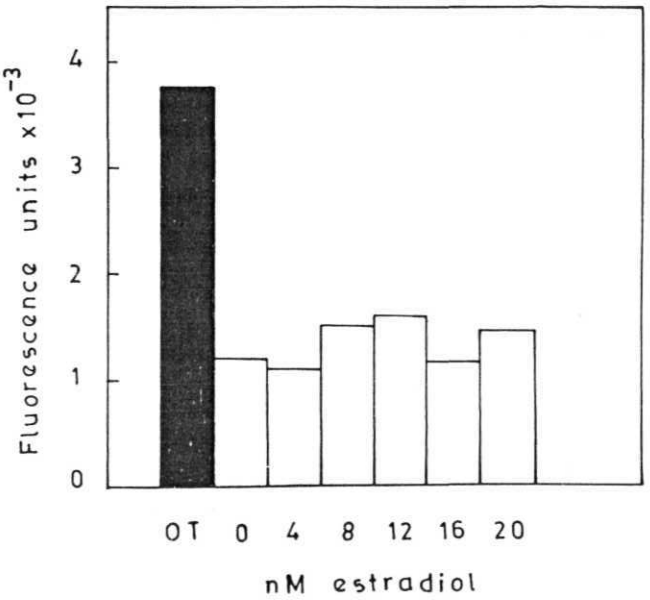

Fluorescence units x 10^{-3}
4
3
2
1
0
OT 0 4 8 12 16 20
nM estradiol

Figure **8**: Cross reactivity of naER and E-RAF with anti polyaspartate IgG (A and B respectively) . C and D are the blots of a and β fragments respectively showing the cross reactivity with anti polyaspartate Ig G.

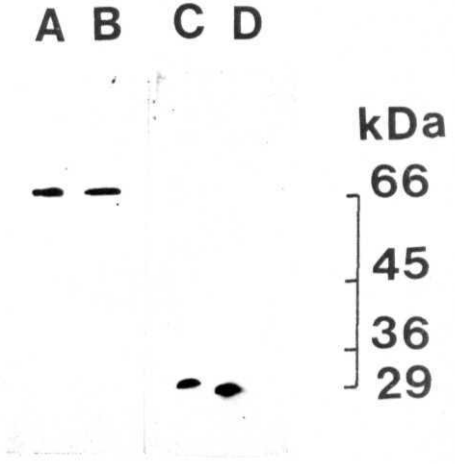
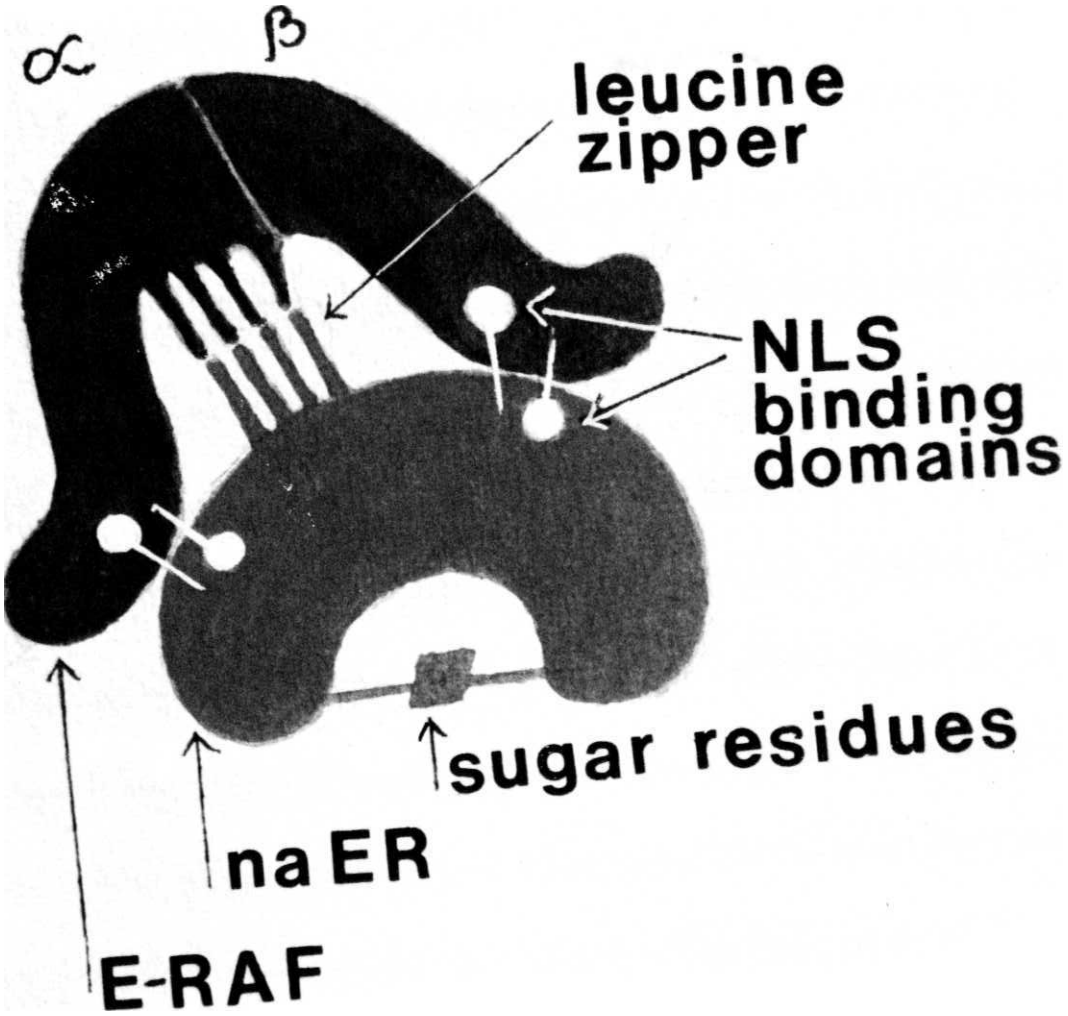

A B C D
kDa
66
45
36
29

Figure 9: Interdependence of naER and E-RAF for nuclear transport. H-estradiol bound-naER was incubated with increasing concentrations of E-RAF (0-100μg) in the medium that contained isolated goat uterine nuclei in order to study the E-RAF dependent transport of naER (Fig. 9A). In the other experiment FITC-E-RAF was incubated with varying concentrations of naER-E complex (0-100μg) along with nuclei in order to measure the naER dependent nuclear transport of E-RAF. The Fluorescence associated with the high salt extracts of the nuclei, following washing was measured (Fig. 9B).

Ⓐ
^3H-E_2 BOUND TO NUCLEI
c.p.m. x 10^{-3}
2.0
1.0
0
0
20
40
60
80
100
µg E-RAF

Ⓑ
FITC-E-RAF BOUND TO NUCLEI
FLUORESCENCE UNITS x10^{-3}
4.0
2.0
2.0
1.0
0
0
20
40
60
80
100
µg naER

Figure 10: A model displaying the possible sites of interaction between E-RAF and naER. The a and *ft* fragments of E-RAF have been identified. Based on the indirect evidence that leucine dissociates the naER -E-RAF heterodimer, it is being proposed that the leucine residues of a interdigitate with the corresponding residues in the naER, forming a leucine zipper. The naER is a glycoprotein; the sugar residues are displayed in such a way as to show that they are responsible for the maintenance of the structural and functional integrity of the protein. Both naER and E-RAF (a and *ft)* are shown here as possessing regions which recognize the nuclear localization sequences in the other monomer.


α
β
leucine
zipper
NLS
binding
domains
sugar residues
naER
E-RAF

Chapter IV

Hormonal Control of Estrogen Receptor Activation Factor (E-RAF) Synthesis in the Rat Uterus: Development of a Sensitive ELISA Method for the E-RAF Assay

The experimental results presented in this chapter is an attempt to understand the hormonal interpaly involved in estrogen receptor activation factor -biosynthesis in the rat uterus. It was felt that in order to understand the true significance of this protein in estrogen action a knowledge of the factors which regulate its synthesis was necessary. As the results indicate, the synthesis of the protein is under the exclusive control either of estradiol or of progesterone. Another part of this study was the developement of an ELISA for the measurement of E-RAF in reproductive tisssue. Using this method the E-RAF level in the rat uterus under certain specific physiological conditions has been estimated.

Immunologqical cross relatedness between E-RAF II and ER:

Both estrogen receptor and E-RAF II were subjected to CNBr fragmentation. The CNBr peptides were allowed to bind to anti ER IgG coupled to Sepharose in a column and both the flow through and the 4M $MgCl_2$ eluate fractions were collected. The peptide fragments were transferred to nitrocellulose which in turn was exposed to anti E-RAF IgG. The antibody cross reacted with E-RAF specific fragment in ER having a molecular mass of about 55 kDa and also with the uncleaved ER showing immunological cross relatedness between the two proteins (Fig 1) In E-RAF as in ER antigenicity was retained by the 55kDa fragment.

The purpose behind the presentation of this experimental results was to show the existence of common antigenic determinants in E-RAF and ER. This was necessary to show the importance of the DE-52-mediated separation of the ER and E-RAF

carried out during the amino acid incorporation studies.

Effect of ovariectomy or ovariectomy-coupled with adrenalectomy on the uterine E-RAF synthesis;

Cycling female rats were subjected either to bilateral ovariectomy or to a combination of ovariectomy and adrenalectomy. The animals were killed at 24h intervals from the day zero onwards. The uteri were incubated in a short term tissue culture medium containing labeled aminoacids and the radioactivity associated with the immuno precipitated E-RAF was examined. E-RAF synthesis in the uterus registered a decline immediately following ovariectomy and reached a basal level on day 3 post ovariectomy. Subsequently, a steady increase in E-RAF synthesis took place which reached the peak activity on day 5 following ovariectomy. This activity also declined, reaching the basal level, towards the end of the first week following surgery (Fig 2A).

It was interesting to note that in the animals subjected to ovariectomy and adrenalectomy the peak synthetic activity of day 5 was not detected (Fig 2B).

Influence of exogenous estradiol or of progesterone on E-RAF synthesis:

Rats were ovariectomized and maintained for a period of one week following which they received subcutaneous implants of estradiol- bees wax pellets (2 pellets per rat). The pellets had a hormone to wax ratio of 1: 1000. Another group of animals were subjected to ovariectomy and adrenalectomy simultaneously. These animals, maintained on saline and food for one week, received

progesterone- wax pellets having a hormone to wax ratio of 1: 200. The uterine E-RAF synthesis was studied in these rats at regular 24 hour intervals following the hormone- wax implantation. Exposure of the uteri to estradiol resulted in a gradual increase in the synthesis of E-RAF, reaching a peak on the day 4 following hormonal exposure (Fig 3A). The synthetic activity registered a decline subsequently. The E-RAF synthesis in response to the exposure of the uteri to progesterone followed an altogether different pattern. A sharp rise in E-RAF synthesis was noticed on day 5 with practically no synthetic activity being detected either before or after this crucial interval (Fig. 3B). The circulating level of estradiol in rats exposed to subcutaneous implants of estradiol- bees wax pellets was measured using estradiol RIA. A linear increase that occurred in the circulating hormone level was seen during the first 4 days after the exposure of the rats to estradiol-wax pellets (Fig 4). No further increase in the circulating hormone concentration was noticed subsequently, after reaching a level of 160 pg/ ml serum. This was compared with the average circulating estradiol level of proestrus rats. The value - 120 pg/ ml serum was significantly lower than that noticed in the animals exposed to estradiol-wax pellets. It has not been possible to carry out progesterone RIA in order to measure circulating levels of progesterone.

