Immunocytochemical study of estrogen receptor activation factor (E-RAF) and the proteins that interact with nuclear estrogen receptor II (nER II) in epithelial endometrial cells, in the presence and in the absence of estradiol

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The localization and abundance of the estrogen receptor activation factor (E-RAF) and a small nuclear ribonucleoprotein (snRNP) complex containing three proteins, *p32*, *p55* and p60, which interact with the nuclear estrogen receptor II (nER II), have been studied in rat endometrial epithelial cells by means of immunofluorescence and high resolution quantitative immunocytochemistry. In the cytoplasm E-RAF is associated with the rough endoplasmic reticulum. In the nucleus it is mainly localized at the interchromatin space, and surrounding the clumps of compact or semi-condensed chromatin. Quantitative analyses show that the abundance of E-RAF in the nucleus increases after ovariectomy and decreases 3 minutes after estradiol administration. These results are in agreement with the currently available biochemical data. Double immunolocalizations demonstrate that p32, p55, p60 co-localize with other splicing-related protein. High resolution immunolocalization shows that p32, p55, p60 are associated with perichromatin fibrils (co-transcriptional splicing) and with clusters of interchromatin granules (storage of splicing-related molecules). The nuclear abundance of the snRNP complex decreases with ovariectomy, increases within 3 minutes after estradiol administration and remains higher than that in ovariectomized animals for 27 minutes. These results strongly support the previous data on the role of nER-II in the regulation of mRNA transcription and its export from the nucleus to the cytoplasm.

Key words: nuclear estrogen receptor II, estrogen receptor activation factor, post-transcription regulation of gene expression, splicing, nuclear pore, estradiol

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Jensen and Jacobsen (1962) proposed for the first time that the effects of estradiol are mediated by a receptor protein. Estradiol receptor (ER) was extensively studied (Gorski *et al.,* 1984) and eventually cloned (Green *et al.,* 1986; Greene et al., 1986; Krust *et al.,* 1986; Koike *et al.,* 1987; White *et al.,* 1987). A second ER was cloned from a rat prostate cDNA library (Kuiper *et al.,* 1996, 1998). Now they are called ER· and ER, respectively. Both proteins are highly homologous particularly in the DNA binding region.

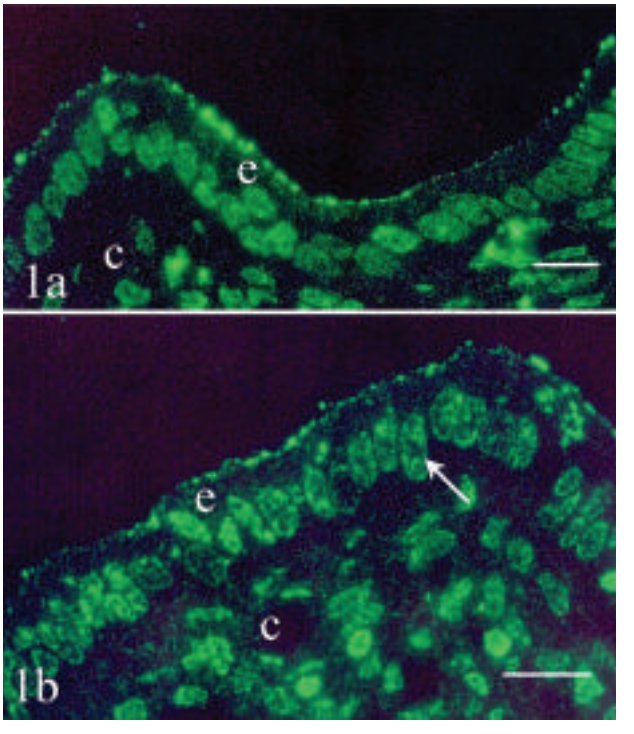
An alternative form of ER was purified from the goat uterus by Karthikeyan and Thampan (1996). This receptor, identified as the non-activated estrogen receptor (naER) is a 66 kDa glycoprotein with tyrosine kinase activity, mainly located in the cell membrane. It binds estradiol with high affinity, but has no capacity to interact with DNA on its own (Anuradha *et al.,* 1994). Hormone binding to naER causes receptor internalization (Karthikeyan and Thampan, 1996). The transport of naER into the nucleus is mediated by a 58kDa cytosolic protein that recognizes its nuclear localization signals (Sreeja and Thampan, 2004). In the nucleus the naER dimerizes with a DNA-binding protein, the estrogen receptor activation factor (E-RAF), which does not bind estradiol. The heterodimer binds to the DNA (Thampan, 1987, 1989). E-RAF is a 62kDa protein existing in two molecular forms, E-RAF I and E-RAF II. E-RAF II destabilizes DNA double helix and enhances transcription *in vitro*, while E-RAF I does the opposite function (Thampan, 1989). In the nucleus a 62-kDa protein transforms the naER to nuclear estrogen receptor II (nER-II). The transformation is achieved through deglycosylation (Karthikeyan and Thampan, 1995; Jaya and Thampan, 2000). The nER-II is a 66 kDa protein which does not dimerize with E-RAF. It binds to

