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 interplay 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 development of an ELISA for the measurement of E-RAF in reproductive tissue. Using this method the E-RAF level in the rat uterus under certain specific physiological conditions has been estimated.

**Immunological 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 MgCl₂ 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 amino acids and the radioactivity associated with the immunoprecipitated 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 0.8ng/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 immunologically 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 MgCl₂ 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 MgCl₂ elution of E-RAF peptides bound to anti-ER Sepharose column.
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 IgG.
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 IgG.
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.
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.
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.
**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.
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.