Additional studies on the hormonal specificity associated with the E-RAF biosynthesis:

It was of interest to observe how specific the estradiol mediated and the progesterone mediated synthetic activities were.

For this purpose ovariectomized rats were exposed to subcutaneous implants of Tamoxifen, either alone or in combination with estradiol. Similarly rats were subjected to ovariectomy and adrenalectomy simultaneously and were exposed to bees wax implants of testosterone or of dexamethasone. The treated animals belonging to both the categories, were killed on day 5 following the hormone- wax implantation.

Tamoxifen on its own was ineffective in stimulating E-RAF synthesis (Fig 5A) . At the same time the anti estrogen effectively blocked the estradiol-mediated rise in E-RAF synthesis. Similarly, testosterone and dexamethasone failed to evoke any positive response in the uterine synthesis of E-RAF (Fig 5B).

Effect of estrogen-progestogen combination on the uterine E-RAF synthesis in normal rats:

Rats belonging to the pro estrus stage of the estrus cycle were selected for this purpose. Two types of hormonal combinations were employed. In the first the animals were exposed to subcutaneous implants of estradiol- wax (1: 1000) or of progesterone- wax (1: 200). In the second the oral contraceptive, MALA-N, which is a mixture of Ethinyl estradiol and Norgestrel (300μg and 30μg respectively, per tablet) was employed. The bees wax implants made for the latter purpose contained a hormone to wax ratio of 1: 1000 with respect to Ethinyl estradiol. The animals were sacrificed at regular intervals and the E-RAF synthesis examined.

The pattern followed in the study involving estradiol and

progesterone (Fig 6A) and that involving MALA-N (Fig 6B) were almost identical. The synthetic activity declined immediately after the in vivo exposure of the uterus to the estrogen-progestogen combination, reaching a total decline by **day** 4 following the treatment. There was no E-RAF synthesis noticed during the remaining part of the hormone exposure regime.

Measurement of E-RAF in the pregnant rat uterus:

An ELISA method was developed in order to determine **the** concentration of E-RAF in tissue samples. The method developed is sensitive; the standard graph (Figure 7A) shows a linear relationship between the response and the concentration of E-RAF employed.

Using this method the concentration of E-RAF in the uteri and fetus of rats was measured during pregnancy. The results show a gradual increase in the concentration of E-RAF in the uteri during the first half of pregnancy, reaching a peak on day 10 (Fig 10B). The activity registered a steep decline almost immediately and the synthesis of E-RAF reached the basal level on day 16 of pregnancy. The E-RAF concentration in the fetus during **the** first two weeks of pregnancy was virtually undetectable. **A** sharp increase in the immunoassayable concentrations of E-RAF was observed on day 16. The fetal E-RAF titer continued to increase till the day of parturition.

The literature survey reveals that the plasma progesterone level in rats rise sharply shortly before implantation and the level remains elevated until day 16 of pregnancy reaching a peak (140 ng/ml) at this stage (Morishige et al., 1973). Immediately

following this, a sharp decline in progesterone concentration results, reaching the basal level of 20 ng/ml plasma. Estradiol levels in the ovarian venous plasma under these conditions showed a secretion rate of about o.8ng/ hour/ ovary. This basal level, detected during the first 15 days of pregnancy, rises sharply on the day 16 and remain elevated till parturition (Shaikh, 1971).

Cyclic changes in E-RAF levels in rat uteri during estrus cycle:

The uterine concentration of E-RAF during estrus cycle was measured following the ELISA. The E-RAF concentration in the uteri remained high during the estrus stage of the cycle. During proestrus the E-RAF concentration was about a half of that detected during the estrus. During metestrus and diestrus the E-RAF levels remained very low (Figure 8).

DISCUSSION:

A preliminary study was conducted earlier in our laboratory in order to understand the hormonal regulation of the synthesis gap junction proteins and E-RAF in the rat uterus (Anuradha & Thampan 1993). A refinement brought about in this study in comparison to that reported earlier was the DE-52 chromatography **and** collection of the DE-52 flow-through fractions of the high speed supernatant prepared from the uteri incubated in vitro. The method was introduced in order to separate E-RAF from the regular ER and the naER as it is known that the regular ER and naER remains firmly bound to the DE-52 matrix while E-RAF appears in the DE-52 flow-through fraction. The three proteins are immmunologically cross reactive (Zafar & Thampan 1993). We felt that it should be possible to bring about selective immunoprecipitation of E-RAF, free from being contaminated by naER and the regular ER by this method.

The first study on the effect of ovariectomy on the uterine E-RAF synthesis indicated that there are two factors that **are** involved in the process. The immediate decline in E-RAF synthesis following ovariectomy, which continued upto day 4 post ovariectomy, indicated that the synthesis was dependent upon one ovarian hormone. The sharp increase in E-RAF synthesis on day 5 after surgery brought into focus the second factor that regulates the protein biosynthesis. Since the comparison was made with a control rat belonging to the proestrus stage of the estrus cycle, it was not difficult to infer that the initial decline in E-RAF synthesis was due to the decline in circulating estradiol.

The sharp increase in the synthetic activity observed on day 5 post-ovariectomy could be interpreted as being due to one of the following reasons:

1. loss of an inhibitory material; 2. release of a hormone from an extra ovarian source.

Resko's observations (Resko, 1969) on the release of adrenal progesterone in rats immediately following ovariectomy gave an explanation in support of both the surmises mentioned above. He observed an enhancement in adrenal progesterone production and release following ovariectomy, apparently indicating that the ovarian estrogen had an inhibitory effect on the adrenal progesterone release into the circulation if not on production. The results of the experiment in which the rats were subjected to both ovariectomy and adrenalectomy confirmed this assumption since the peak synthetic activity of day 5 following ovariectomy in these rats disappeared completely.

The progesterone mediated stimulatory effect on E-RAF synthesis was restricted to a specific interval following in vivo hormone exposure, probably suggesting that the activity was dependent on a threshold level of circulating progesterone. Hormone levels either below or above this did not evoke a response. On the other hand, estradiol- mediated effect was spread over a longer interval. The increase in the uterine protein synthesis following its exposure to estradiol was gradual. The estradiol dependence in E-RAF synthesis is further highlighted by the fact that the anti estrogen, Tamoxifen, brought about a total inhibition in the estradiol mediated

protein synthesis. Further, testosterone and dexamethasone were unable to stimulate the E-RAF synthesis thereby to reaffirming the hormone specificity that is associated with this synthetic activity.

A totally different picture was obtained when E-RAF synthesis was examined in normal rats exposed in vivo to a mixture of estradiol and progesterone through subcutaneous bees wax implants. The synthetic activity declined as the days progressed following hormone treatment. This was clearly indicative of the fact that E-RAF synthesis was dependent on a highly balanced dose of ovarian hormones in vivo. Exposure of rats to the exogenous hormones apparently disrupted this delicate internal hormone balance possibly through the blocking of the hypothalamo- hypophysial axis. It appeared from this data that there existed a non responsive, latent phase during which additional exposure to the hormone could not evoke a stimulatory response in the uterus.

Mala-N is an oral contraceptive that is now being used by many women in India. It is mixture of a synthetic estrogen (Ethinylestradiol) and a synthetic progestogen (Norgestrel). The results of the experiment in which the rats were exposed in vivo to this hormonal mixture was identical to those of the experiment in which the rats were exposed to a mixture of estradiol and progesterone. It is therefore possible to conclude that exposure to this oral contraceptive has the inherent danger of interfering with the hypothalamo hypophysial axis. The drastic inhibitory effect observed on the uterine synthesis of E-RAF under such

circumstances is difficult to be ignored. Unfortunately, the research on the role of this protein in estrogen action has not captured the imagination of workers in other laboratories. We are also in no position at this stage to comment upon the biological consequences of such a block in E-RAF synthesis in women.

The sensitive ELISA method for the measurement of E-RAF in the rat uterus was helpful in measuring the E-RAF titers during pregnancy. It is known that the uterus is influenced predominantly by progesterone during the major part of pregnancy. As is evident from figure 3A the progesterone-dependent synthesis of uterine E-RAF is influenced by a threshold concentration of the hormone. The protein synthesis is not stimulated by a concentration of progesterone either above or below this threshold level. Although we do not have a RIA data to show the circulating progesterone concentration during this period, it appears logical to infer that the peak in endogenous E-RAF level noticed in the uterus during mid pregnancy was influenced by this highly specified level in circulating progesterone. A steep decline in the E-RAF titer takes place during later pregnancy, a period marked by decreasing levels of progesterone and increasing levels of estradiol in circulation (16, 17). Possibly, the increasing titers of immunoassayable E-RAF detected in the fetus during this period is an estrogen dependent phenomenon.

Looking at the extensive changes that the uterine E-RAF level undergoes during the various hormonal exposures it is possible to speculate that this protein has the potential to be identified as a marker protein in the diagnosis of estrogen

dependent cancers of the uterus and the breast in humans. This view is supported by the data that the E-RAF shares common antigenic determinants with the c-fos oncoprotein (Anuradha, 1992) . It is anticipated that the E-RAF ELISA will be of use in evaluating mammary and uterine cancers before they are exposed to the therapeutic regimen.

Figure 1: CNBr peptides of ER (A) and E-RAF (B) and their cross reactivity with anti E-RAF IgG (C - H).

lane C: CNBr peptide of ER

D: CNBr peptides of ER appearing in flow-through fraction of anti-ER Sepharose column.

E: 4M MgCl elution of ER peptides bound to anti-ER Sepharose column.

F: CNBr peptides of E-RAF.

G: E-RAF peptides appearing in the flow-through fraction of anti-ER Sepharose column.

H: 4M MgCl elution of E-RAF peptides bound to anti-ER Sepharo column.

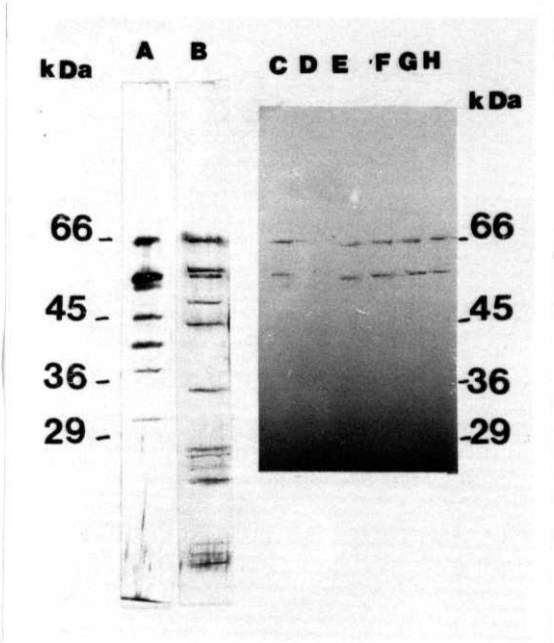

kDa
A
B
C D E F G H
kDa
66
45
36
29
66
45
36
29

Figure 2: Effect of ovariectomy (A) or ovariectomy combined with adrenalectomy (D) on the E-RAF synthesis by the rat uterus. The uteri, removed at intervals following surgery were incubated with a labelled amino acid mixture, as described in the text. The radioactivity associated with the proteins, immuno precipitated by monospecific rabbit anti goat uterine E-RAF IgG, was estimated. The data is the mean + S.E. of 4 independent determinations. The open circles represent the control data from experiments where the labelled proteins were first exposed to rabbit pre immune IgG and subsequently to goat anti rabbit Ig G.