RNA and interacts with three proteins in the small nuclear ribonucleoprotein complexes (snRNPs), p32, p55, and p60. The three proteins bind to the nuclear pore complex. nER-II along with p32 and p55 forms a Mg^{++} ATPase complex. The activation of this complex appears to be related to the export of RNP from the nucleus to the cytoplasm (Sebastian and Thampan, 2002 a, b).

Although the association of estradiol receptor complex with ribonucleoprotein had been described earlier (Liang and Liao, 1974), changes in the rate of nuclear export of RNA after variations of estradiol exposure were first demonstrated in uterine epithelial cells in culture using high resolution quantitative autoradiography (Vázquez-Nin *et al.,* 1979). Biochemical studies showed the association of estrogen receptor with pre-messenger ribonucleoproteins (RNP) (Thampan, 1985, 1988). The use of high resolution immunolocalization demonstrated the association of estradiol receptor with nuclear hnRNP-containing structures (Vázquez-Nin *et al.,* 1991), such as perichromatin granules and fibrils characterized for the first time by Monneron and Bernhard (1969). In the present study, we utilized immunocytochemical methods at the light and electron microscope to analyze the changes in the localization and abundance of E-RAF and the nER-II binding proteins. These approaches provide high resolution specific localizations and quantitative date that were compared with previous biochemical results in order to contribute to the understanding of the cytophysiological role of these proteins in transcription and post-transcriptional events. The experimental animals were normal rats and ovariectomized rats which were not exposed to estradiol and also ovariectomized rats treated with estradiol for varying intervals.

Materials and Methods

Two months old female Wistar rats were used in the present immunoelectronmicroscopic study. Three groups of animals were employed: a) normal rats; b) animals ovariectomized 8 days before the study; c) rats 8 days after ovariectomy and injected intraperitoneally with 3μg estradiol per rat. The hormone- treated animals were sacrificed 3, 9, 18, 27 and 36 minutes after estradiol administration. Samples of uterus, about $1mm^3$ in size, containing endometrial epithelium, were dissected out and the adhering fat and connective tissue elements were removed. The tissue fragments were fixed in 4% paraformaldehyde in phosphate buffer at pH 7.3 for 2 hours at 4°C. The samples were embedded in an epoxy resin (Glycide ether 100, Merck, Darmstadt, Germany). Uranyl acetate-EDTA-lead citrate preferential staining for RNP-containing particles was performed according to Bernhard (1969). The ultrathin sections were mounted in Formvar coated nickel grids, and processed for postembedding immunolocalization as described previously (Biggiogera *et al.,* 1989; Vázquez-Nin *et al.,* 1990), except for the use of an epoxy embedding resin instead of an acrylic one. Antibodies were raised in rabbits against goat uterine E-RAF (Thampan, 1987) and against three proteins, p32, p55 and p66 isolated from goat uterine snRNP complex (Sebastian and Thampan, 2002 b). The high resolution immunolocalizations were performed using two rabbit anti-sera: one developed against E-RAF and the other recognizing p32, p55 and p60 of snRNPs. These sera were localized with