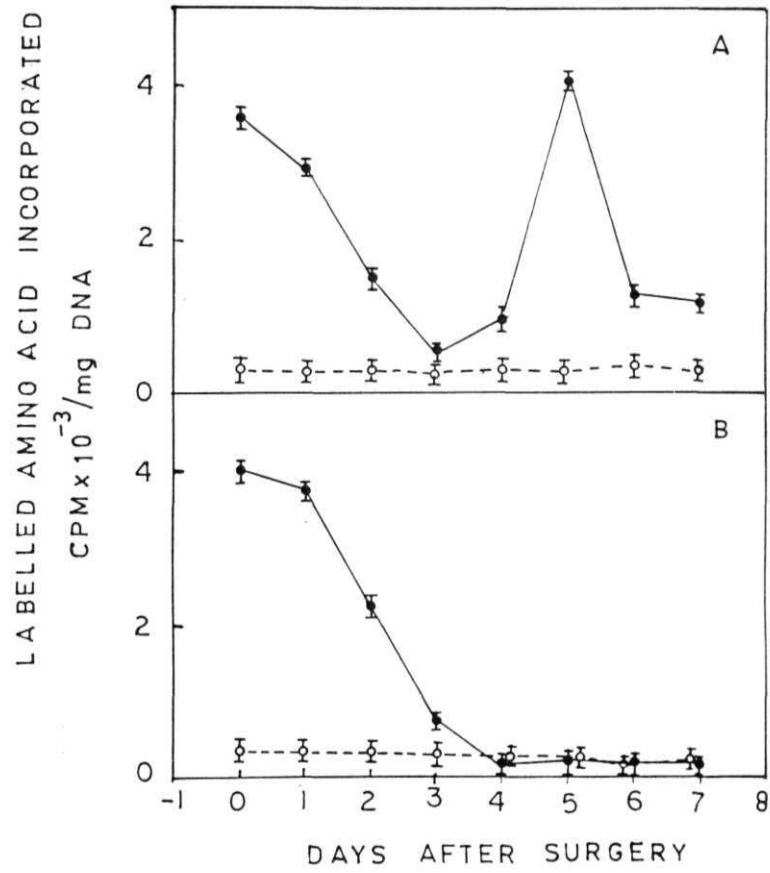

LABELLED AMINO ACID INCORPORATED
CPM x 10^-3/mg DNA
A
B
4
2
0
-1
0
1
2
3
4
5
6
7
8
DAYS AFTER SURGERY

Figure 3.: Effect of exogenous estradiol or of progesterone on the uterine E-RAF synthesis. (A) Rats were subjected to bilateral ovariectomy. One week after the surgery each animal received two subcutaneous implants of estradiol- bees wax pellets with a hormone to wax ratio of 1:1000. The animals were killed at 24 hour intervals and the uteri were incubated with the labeled amino acid mixture as described in "methods". The radioactivity associated with the proteins immuno precipitated by monospecific rabbit anti goat E-RAF IgG was measured. The data is the mean + S.E. of 4 independent determinations. (B) The rats were subjected to ovariectomy and adrenalectomy simultaneously and , one week after the surgery, were implanted with progesterone- bees wax pellets (1:200 ratio of hormone to wax). The remaining experimental steps were the same as in (A) . The open circles represent the control data from experiments where the labeled proteins were first exposed to rabbit pre immune IgG and subsequently to goat anti rabbit Ig G.

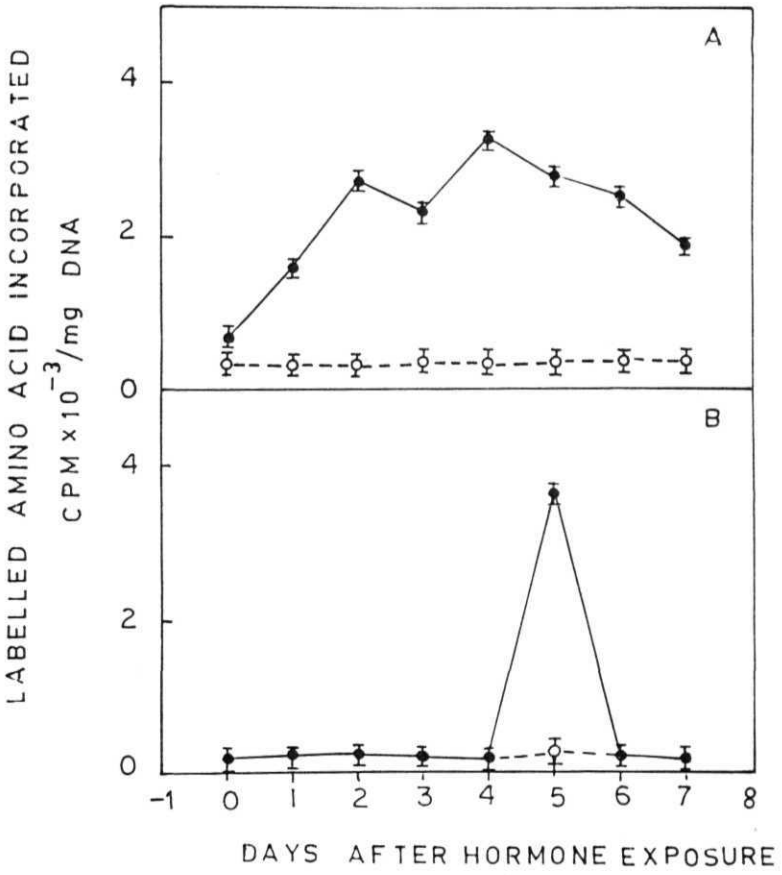

A
B
4
2
0
4
2
0
-1
0
1
2
3
4
5
6
7
8
LABELLED AMINO ACID INCORPORATED
CPM x 10^{-3}/mg DNA
DAYS AFTER HORMONE EXPOSURE

Figure 4.: Concentration of estradiol in the rat serum following implantation of the ovariectomized rats with estradiol wax pellets a week after the surgery. The animals received subcutaneous implants of two estradiol bees wax pellets with a hormone to wax ratio of 1: 1000 and were killed at 24 hour intervals. The serum was collected for the estimation of circulating estradiol using radioimmunoassay. PE indicates the hormone concentration in the proestrus rat (Fig. 4B).

Fig 4A is the standard graph of estradiol RIA.

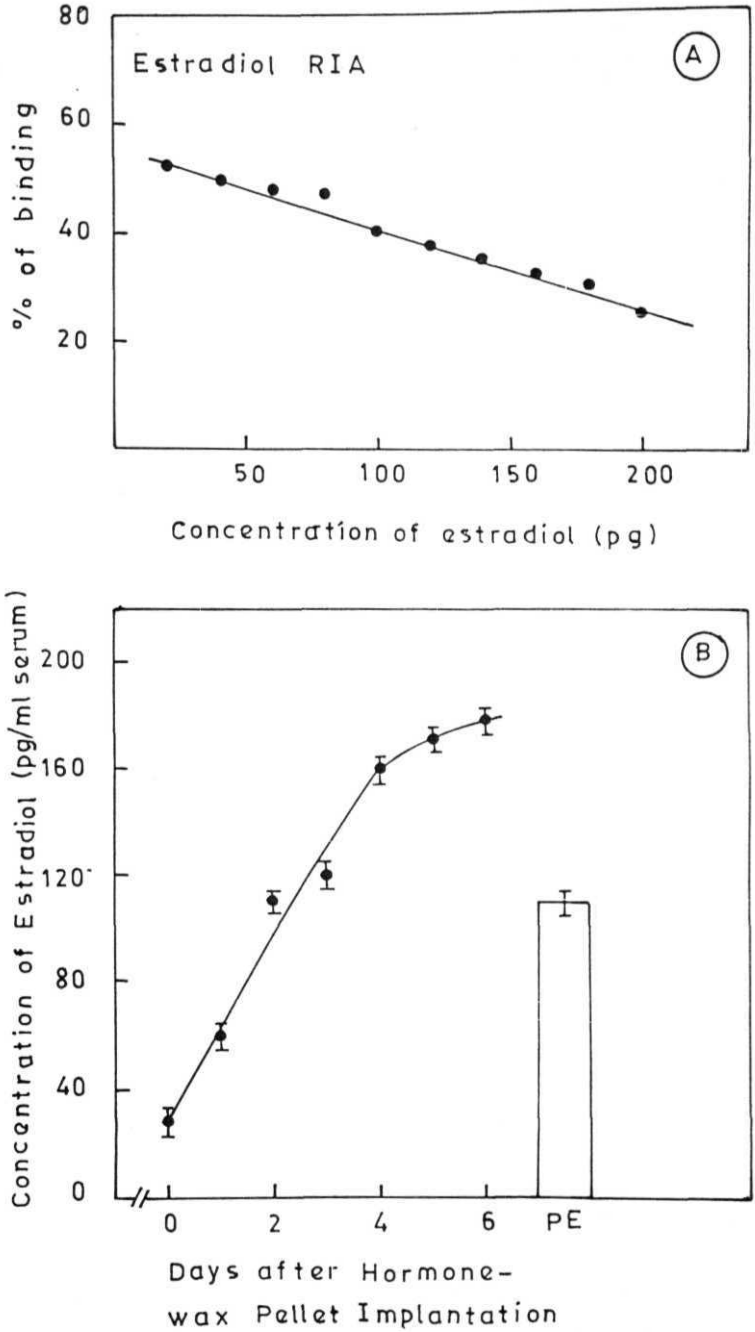

Estradiol RIA
A
% of binding
80
60
40
20
50
100
150
200
Concentration of estradiol (pg)
B
Concentration of Estradiol (pg/ml serum)
200
160
120
80
40
0
0
2
4
6
PE
Days after Hormone-
wax Pellet Implantation

Figure 5: Hormonal specificity involved in E-RAF synthesis. (Upper panel) Rats were ovariectomized and one week after the surgery, were implanted subcutaneously with two control bees wax pellets(C), estradiol- bees wax pellets (E), Tamoxifen- bees wax pellets (Tx) or a combination of Tamoxifen- bees wax and estradiol- bees wax pellets (TxE). (Lower panel) The animals were subjected to ovariectomy and adrenalectomy. The rats received two subcutaneous implants of bees wax (C), progesterone- bees wax (P), testosterone- bees wax (T) or Dexamethasone- bees wax pellets (Dx) . The animals were exposed to the hormone for a period of 5 days following which they were killed. The uteri were incubated with the labeled amino acid mixture and the radioactivity associated with the proteins immuno precipitated by monospecific, rabbit anti goat uterine E-RAF IgG measured. The data is the mean + S.E. of 4 independent determinations.

Labelled Amino acids Incorporated C.P. M.x 10^{-3}/mg DNA
A
4
3
2
1
0
C
E
Tx
TxE
B
4
3
2
1
0
C
P
T
Dx

Figure *6:* Effect of in vivo exposure of rats to an estrogen-progestogen combination on the E-RAF synthesis. Rats belonging to the proestrus stage of the estrus cycle were selected for this study. The animals received (A) 2 pellets of estradiol- bees wax and 2 pellets of progesterone- bees wax; (B) 2 pellets of Mala-N (ethanol extract- bees wax, 1:1000 ratio with reference to ethinyl estradiol and bees wax). The rats were killed at different intervals following hormone exposure and the synthesis of E-RAF studied. The data is the mean + S.E. of 4 independent determinations. The "control" rats received bees wax implants alone.

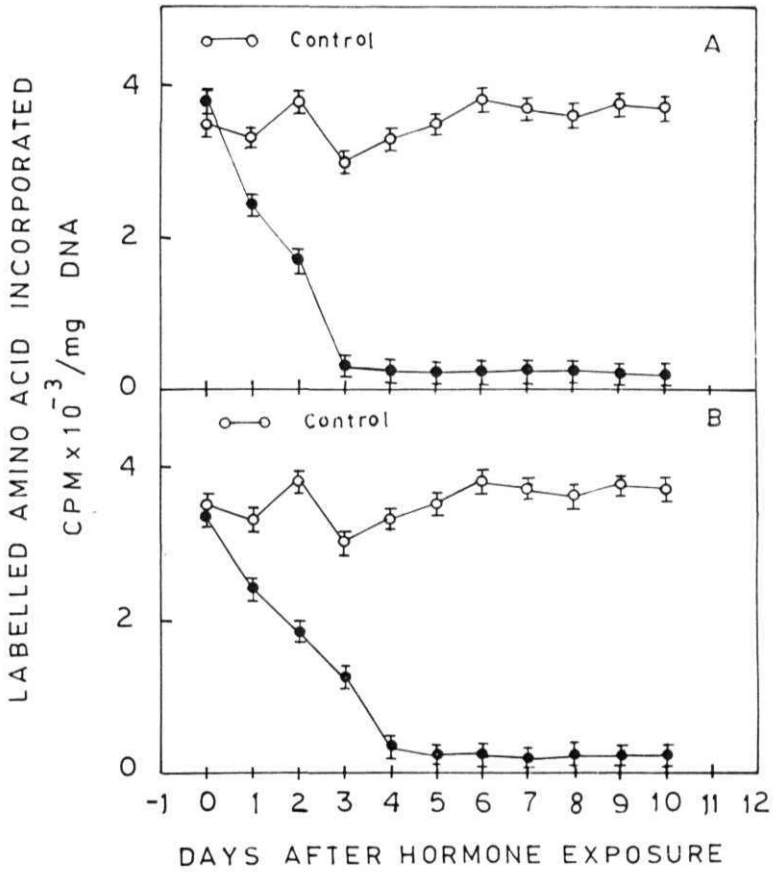

Control
A
Control
B
4
2
0
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
LABELLED AMINO ACID INCORPORATED
CPM x 10^-3/mg DNA
DAYS AFTER HORMONE EXPOSURE

Figure 7: Concentration of E-RAF during pregnancy in the rat uterus and fetus as measured by the ELISA. A) Standard graph for ELISA showing linear relationship between the response and E-RAF concentration. B) Open circles showing the concentration of E-RAF in the fetus during pregnancy. The closed circles represent the concentration of E-RAF in the pregnant uterus. The concentration of protein is expressed in terms of ng per mg DNA. The data is the mean ± S.E. of 4 independent determinations.

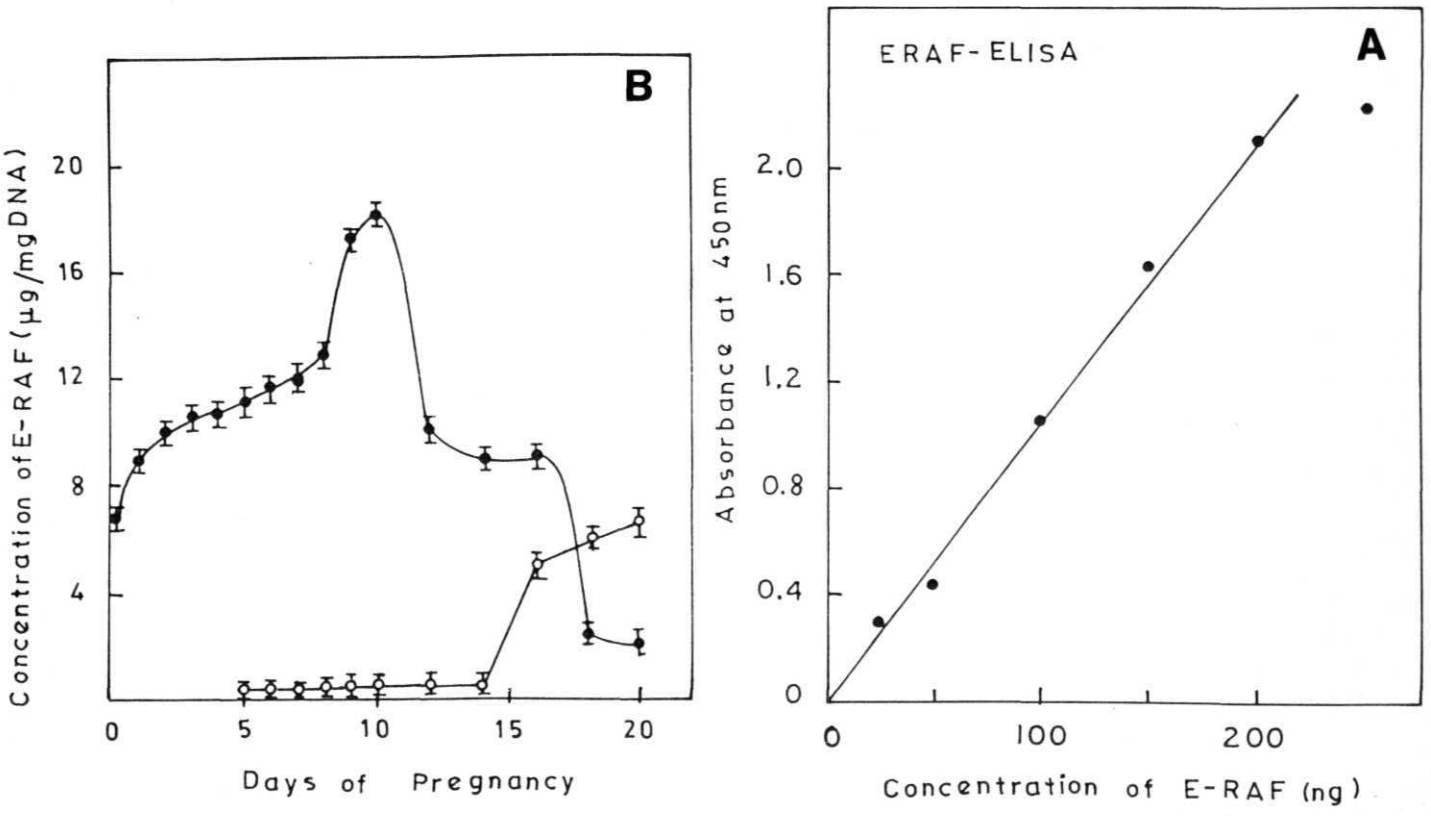
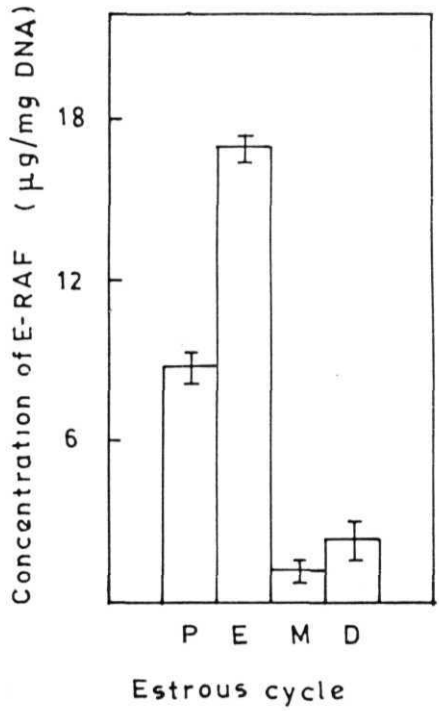

ERAF-ELISA
A
Absorbance at 450nm
2.0
1.6
1.2
0.8
0.4
0
0
100
200
Concentration of E-RAF (ng)
B
Concentration of E-RAF (µg/mg DNA)
20
16
12
8
4
0
5
10
15
20
Days of Pregnancy

Figure 8: Concentration of E-RAF in rat uterus during different stages of the estrus cycle, as measured by the ELISA. The data is the mean ± S. E. of 4 independent determinations. P = Proestrus; E = Estrus; M = Metestrus; D = Diestrus stage of estrus cycle.


Concentration of E-RAF (μg/mg DNA)
18
12
6
P
E
M
D
Estrous cycle

Appendix

Dual Hormonal Involvement in the Regulation of Rat Uterine Collagenase Activity

Collagenases are metalloproteinases synthesized by fibroblasts (chua et al., 1985), rat skin (Nagai et al., 1972), embryonic chicks (Sakamoto et al.,), rat uterus (Weeks et al., 1976)and other tissues involved in the degradation of collagen. Earlier studies on the collagen produced by the rat uterus both in vivo (Ryan et al., 1972) and in vitro (Jeffrey et al., 1970) emphasized the importance of this enzyme in the initiation of the breakdown process of the collagen molecules during the process of uterine involution.

Tyree et al., (Tyree et al., 1980) reported on the existence of latent collagenases in the medium of cultured involuting uterine explants and presented the evidence that progesterone prevented the conversion of the inactive collagenase to the active species. Whal, (Whal, 1977) on other hand, showed that both estradiol and progesterone were effective in the regulation of collagenase production by cultured macrophages. Other reports suggested that the dilation of cervix in pigs was achieved by estradiol- mediated degradation of type I collagen in situ by collagenase (Rajabi et al., 1990). It was also reported that apart from estradiol and progesterone, prostaglandin intermediates were also involved in collagenase production in the cervical cell cultures of pregnant guinea pigs (Rajabi et al., 1991). In the present study attempts are being made in order to

highlight the involvement of estradiol and progesterone in the regulation of rat uterine collagenase activity using each of the three collagen types (Type I,III, and V) that are predominant in the uterine wall.

RESULTS AND DISCUSSION:

Fig.1 shows the changes in the collagenase activity associated with the rat uterus in response to ovariectomy or a combination of ovariectomy and adrenalectomy over a period of one week. Assays were carried out at 24 hours intervals using the three collagen types viz type I, III and V. The activity of the collagenase that used type I collagen as substrate did not show any clear difference in the study comparing the ovariectomized and ovariectomized + adrenalectomized rats (Fig. 1A). The only difference that was apparent was that while in the ovariectomized rats the activity after day 5 post surgery persisted, it registered a decline in the rats subjected to both ovariectomy and adrenalectomy. On the other hand, the enzyme activity that required type III collagen as the substrate showed a clear difference between the two groups (Fig 1B) . The peak activity detected on days 2 to 4 post ovariectomy was no longer visible in the rats subjected to ovariectomy and adrenalectomy. The collagenase activity which used type V collagen as substrate also did not differentiate between the two experimental conditions, ovariectomy and ovariectomy + adrenalectomy (Fig 1C). Resko (Resko, 1969) reported that following ovariectomy the

plasma progesterone levels showed an increase. This was shown to be due to the enhanced secretion of progesterone from the adrenals, under the influence of ACTH. Based on this it was assumed that the changes in collagenase activity that required type III collagen as the substrate, noticed in the ovariectomy + adrenalectomized rats, could have been due to the influence of adrenal progesterone. Experiments were carried out in order to examine this assumption. In these experiments the animals used were subjected to both ovariectomy and adrenalectomy and were provided with subcutaneous implants of estradiol-beeswax pellets (1:1000 hormone to wax ratio) or progesterone beeswax pellets (1:200 hormone to wax ratio). The hormone exposure of animals was carried out one week after the rats were subjected to ovariectomy and adrenalectomy simultaneously.