Figures 1. Immunofluorescent localization of E-RAF in 1 μm thick sections of endometrium (see Material and methods). The cytoplasms of epithelial endometrial cells (e) are faintly fluorescent. Nuclear labeling is intense. Brilliant spot are sparse in the nucleoplasm and associated with the nuclear envelope. c- Connective tissue cells are also labeled. Unspecific labeling is associated with the luminal surface of the epithelial cells. A Normal animal. Bar 10 μm. B Ovariectomized animal. Some dark areas correspond to the nucleolus (arrow). Bar 10 μm.

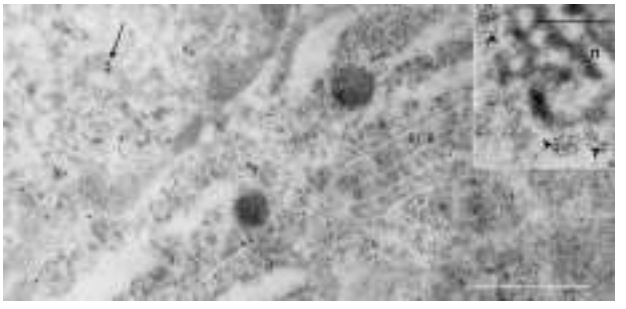


Figure 2. Endometrial epithelial cell labeled with anti-E-RAF and stained with uranyl acetate and lead citrate. The nuclear labeling is preferentially located at the interchromatin space (arrow). Gold grains are also associated with the periphery of compact chromatin. The cytoplasmic labeling is primarily located at the endoplasmic reticulum (RER) and in the vicinity of the nucleus. The inset shows that the nucleolus (n) is unlabeled. The semi-condensed chromatin surrounding the nucleolus is labeled (arrow heads). Bars 0.5 μm.

a goat anti-rabbit IgG antibody coupled to 12 or 18 nm gold particles (Jackson Res. Lab., Pennsylvania, USA). For double immunolocalization anti-SC35 monoclonal antibody was purchased from Santa Cruz Biotechnology (California, USA). The secondary monoclonal goat anti-mouse antibody was conjugated with 5 or 10 nm gold particles (Sigma, St. Louis Missouri, USA). Immunofluorescence was carried out on 0.5-1 μm thick sections of samples fixed and embedded for electron microscope or they were performed on 5-10 μm thick sections of paraformaldehyde fixed and paraffin embedded tissues. The further method allows a resolution along Z axis similar to that obtained with confocal microscope and better resolution in X and Y axes. The sections of paraffin embedded tissues were further further cut with a laser confocal microscope (Biorad MRC 1024).

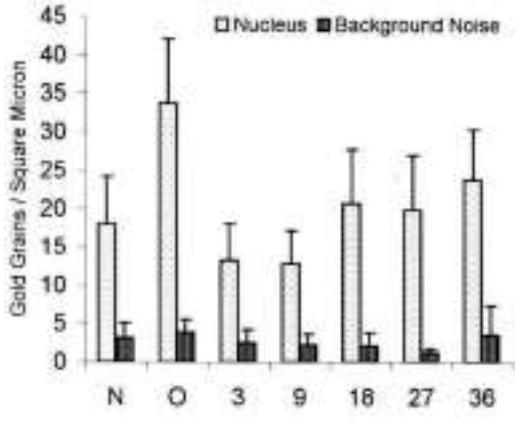


Figure 3. Changes in the nuclear density of labeling of anti-E-RAF in endometrial epithelial cells. The bars represent standard deviation. Abscissa: N- Normal; O-Ovariectomized rats. 3, 9, 18, 27, 36 – Minutes passed between the administration of estradiol to ovariectomized animals and the fixation of tissues.

This procedure was mainly used for double immunolocalizations. The secondary antibody was a goat anti-rabbit IgG (H+L) coupled to FITC for the localization of E-RAF and p32, 55, 60 (GIBCO, Gaitherburg, USA) and goat anti-mouse IgG+IgM (H+L) coupled to exas red (Jackson Immuno Research Lab. Baltimore USA) to localize SC35. Control experiments of both types of immunolocalizations were carried out with pre-immune serum or with the secondary antibody in the absence of the primary antibody. Images of nuclei and cytoplasm of were selected systematically, registered in video tape, digitized and analyzed using the program Imagenia 5000 obtained from Biocom (Paris, France). Thirty cells per rat and 30 rats per experimental group were studied. The significance of the differences was estimated by variance analysis.

Results

Indirect immunofluorescence using anti-E-RAF shows that the distribution of this protein is similar in endometrial epithelial cells of control and treated animals. The cytoplasm is labeled to a low extent. Brilliant points and a diffuse labeling are distributed inside the nuclei. The nucleolus is not fluorescent. An irregular labeling is present at the apical surface of some epithelial endometrial cells (Figures 1A, B). This labeling is also present in control experiments performed with pre-immune rabbit antiserum; thus it is considered to be an unspecific labeling. Electron microscopic immunolocalization of E-RAF demonstrates an intense labeling of the interchromatin space, in contact with the periphery of the clumps of compact chromatin and at the nuclear pores. The nucleolus remains practically devoid of gold grains (Figure 2). This feature pro-

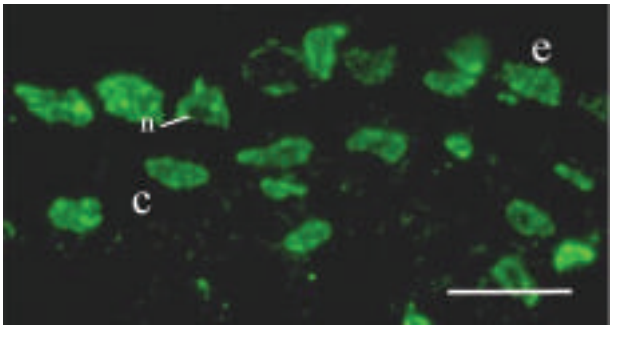


Figure 4. 1 μm thick section. Endometrial epithelial cells (e) are labeled with an anti-snRNP proteins p32, p55 and p60. Nuclei are intensively labeled. Strongly fluorescent dots and spread labeling can be seen. The nucleoli are not fluorescent (n). The cytoplasms are not labeled. c- Connective tissue cells. Bar 10 μm.

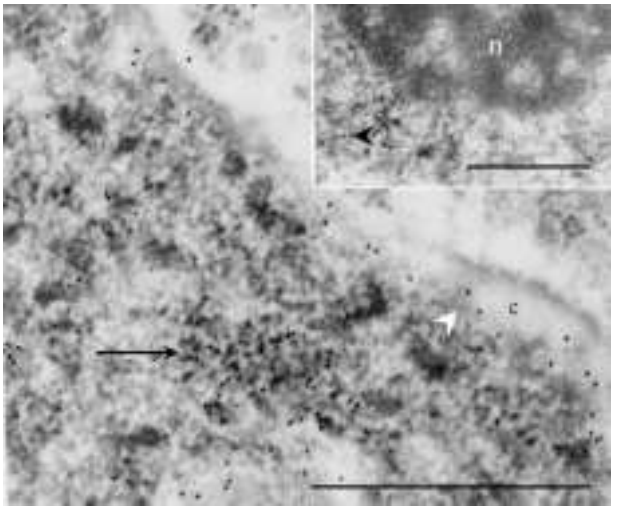


Figure 5. Immunoelectron micrographs showing the distribution of snRNP proteins p32, p55 and p60. Uranyl acetate-EDTA-lead citrate staining procedure preferential for RNP. Chromatin is clear gray (c). A cluster of dark stained interchromatin granules are labeled (arrow). Gold granules are also seen associated with EDTA-positive perichromatin region surrounding the clumps of chromatin (white arrow head). The inset shows a nucleolus (n) devoid of labeling. The arrow head points to gold grains associated to semi-condensed chromatin. General staining. Bars 0.5 μm.