The collagenase activities which used type I and V collagens as substrates responded positively to the treatment with both progesterone and estradiol (Fig 2A and C). The only difference noticed was that the positive response to progesterone appeared earlier than that to estradiol. The progesterone dependent effect declined as the estradiol dependent response registered an increase particularly in the case of the enzyme which used type I collagen as the substrate. The most convincing result was shown by the enzyme system that used type III collagen substrate. The activity was shown to be influenced predominantly by progesterone in the time dependent hormone exposure study (Fig 2B). An

immediate enhancement in this activity was noticed along with the *in vivo* exposure of the animals to progesterone. This activity dropped for a brief interval on the day 6 of hormone treatment when the short lived peak associated with the estradiol-dependent activity surfaced.

What appears is a progesterone-estradiol antagonism in the stimulation of different collagenase types in the rat uterus (Prem Kumar and Thampan, 1992). When the progesterone effect is at its peak, estradiol action remains low and the *vice versa*. There is a clear preference for the progesterone-stimulated system over the estradiol-dependent one in the regulation of the collagenase activity which uses type III collagen as the substrate.

Fig *1:* Effect of ovariectomy or of ovariectomy + adrenalectomy on the rat uterine collagenase activity. Uterine collagenase was assayed at different intervals following the surgery. The collagenase substrates used in the enzyme assay were type I (Fig 1A), type III (Fig 1B) and type V (Fig 1C). The data are mean + SD of at least 4 independent determinations.

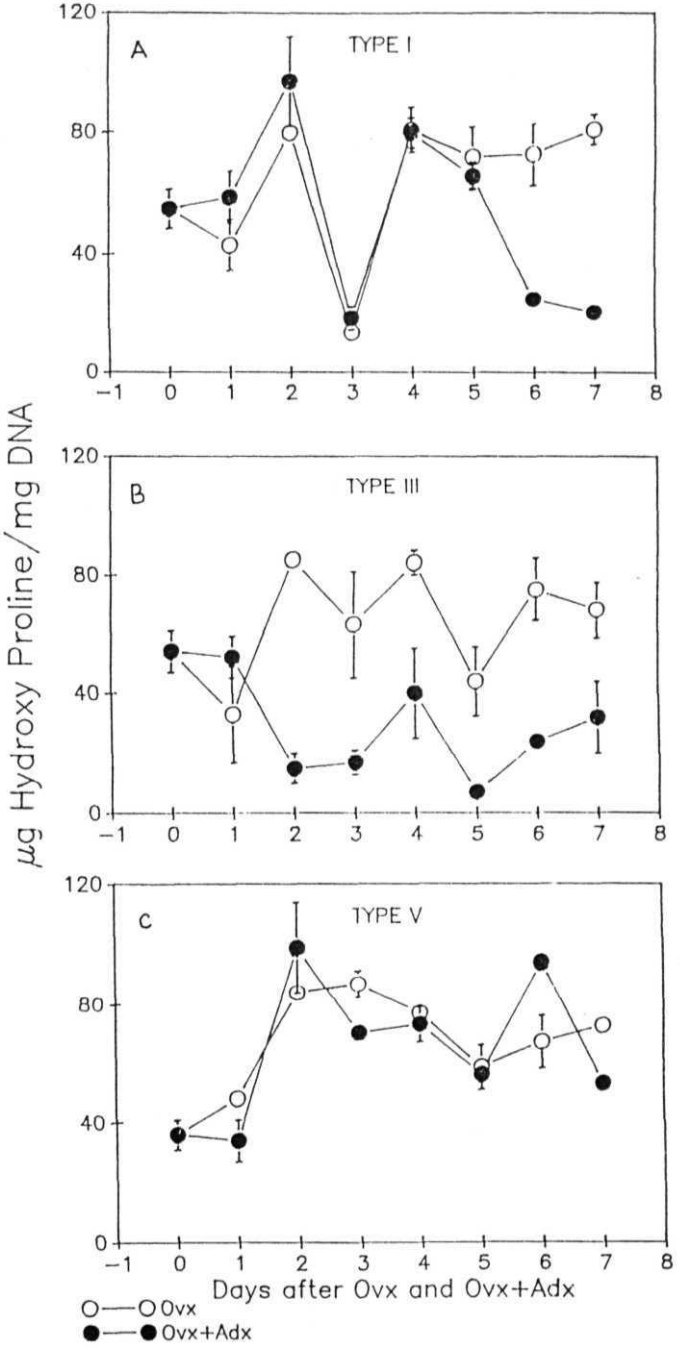
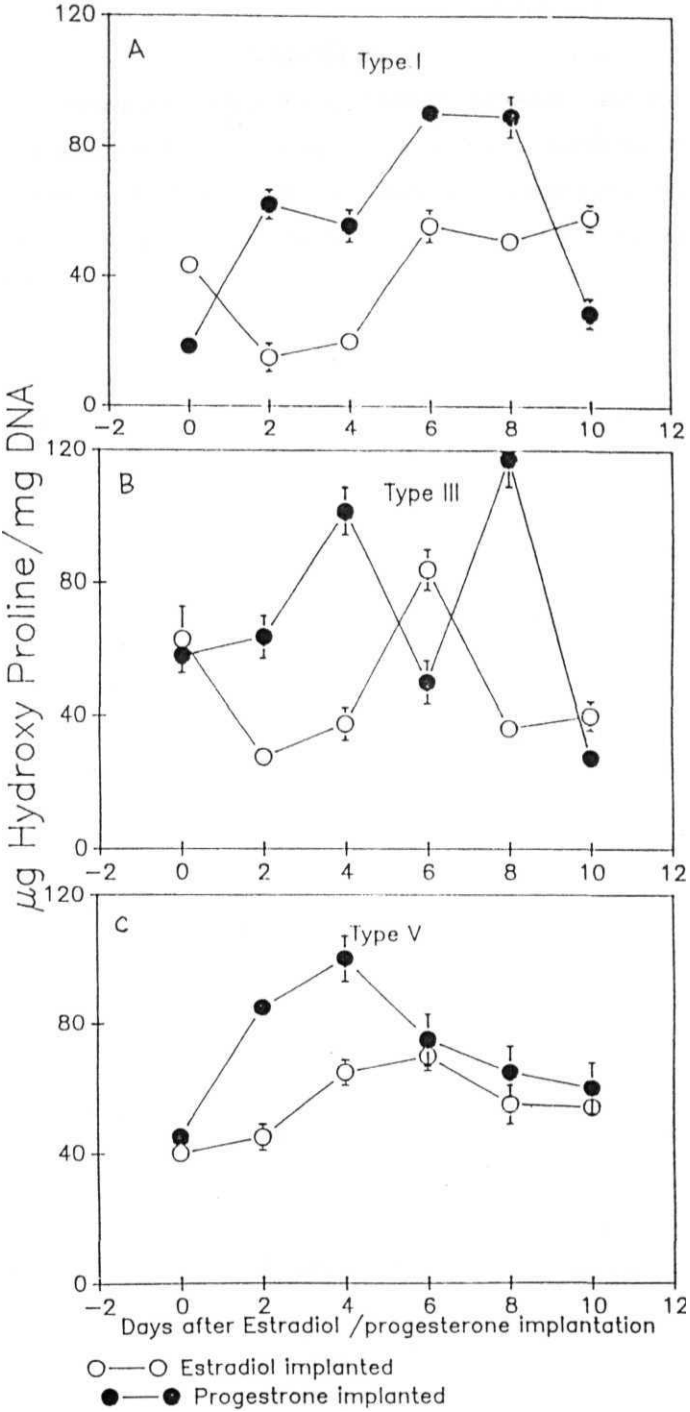

A
TYPE I
B
TYPE III
C
TYPE V
μg Hydroxy Proline/mg DNA
Days after Ovx and Ovx+Adx
O—O Ovx
●—● Ovx+Adx

Fig *2:* Effect of exogenous estradiol or of progesterone on the rat uterine collagenase activity in rats subjected to ovariectomy and adrenalectomy simultaneously . Hormone bees wax pellets were implanted in rats subcutaneously one week after the animals were subjected to the surgical intervention. The collagen substrates used in the enzyme assays were type I (Fig 2A), type III (Fig 2B) and type V (Fig 2C) . The data are mean ± SD of at least 4 independent determinations.


A
Type I
B
Type III
C
Type V
μg Hydroxy Proline/mg DNA
Days after Estradiol /progesterone implantation
Estradiol implanted
Progestrone implanted

CONCLUSIONS

1) Estrogen receptor activation factor (E-RAF) is a 66kDa receptor accessory protein having no capacity to bind to DNA. It dimerizes with a special class of estrogen receptor, the non activated estrogen receptor (naER) that has no capacity to bind to DNA on its own.

2) A 80 kDa calcium activated neutral protease (CANP), purified to homogeneity from the goat uterine cytosol cleaves the E-RAF into two near identical fragments α and β, of molecular mass 32 and 30 Kda respectively. The enzyme, under these conditions did not succeed in cleaving naER.

3) α-E-RAF retains the DNA-binding activity while the β fragment does not bind to DNA. Incubation of these fragments, independently with purified naER revealed that the a was capable of dimerization with naER.

4) Conditions under which the E-RAF-naER heterodimer dissociated into individual units were examined. While very high ionic strength could not dissociate the two monomeric units, leucine, at a concentration of 10mM succeded in dissociating them; all other amino acids tested in this process provided negative results. This was possibly an indirect evidence for the involvement of leucine zippers in the naER-E-RAF dimerization. It has also been shown that the dimerization domain is carried by E-RAF a.

5) anti Poly aspartate IgG has been used as a tool in detecting proteins having capacity to bind to nuclear localization sequence (NLS) in proteins which need to migrate to the nucleus. It has been shown that the antibody cross reacts with the naER as well

as the a and β fragments of the E-RAF. This possibly indicated **that** the NLS in one unit was recognized by its counterpart in the heterodimer. Migration of E-RAF and naER into nucleus failed to take place as independent units. 6) Hormonal control of E-RAF biosynthesis in the rat uterus has been analyzed. The synthesis is dependent on the exposure of the uteri either to estradiol or to progesterone. Exposure of rats in vivo to Mala-N an oral contraceptive, has been shown to inhibit E-RAF biosynthesis.

7) An ELISA method has been developed for the measurement of E-RAF in reproductive tissues. Using this method E-RAF analysis was made in rats belonging to two physiological states: (a) pregnancy; (b) the four stages of estrus cycle.

8) The studies revealed that E-RAF level in the uterus reached a peak towards a critical period during pregnancy when serum progesterone level is known to be high. towards the late pregnancy as the uterine E-RAF level declined, a corresponding increase in the fetal E-RAF level became apparent.

9) DMBA, in the presence of estradiol, produce a dramatic increase in the level of E-RAF, following exposure to very low doses of the carcinogen (data not shown).

10) The question remains : can E-RAF assay be carried out as a routine diagnostic exercise in evaluating estrogen-dependent cancers of uterus and the mammary gland.

References

Adam S. A., Lobl, T. J., Mitchell, M. A., and Gerace, L. (1989).
Identification of specific binding proteins for a nuclear location sequence.
Nature 337: 276-279.

Adam, S. A. and Gerace, L (1991).
Cytosolic proteins that specifically bind nuclear localization signals are receptors for nuclear import.
Cell 66: 837-847.

Alberts, B. M., and Herrick, G. (1971)
DNA-cellulose chromatography.
Methods Enzymol 21: 198-217.

Anuradha, P. (1992)
Hormonal Regulation of the synthesis of three classes of proteins in the rat uterus: Collagenases, Gap junction proteins and Estrogen receptor activation factors Ph. D. Thesis, University of Hyderabad.

Anuradha, P. and Thampan, R. V. (1993)
Hormonal regulation of gap junction proteins and estrogen receptor activation factors in the rat uterus.
Membrane Biochemistry 10, 119- 127.

Anuradha, P., Khan, S.M., Kartikeyan, N. and Thampan, R.V. (1994)
The nonactivated estrogen receptor (naER) of the goat uterus is a tyrosine kinase.
Arch. Biochem and Biophys, 309: 195-204.

Atkinson, H.C., Ratajesak, T. and Hahnel, R. (1990)
Interaction of Bovine estrogen receptor with immobilised Zinc.
Steroids 55: 405-409.

Azanza, J-A., Raymond, J., Robin, J-M., Cottin, P. and Ducastaing, A. (1979)
Purification and some physico-chemical and enzymatic properties of a calcium ion-activated neutral proteinase from rabbit skeletal muscle.
Biochem. J 183: 339-347.

Bala Nirmala, P. (1994)
Protein-protein interactions in estrogen receptor biology: Involvemnent of a nuclear localization sequence binding protein and Ubiquitin. Ph. D. Thesis, University of Hyderabad.

Bantien, E.E. (1987)
Steroid hormone antagonists at the receptor level: A role of heat shock protein MW 90,000 (hsp 90)
J Cell Biochem. 35: 161-174.

Baniahmad, A., Steiner, C., Kohne, A.C. and Renkawitz, R. (1990)

Modular structure of a Chicken Lysozyme silencer: Involvement of unusual thyroid hormone receptor binding site.
Cell. 61: 505-514.

Batra, S. and Sjogren, C. (1982)
Effect of estrogen treatment on calcium uptake by the rat uterine smooth muscle.
Life Sciences 32: 315-319.

Beato, M. (1989)
Gene regulation by steroid hormones.
Cell 56: 335-344.

Benbrook, D., Lernhardt, E. and Pfahl, M. (1988)
A retinoic acid receptor identified from a hepatocellular carcinoma.
Nature 333: 669-672.

Berg, P. (1986)
Protein metal binding domains in nucleic acid binding proteins.
Science 232: 485-487.

Binart, N., Chambrand, B., Dumas B., Rowlands, D.A., Bigogne, C., Levin, J.M., Gamier, J., Bantie, E.E. and Canelli, M.G. (1989)
The C-DNA derived aminoacid seguence of chick heat shock protein MW 90,000 (hsp 90) reveals a DNA like structure: Potential site of interaction with steroid receptors.
Biochem Biophys Res Commun 159: 140-147.

Blum, H., Beir, H. and Gross, H. J. (1987)
Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels.
Electrophoresis 8: 93-99.

Bradford, M. (1976)
A rapid and sensitive method for quantitation of microgram qunatities of protein utilising the principle of protein-dye binding.
Anal. Biochem. 72: 248-254.

Brand, N., Petkovich, M., Krust, A., Chambon, P., Dethe, H., Marachio, A., Tiollaic, P. and Deejan, A. (1988)
Identification of second human retinoic acid and thyroid hormone receptors.
EMBO. J. 11: 1409-1418.

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Stunzunbecker, L.J., G rippo, J.F., and Hunziker, W. (1993)
Two nuclear signalling pathways for vitamin D.
Nature 361: 657-660.

Chua, C. C, Geinman, D. E., Keller, G.H. and Lada, R. L. (1985)
Induction of collagenase secretion in human fibroblast cultures by growth promoting factors.
J. Biol. Chem. 260: 5213-5216.

Croall, D. E., and Demartino, G. N. (1983)
Purification and characterization of calcium-dependent proteases from rat heart.
J. Biol. Chem. 258: 5661-5665.

Croall, D. E., and Demartino, G. H. (1984)
Comparison of two calcium dependent proteinases from bovine heart.
Biochem. Biophys.Acta 788: 348-355.

Croall, D.E., Demartino, G. N. (1991)
Calcium-activated neutral protease (calpain) system: Structure, function and regulation.
Physiological reviews 71: 813-847.

Darling, D.S., Beebe, J.S., Burnside, J., Winslow, E.R. and Chin, W.W. (1991)
3, 5, 3'- Triiodothyronine (T_3) receptor auxiliary protein (TRAP) binds DNA and forms heterodimers with the T receptor.
Mol. Endocrinol 5: 85-93.

Davey, J., Dimmock, N. J., Colman, A. (1985)
Identification of the sequence responsible for the nuclear accumilation of of the influenza virus nucleoprotein in xenopus oocyte
Cell 40: 667-675.

Demartino, G. N. (1981)
Calcium-dependent proteolytic activity in rat liver: identification of two proteases with different calcium requirements.
Arch. Biochem. Biophys. 211: 253-257.

Demartino, G. N. and Croall. D. E. (1983)
Purification and characterization of calcium-dependent protease from rat liver.
Biochemistry 22: 6287-6291.

De Robertis, E. M. (1983)
Nucleocytoplasmic segregation of proteins and RNAs.
Cell 32: 1021-1025.

Diankun, G. P., Fairall, 1. and Khung, A. (1986)
EXAFS study of the zinc binding sites in the protein transcriptional factor IIIA.
Nature 324: 698-699.

Dice, J. F., (1987)
Molecular determinants of protein half-lives in eukaryotic cells.
FASEB. J. 1: 349-357.

Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982)
A polypeptide domain that specifies migration of nucleoplasm into

the nucleus.
Cell 30: 449-558.

Dingwall, C., and Laskey, R. A. (1986)
Protein import into the cell nucleus.
Ann. Rev. Cell. Biol 2: 367-390.

Emori, Y., Kawasaki, H., Imajoh, S., Kawashima, S. and Suzuki, K. (1986)
Isolation and sequence analysis of cDNA clones for the small subunit of rabbit calcium dependent protease.
J. Biol. Chem. 261: 9472-9476.

Evans, R.M. (1988)
The steroid and Thyroid hormone receptor superfamily.
Science 240: 889-895.

Evans, R.M. and Hollenberg, S.M. (1988)
Zinc fingers: gilt by association
Cell 52: 1-3

Exton, J. H. (1988)
Mechanism of actions of calcium-mobilizing agonists: some variations on a young theme.
FASEB. J. 2: 2670-2676.

Fawell, S.E., Lees, J.A., White, R. and Parker, M.G (1990 a)
Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor.
Cell 60: 953-962.

Fawell, S.E., White, R., Hoare, S., Syndehham, M., Page, M. and Parker, M.G (1990 b)
Inhibition of estrogen receptor - DNA binding by the pure anti estrogen ICI 164, 384 appears to be mediated by impaired receptor dimerization.
Proc. Natl. Acad. Sci., USA 87: 6883-6887.

Feldherr, C. M., and Pomerantz, J. (1978).
Mechanism for the selection of nuclear polypeptide in xenopus.
J. Cell Biol. 78: 168-175.

Feldherr, C. M., Cohen, R. J., and Ogburn, J. A., (1983)
Evidence for mediated protein uptake by amphibian oocyte nuclei.
J. Cell. Biol. 96: 1486-1490.

Forman, B.M., Casanova, J., Raaka, B.M., Ghysdael, J. and Samuel, H.H. (1992)
Half site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers or heterodimers.
Mol. Endocrinol 6: 429-442.

Forster, T. (1948)
Ann. Physik 2: 55-75.

Furey , W. F., Robbins, A. H., Clancy, L. L. , Winge, D. R., Wang, B. C. and Scout, C. D. (1986)
Crystal structure of Cd, Zn metallothionein.
Science 231: 704-710.

Giguere, V., Ong, E.S., Segui, P. and Evans, R.M (1987)
Identification of receptor for morphogen retinoic acid.
Nature 330: 624-629.

Goldberg, A. L. and Dice, J. F. (1974)
Intracellular protein degradation in mammalian and bacterial cells.
Ann. Rev. Biochem. 43: 835-869.

Goldberg, A. L. and St. John, A. C., (1976)
Intracellular protein degradation in mammalian and bacterial cells: Part 2.
Ann. Rev. Biochem. 45: 747-803.

Goldfarb, D. S., Gariepy, J., Schoolnik, G. and Kornberg , R. D. (1986)
Synthetic peptides as nuclear localization signals.
Nature 322: 642-644.

Gordon, M. S. and Notides, A. C. (1986)
Computer modelling of estradiol interactions with estrogen receptor.
J. Steroid Biochem 25: 177-182.

Green, S. and Chambon, P (1986)
A superfamily of potentially oncogenic receptors.
Nature 324: 615-617

Green, S. and Chambon, P (1987)
Estradiol induction of a gluco corticoid responsive gene by a chimaeric receptor.
Nature 325: 75-78.

Green, S., Kumar, V., Thenlaz., I. Wahli, W. and Chambon, P (1988)
The N-terminal DNA binding zinc finger of the oestrogen and glucocorticoid receptors determines the target gene specificity.
EMBO. J 7: 3037-3044.

Hamada, K., Gleason, S.L., Levi, B-Z., Hirschfeld, S., Appella, E and Ozato, K (1989)
H-2 RII BP, a member of nuclear hormone receptor superfamily that binds both the regulatory element of major histocompatibility Class I genes and the estrogen response element.
Proc. Natl. Acad. Sci., USA 86: 8289-8293.

Hai, T. and Curran, T (1991)
Cross family dimerization of transcription factors FOS/JUN and ATF/CREBP alters the DNA binding specificity.

Proc. Natl. Acad. Sci., USA 88: 3720-3724.

Hatanaka, M., Sasaki, T., Kikuchi, T. and Murachi, T. (1985)
Evidence for the hetrodimeric nature of calpain molecules as studied by cross-linking modification.
Biochem. Int. 10: 187-193.

Hershko, A. and Ciechanover, A. (1986)
The ubiquitin pathway for the degradation of intracellular proteins.
Prog. Nucleic Acid Res. Mol. Biol. 33: 19-56.

Holloway, J.M., Glass, C.K., Alder, S., Nelson, C.A. and Rosenfeld, M.G (1990)
The C'-terminal interaction domain of the thyroid hormone receptor confers the ability of the DNA site to dictate positive or negative transcriptional activity.
Proc. Natl. Acad. Sci. USA 87: 8160-8164.

Huckaby, C. S., Conneely, O. M., Beattie,W. G., Dobson, A. D. W., Tsai, M-J. and O'Malley, S. W. (1987)
Structure of chromosomal chicken progeserone receptor gene.
Proc. Natl. Acad. Sci. 84: 8380-8384.

Iano, K., Haino, M., Iwasaki, M., Oho, N., Horigma, T. and Sugano, H. (1990)
Reconstitution of 9s estrogen receptor with heat shock protein 90.
FEBS Letters 267: 157-159.

Inomata, M., Kasai, Y., Nakamura, M. and Kawashima, S. (1988)
Activation mechanism of calcium-activated neutral protease. Evidence for the existence of intramolecular and intermolecular autolyses.
J. Biol. Chem. 263: 1759-1766.

Ishura, S., Murofuschi, H., Suzuki, K. and Imahori, K., (1978)
Studies of calcium activated neutral protease from chicken skeletal muscle.
J. Biol. Chem. 84: 225-230.

Jacob, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.G, Binart, N., Mester, J and Baulien, E.E (1984)
Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones.
Nature 308: 850-853.

Jeffrey, J. J. and Gross, J. (1970)
Isolation and characterization of collagenase from post-partum rat uterus.
Biochemistry. 9: 269-278.