vides an internal negative control. In the cytoplasm the gold grains are mainly associated with clusters of rough endoplasmic reticulum or are located in the vicinity of the nucleus (Figure 2). Semi-condensed chromatin is also labeled (Figure 2 inset). The nucleolus remains practically devoid of gold grains (Figure 2 inset). This feature provides an internal negative control. In the cytoplasm the gold grains are mainly associated with clusters of rough endoplasmic reticulum cisternae or are located in the vicinity of the nucleus (Figure 2). Ovariectomy and estradiol administration do not change the general pattern of the distribution of the gold grains.

The quantitative analysis of the changes in the abundance of E-RAF showed that ovariectomy caused significant increase in the nuclear labeling, while the restitution of the hormone induced a very rapid decrease in labeling. The nuclear concentration of E-RAF increased slowly between 9 and 36 minutes of estrogen treatment (Figure 3). The increase observed after ovariectomy, the decrease that accompanied the restitution of hormone, and the difference observed between 9 and 36 min of treatment are statistically significant ($p<0.001$).

Immunofluorescence assays with anti-snRNPs, p32, p55 and p60 revealed intensively labeled spots and a scattered low level signal in nucleus of endometrial epithelial cells. Nucleoli are not labeled (Figure 4). This distribution is similar in the normal and ovariectomized animals with or without treatment. High resolution immunolocalization demonstrates that in the cytoplasm the label is mainly associated with the ribosomes. Mitochondria, vesicles and other cytoplasmic structures are not labeled. Immunocytochemical localizations of p32, p55 and p60 followed by a preferential staining for RNPs (Bernhard 1969), provided evidence for the presence of these proteins in the perichromatin fibrils surrounding the clumps of compact chromatin and at the clusters of interchromatin granules (Figure 5). The nucleolus is not labeled above the background level (Figure 5 inset).

Double immunofluorescent localization of p32. p55, p60 and the splicing factor SC35 demonstrates extensive co-localization in most of the nuclear space reconstructed by superposition of confocal sections (Figure 6). The study of one micrometer thick confocal optical sections demonstrate that SC35 splicing factor and the three proteins

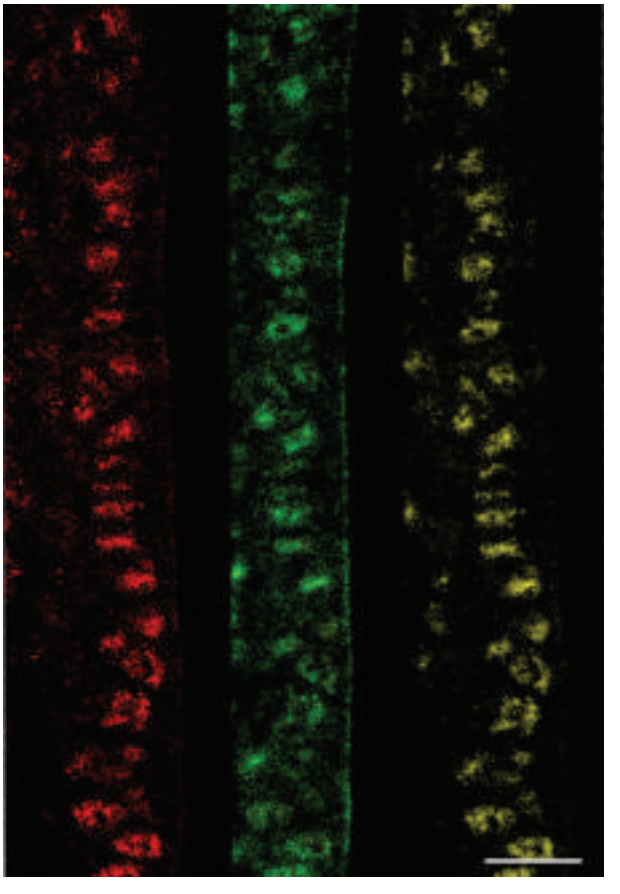


Figure 6. Double immunolabeling, red - SC 35; green – p 32, 55, 60. Confocal section of epithelial endometrial cells of a normal rat. The superposition of both localizations is shown on the right. The yellow shades indicate the colocalizations. Bar 10 μm.