Jensen, E.V. and De Sombre, E.R (1973)
Estrogen receptor interaction.
Science 182: 126-134.

Johnson, M. (1987)
Genetic evidence that zinc is an essential co-factor in the DNA binding domain of GAL4.
Nature 328: 353-355.

Kalderon , D. , Richardson, W. D. , Markham, A. F., and Smith, A. E., (1984)
Sequence requirements for nuclear location simian virus 40 large -T Antigen.
Nature 311: 33-38.

Kasper, C. B. (1970)
Fragmentation of proteins for sequence studies and seperation of peptide mixtures, in protein sequence determination.
Protein sequence determination (Needeman, S. B. , Ed) pp 137-184 Chapman and Hall, London.

Karthikeyan, N. (1994)
Estrogen receptors of goat uterus: Emphasis on a receptor form that enters the nucleus as constituent of a Heterodimer. Ph. D. Thesis, University of Hyderabad.

King, W.J. and Greene, G.L. (1984)
Monoclonal antibodies localize oestrogen receptors in the nuclei of target cells.
Nature 307: 745-747.

King, R.J.B. (1986)
Receptor structure: A personal assessment of the current status.
J. Steroid Biochem. 25: 451-454.

King, R.J.B. (1987)
Structure and function of steroid receptors.
J. Endocrinol 114: 341-349.

Kitahara, A., Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Hatanaka, M. and Murachi, T. (1984)
Large scale purification of porcine calpain I and calpan II and comparison of proteolytic fragments of their subunits.
J. Biochem. 95: 1759-1766.

Kouzarides, T. and Ziff, E. (1988)
The role of the leucine zipper in the fos-jun interaction.
Nature 336: 646-651.

Krestsinger, R. H. and Nockolds, C. E., (1973)
Carp muscle calcium binding protein II. Structure determination and general description.
J. Biol. Chem. 248: 3313-3326.

Krust, A., Green, S., Argos, P., Kumar, V., Waller, P., Bornet, J.M. and Chambon, P. (1986)
The chicken oestrogen receptor sequence: homolog with V-erb A and

the human oestrogen and glucocorticoid receptors.
EMBO. J 5: 891-897.

Krust, A., Kastuer, P.H., Petkovich, M. , Zelent, A. and Chambon, P. (1989)
A third human retinoic acid receptor, h RAR - gamma.
Proc. Natl. Acad. Sci., USA 86: 5310-5314.

Kumar, V., Green, S., Staub, A. and Chambon, P (1986)
Localization of the estradiol binding putative DNA binding domains of the human estrogen receptor.
EMBO. J 5: 2231-2236.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R. and Chambon, P. (1987)
Functional domains of human estrogen receptor.
Cell 51: 941-951.

Kumar, V. and Chambon, P. (1988)
The estrogen receptor binds tightly to its responsive element as a ligand induced homodimer.
Cell 55: 145-156.

Lacasse. E. C. , Lochnan, H. A., Walker, P. and LeFebvre, Y. A.(1993)
Identification of binding proteins for nuclear localization signals of the glucocorticoid and thyroid hormone receptors.
Endocrinology 132: 1017-1025.

Laemmli, U. K. (1970)
Clevage of structural proteins during the assembly of the head of bacteria T4.
Nature 227: 680-685.

Landschulz, W.H., Johnson, P.F. and Mc Knight, S.L. (1988)
The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins.
Science 240: 1759-1764.

Landschulz, W.H., Johnson, P.F. and Mc Knight, S.L. (1989)
The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite.
Science 243: 1681-1688.

Lannigau, D.A. and Notides, A.C. (1990)
A novel mechanism for eukaryotic gene expression: The involvement of DNA tertiary structure in estrogen receptor recognition of its target nucleotide seguence.
Biochemical pharmacology 40: 2579-2585.

Lazar M.A. and Chin, W.W. (1990)
Nuclear thyroid hormone receptors.
J. Clin. Invest. 86: 1777-1782

Lazar, M.A., Berrodiu, T.J. and Harding, H.P. (1991)
Differential DNA binding by monomeric, homodimeric and potentially heteromeric forms of thyroid hormone recptor.
Mol. Cell Biol. 11: 5005-5015.

Liao, J., Ozono, K., Sone, T., Mc Donnel, D.P. and Pike, J.W. (1990)
Vitamin D receptor interaction with specific DNA requires; a nuclear protein and 1,25-dihydroxy vitamin D .
Proc. Natl. Acad. Sci. USA 87: 9751-9755.

Lindstet, A.D., West, N.B. and Brenner, R.M. (1986)
Analysis of monomeric-dimeric states of the estrogen receptor with monoclonal antiestrophilin.
J. Steroid Biochem. 24: 677-686.

Malik, M. N., Fenko, M. D., Iqbal, K. and Wishniewski, H. M. (1983)
Purification and characterization of two forms of Calcium-activated neutral protease from calf brain.
J. Biol. Chem. 258: 8955-8962.

Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990)
Nuclear receptor that identifies a novel retinoic acid response pathway.
Nature 345: 224-229.

Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S. and Evans, R.M. (1991)
A direct repeat in the cellular retinol binding protein type II gene confers differential regulation by RXR and RAR.
Cell 66: 555-561.

March, S. C, Parikh, I. and Cuatrecasas, P. (1974)
A simplified method of cyanogen bromide activation of agarose for affinity chromatography.
Anal. Biochem. 60: 149-152.

Marks, M.S., Hellenbeck,P.L., Nagata, T., Sagars, J.H., Appella, E., Nikodem, V.M. and Ozato, K. (1992)
H-2RIIBP (RXR_α) heterodimerization provides a mechanism for combinational diversity in the regulation of retinoic acid and thyroid hormone responsive genes.
EMBO. J 11: 1419-1435.

Marmur, J. (1961)
A procedure for isolation of DNA from microorganisms.
J. Mol. Biol. 3, 208-218.

Melloni, E., Pontremoli, S., Michetti, M., Sacco, 0., Sparatore, B. and Horecker, B. C., (1985)
Binding of protein kinase C to neutrophil membranes in the presence of Calcium and its activation by calcium-requiring proteinase.

Proc. Natl. Acad. Sci. USA 82: 6435-6439.

Melloni, E., Salamoni,F., Sparatore, B., Michetti, M. and Pontemoli, S. (1986)
Calcium-dependent neutral proteinase from human erythrocytes: activation by Calcium ions and substrate and regulation by the endogenous inhibitor.
Eur. J. biochem. 159: 387-392.

Miller, E. J. and Rhodes, R. K. (1982)
Preparation and characterization of different types of collagen.
Methods in Enzymology (Cunningham, L. W. and Frederiksen, D. W. Eds), 82: 33-64, Academic Press, New York.

Miller, M. A., Mullick, A., Greene, G. L. and Katzenellenbogen, B. S. (1985)
Characterization of subunit nature of nuclear estrogen receptors by chemical cross linking and dense amino acid labelling.
Endocrinology. 117, 515-522.

Miller, J., Mclachlan, A.D. and King, A. (1985)
Repetitive zinc binding domains in the protein transcription IIIA from xenopus oocytes.
EMBO. J 4: 1609-1614.

Morishige, W. K., Pepe, A. P., and Rothchild, I. (1973)
Serum leutenizing hormone, prolactin and progesterone levels during pregnancy in the rat.
Endocrinology 5: 1527-1530.

Muller, R.E., Traish, A.M. and Wotiz, H.H. (1983)
Estrogen receptor activation precedes transformation.
J. Biol. Chem. 258: 9227-9232.

Murakami, T., Ueda, M., Hamakubo, T. and Murachi, T. (1988)
Identification of both clapains I and II in nucleated chicken erythrocytes.
J. Biochem, 103: 168-171.

Murray, M.B. and Towle, H.C. (1989)
Identification of nuclear factors that enhance binding of thyroid hormone receptors to a thyroid hormone response element.
Mol. Endocrinol. 3: 1434-1442.

Nagai, Y., Hori, H. (1972)
Entrapment of collagen in polyacrylamide matrix and its application in the purification of animal collagenase.
Biochim. Biophys. Acta. 263: 564-573.

Nargessi, R. M., and Landon, J. (1981)
Indirect quenching fluoro immunoassay
Methods in enzymol (ed. . Langone, J, J. and Vunakis, H. V.) 74: 64-79.

Neuhaus, D., Nakaseko, Y., Nagai, K. and Klung, A. (1990)
Sequence specific [H] NMR resonance assingnments and secondary structure identification for 1 and 2 zinc finger constructs from SW15. A hydrophobic core involving four invariant residues.
FEBS Letters 262: 179-184.

Niswender, G. D., Akbar, A. M. and Nett, T. M. (1975)
Use of specific antibodies for qunatification of steroid hormones.
Methods in enzymology vol. 36 (O'Malley, B.W. and Hardman, J.G. ed.) 16-34.

Notides, A.C. and Nielson, S. (1974)
The molecular mechanism of the in vitro 4S to 5S transformation of the uterine estrogen receptor.
J. Biol. Chem. 249: 1866-1873.

Oas, T.G., Mc Intosh, L.P., O'Shea, E.K., Dahlquist, F.W. and Kim, P.S. (1990)
Secondary structure of a leucine zipper determined by nuclear magnetic resonance spectroscopy.
Biochemistry 29: 2891-2894.

O'Donnell, A.L., Rosen, E.D., Darling, D.S. and Kooing, R.J. (1991)
Thyroid hormone mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein.
Mol. Endocrinol. 5: 94-99.

O'Malley, B.W. and Mean, A.R. (1974)
Female steroid hormones and target cell nuclei.
Science 183: 610-620.

O'Shea, E.K., Rotkowski, R., Stafford, W.F. and Kim, P.S. (1989)
Evidence that leucine zipper is a coiled coil.
Science, 243: 538-540.

O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991)
X-ray structure of the GCN 4 leucine zipper, a two stranded parallel coiled coil.
Science 254: 539-544.

Otsuka, Y. and Tanaka, H. (1983)
Purification of new calcium activated protease (low calcium requiring form) and comparison to high calcium requiring form.
Biochem. Biophys. Res. Commun. 111: 700-109.

Petkovich, M., Brand, N.J., Krust, A. and Chambon, P. (1987)
A nuclear human retinoic acid receptor which belongs to the family of nuclear receptors.
Nature 330: 444-450.

Picard, D. and Yamamoto, K. R. (1987).
Two signals mediate hormone dependent nuclear localization of the glucocorticoid receptor.
EMBO J. 6: 3333-3340.

Picard, D., Kumar, V., Chambon, P. and Yamamoto, K. R., (1990).
Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors.
Cell Regul. 1: 291-299.

Prem Kumar, M. and Thampan, R. V. (199 2)
Dual hormonal involvement in the regulation of rat uterine collagenase activity.
Biochem. Int. 28: 975-980.

Puca, G. A., Nola, E., Sica, V. and Bresciani, F., (1977)
Estrogen binding proteins of calf uterus: Molecular and functional characterization of the receptor transformation factor: a Calcium-activated protease.
J. Biol.Chem. 252: 1358-1366.

Raam, S., Lauretano, A. M. , Vrabel, D. M. , Pappas, C. A., and Tamura, H. (1988).
Nuclear localisation of hormone-free estrogen receptors by monoclonal antibodies could be a tissue-fixation dependent artifact.
Steroids. 51:425-439.

Rajabi, M. R. , Dodge, G. R., Solomon, S. and Poole, A. R. (1990)
Dilation of uterine cervical parturition in guinea pig I. Mechanisms.
Proceedigs of the 37th Annual Meeting of the Society for Gynecologic Investigation. St. Louis p201 (Abstract).

Rajabi, M. R., Solomon, S. and Poole, A. R. (1991)
Hormonal regulation of interstitial collagenase in the uterine cervix of pregnant guinea pig.
Endocrinology. 128: 863-871.

Rasmussen, H. and Barrett, P. Q. (1984)
Calcium messenger system: an integrated view.
Physiol. Rev. 64: 938-984.

Rasmussen, H., Takuwa, Y. Park, S. (1987)
Protein kinase C in the regulation of smooth muscle contraction.
FASEB. J. 1: 177-185.

Rauscher, F. J, III, Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tijan, R., and Franza Jr., B. R. (1988).
Fos-associated protein P39 is the product of the Proto-oncogene.
Science, 240: 1010-1016.

Resko, J. A. (1969)
Endocrine control of adrenal progesterone secretion in ovariectomized rats.
Science 164: 70-71.

Richardson, W. D., Roberts, B. C., Smith, A. E. (1986)
Nuclear localization signal in polyoma virus large T.
Cell 44: 77-85.

Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991)
Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequences.
Cell 64: 615-623.

Ross, K. T., Moss, V. E., Prahl, J. M., and De Luka, H. F. (1992)
A Nuclear protein essential for binding of rat 1,25-dihydroxy vitamin D3 receptor to its response elements.
Proc. Natl. Acad. Sci USA. 89:256-260.

Ryan, J. .N. and Woessner, J. F. Jr. (1972)
Oestradiol inhibits collagen break down in the involuting rat uterus.
Biochem. J. 127: 705-713.

Sabbah, M., Redevilts, G., Secco, C., and Baulieu, E. E. (1987)
The binding activity of estrogen receptor to DNA and heat shock protein (Mr 90,000) is dependent on receptor-bound metal.
J. Biol. Chem. 262: 8631-8635.

Sakamoto, S., Sakamoto, M., Goldhaber, P.and Glimcher, M. J (1973)
Isolation of tissue collagenase from homogenates of embryonic chick bones.
Biochem. Biophys. Res. Commun. 53: 1102-1108.

Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, T. M. (1988).
Fos associated cellular p39 is related to nuclear transcription factor AP-1.
Cell 54: 553-560.

Schwabe, J. W. R., and Rhodes, D. (1991).
Beyond zinc fingers: Steroid hormone receptors have a novel structural motif for DNA recognition.
TIBS 16: 291-296.

Schuermann, M., Neuberg, M., Hunter, J. B., Jenuwein, T., Rysein, R. P., Brano, R., and Muller, R. (1989)
The leucine repeat motif in fos protein mediates complex formation with Jun/AP-1 and is required for transformation.
Cell. 56: 507-517.

Shaikh, A. A. (1971)
Estrone and estradiol levels in ovarian venous blood from rats during estrus cycle and pregnancy.
Biol. Reprod. 5: 297-300.

Sheridan, P. J. (1975).
Is there an alternative to the cytoplasmic receptor model for the mechanism of action of steroid hormone.
Life sciences 17: 495-502.

Sheridan, P. J., Buchanan, J. M. , Anselmo, V. C., and Martin, P. M. (1979).
Equilibrium: The intracellular distribution of steroid receptors.
Nature. 282: 579-582.

Stryer, L. and Haugland, R. P., (1967)
Proc. Natl. Acad. Sci. USA 58: 719-726.

Stryer, L., (1978)
Fluorescence energy transfer as a spectoscopic ruler.
Ann. Rev. Biochem 47: 819-846.

Sugawara, A., Yen, P. M., Darling, D. S., and Chin, W. W.
Characterization and tissue expression of multiple triiodo thyronine receptor-auxiliary proteins and their relationship to the retinoid X receptors.
Endocrinology. 133: 965-971.

Sullivan, W. P., Vroman, B. P.,Bauer, V. J., Puri, R. K., Riehl, R. M., Pearson, G. R. and Toft, D. 0. (1985)
Isolation of steroid receptor binding proteins from chicken oviduct. Production of monoclonal antibodies.
Biochemistry 24: 4214-4222.

Szego, C. M., and Pietras, R. J. (1984)
Lysosomal functions in cellular activation, propagation of the actions of hormones and other effectors.
Int. Rev. Cytology 88: 1-302.

Thampan, T. N. R. V., and Clark, J. H. (1981).
An Oestrogen receptor activator protein in rat uterine cytosol.
Nature 290: 152-154.

Thampan, R. V. and Clark, J. H. (1983)
Estrogen receptor activation factor.
UCLA symposia on molecular and cell biology series (ed.. Bradshaw, R. A. and Gill, G. N.) Vol 6, 457- 464.

Thampan, R. V. (1985)
The nuclear binding of estradiol stimulates ribonucleoprotein transport in rat uterus.
J. Biol. Chem. 260: 5420-5426.

Thampan, R. V. (1987)
A 62 kDa protein functions as estrogen receptor activation factor

(E-RAF) in the goat uterus.
Mol. Cell. Endocrinol. 53: 119-130.

Thampan, R. V. (1989).
Molecular aspects of estrogen receptor activation factor (E-RAF) function.
Mol. Cell. Endocrinol. 64: 19-34.

Towbin, H., Staehlin, T. and Gordon, J., (1979)
Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.
Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Tsai, S. Y., Carlstedt-Duke., Weiger, N. C., Dahlman, K., Gustafsson, J. A., Tsai, M. J., and O'Malley, B. W. (1987).
Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation.
Cell, 55: 361-369.

Tyree, B., Halme, J. and Jeffery, J. J. (1980)
Latent and active forms of collagenase in rat uterine explant cultures. Regulation of conversion by progestational steroids.
Arch.Biochem. Biophys. 202: 314-317.

Van der Hoven, Th. (1981)
Isolation of hepatic microsomes by polyethylene glycol 6000 fractionation of the post mitochondrial fraction.
Anal. Biochem. 115, 398-402.

Vazquez - Nin, G. H., Echverria, 0. M. , Fakan, S., Traish, A. M., Wotiz, H. H. and Martin, T.E. (1991)
Immunoelectron microscopic localisation of estrogen receptor on pre-mRNA containing constituents of uterine cell nuclei.
Exp. Cell. Res. 192: 396-404.

Vedeckis, W. V., Schrader, W. T. and O'Malley, B. W. (1980a)
Progesterone binding components of chick oviduct: Partial purification and characterization of a Calcium activated neutral protease which hydrolyzes the progesterone receptor
Biochemistry 19: 335-343.

Vedeckis, W. V., Schrader, W. T. and O'Malley, B. W. (1980b)
Progesterone binding components of chick oviduct: Analysis of receptor structure by limited proeolysis.
Biochemistry 19: 343-349.

Voller, A., Bidwell, D. and Bartlett, A. (1987)
Enzyme linked immunosorbent assay.
Immunochemical methods in cell and molecular biology (ed..Mayer, R. J. and Walker, J. H.) pp 359-371.

Walters, M. R., Hunziker, W. and Normann, A. W. (1981 a).
1,25- Dihydroxy Vitamin D3 receptors: Intermediates between triiodo thyronine and steroid hormone receptors.
Trends Biochem Sci. 6: 268-273.

Walters, M. R., Hunziker, W., and Norman, A. W. (1981 b).
A mathematical model describing the subcellular localization of non-membrane bound steroid, seco steroid and thyronine receptors.
J. Steroid. Biochem. 15: 491-496.

Webster, N. J. G., Jin, J. R., Green, S., Hollis, M., and Chambon, P. (1988a).
The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans activator.
Cell. 52: 169-178.

Webster, N. J. G., Green, S., Jin, J. R., and Chambor, P. (1988 b).
The hormone binding domains of the estrogen and glucocorticoid receptor contain a inducible transcription activation function.
Cell. 54: 199-207.

Weeks, J. G., Halme, J. and Woessner, J. F. Jr. (1976)
Extraction of collagenase from involuting rat uterus.
Biochim. Biophys. Acta. 445: 205-214.

Welch, W. J., Feramisco, J. R. (1982).
Purification of major mammalian heat shock proteins.
J. Biol. Chem. 257: 14949-14959.

Welshons, W. V., Lieberman, M. E., and Gorski, J. (1984).
Nuclear localization of unoccupied Oestrogen receptors.
Nature. 307: 747-749.

Whal, L. M. (1977)
Hormonal regulation of macrophase collagenase activity.
Biochem.Biophys. Res. Commun. 74: 838-845.

Woessner, J. F. Jr. (1961)
The determination of hydroxyproline in tissue and protein samples containing small proportins of this imino acid.
Arch. Biochem. Biophys. 93: 4 4 0-447.

Wrange, O., Eriksson, P., and Perlmann, T. (1989).
The purified activated Glucocorticoid receptor is a homodimer.
J. Biol. Chem. 264: 5253-5259.

Yamamoto, K. R. and Alberts, B. (1972).
In Vitro conversion of estradiol receptor protein to its nuclear form: Dependence on hormone and DNA.
Proc. Natl. Acad. Sci. USA. 69: 2105-2109.

Yamamoto, K. R. (1974).
Characterization of the 4S and 5S forms of the estradiol receptor protein and their interactions with deoxyribonucleic acid.
J. Biol. Chem. 249: 7068-7075.

Yamamoto, K. R. (1985).
Steroid receptor regulated transcription of specific genes and gene network.
Ann. Rev. Genet. 19: 209-252.

Yen, P. M., Sugawara, A., and Chiu, W. W. (1992a).
Triiodo thyronine (T3) decreases T3- receptor/retinoic acid receptor but not T3 -Receptor/ Retinoid X Receptor heterodimer binding to DNA.
J. Biol. Chem. 267: 23248-23252.

Yen, P. M., Darling, D. S., Carter, R. L., Forgione, M., Umeda, P. K., and Chiu, W. W. (1992b).
Triiodo thyronine (T3) decreases binding to DNA by T3- receptor homodimers but not receptor- auxiliary protein heterodimers.
J. Biol. Chem. 267:3565-3568.

Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H. and Chambon, P. (1992).
Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors.
EMBO J. 11: 681-3694.

Yoneda, Y., Imamoto-Sonobe, N., Matsuoka, Y., Iwamoto. R., Kiho, Y. and Uchida, T. (1988).
Antibodies to Asp-Asp-Glu-Asp can inhibit transport of nuclear proteins into the nucleus.
Science 242: 275-278.

Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, 0. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K. and Rosenfeld, M. G. (1991).
RXR β: A coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements.
Cell. 67: 1251-1266.

Zafar, A. and Thampan, R. V. (1993)
A four-step, inexpensive protocol for large scale purification of goat uterine estrogen receptor.
Protein expression and Purificcation 4: 534-538.

Zelent, A., Krust, A., Petkovich, M., Kastner, P., and Chambon, P., (1989).
Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin.
Nature. 339:714-717.

Zhang X-K., Trans, PB- V., and Pfahl, M. (1991).
DNA binding and dimerisation determinants for thyroid hormone receptor α and its interaction with a nuclear protein.
Mol. Endocrinol. 5:1909-1920.

Zhang, X- K., Hoffmann, B., Tran, PB- V., Granpuer, G., and Pfahl, M. (1992).

Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors.
Nature, 355: 441-446.

Zimmerman, U-J, P, and Schlaepfer, W. W. (1984)
Multiple forms of Ca-activated protease from rat brain and muscle.
J. Biol. Chem. 259: 3210-3218.