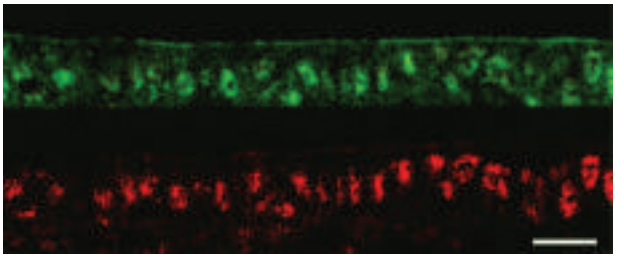


Figure 7. Double immunolabeling, red – SC35; green – p32, 55, 60. One micrometer thick section of epithelial endometrial cells of a normal rat. Bar 10 µm.

–p32, p55 and p60- complex frequently co-localize precisely in the same nuclear zones (Figure 7). Electron microscope double immunolocalizations show that both antibodies co-localize at the perichromatin region, in several places of the interchromatin region (Figure 8), and in clusters of interchromatin granules. It is interesting to note that the complex of snRNPs -p32, p55, and p60- are frequently localized associated with the pores of the nuclear envelope (Figure 8).

The changes observed in the distribution of the snRNP complex were quantified in two nuclear compartments: the interchromatin and the perichromatin region. The external border of perichromatin region was conventionally fixed at 100 nm from the limit of compact chromatin. The proteins were found to be more abundant in the interchromatin space than in the perichromatin zone, except in ovariectomized animals treated with estradiol for 3 and 27 minutes. In these animals there were no significant differences observed between the two regions (Figure 9). Ovariectomy caused a significant decrease in labeling in both compartments. The administration of estradiol induced a very rapid increase in the labeling, exceeding the levels of ovariectomized animals within 3 minutes of estradiol exposure. The concentration of snRNP complex remained higher than that in ovariectomized animals for at least 27 minutes after the injection of estradiol. However, 36 minutes after the hormone restitution a decrease of the labeling was detected (Figure 9).

Discussion

It has been observed that in the presence of estradiol, E-RAF remains in the cytoplasm anchored to a 55 kDa protein (ap 55), which is a high affinity estradiol binding protein. This anchoring is mediated by a 66 kDa transport protein (tp 66). The decline in intracellular concentration of estradiol apparently induces conformational changes in ap 55, causing the detaching of tp 66- E-RAF complex from ap 55 and tp 66 moves E-RAF to the nucleus (Govind *et al.* 2003 a, b). The immunocytochemical evidence presented here clearly supports the biochemical data, indicating the strong presence of E-RAF in the uterine nucleus of ovariectomized rats and its subsequent decline following in vivo exposure to estradiol. The model presented (Figure 10) serves to explain these molecular details. The frequent localization of E-RAF at nuclear pores corresponds to the migration of the protein from cytoplasm to the nucleus.

In the nucleus E-RAF is located in the nucleoplasm and surrounding the clumps of semi-condensed chromatin. Most of the sites of non-nucleolar transcription are located at these sites, especially at the periphery of compact or semi-condensed chromatin (Fakan and Bernhard, 1971; Bachellerie *et al.,* 1975; Cmarko *et al.,* 1999; Puvion and Puvion-Dutilleul, 1996; Fakan, 2004). The association of R-RAF with structures related to the process of transcription supports the proposition that the naER-E-RAF heterodimer binds to RNA-polymerase II at the site of transcription initiation (Govind and Thampan, 2001). The scattered immunocytochemical signal of E-RAF in the nucle-

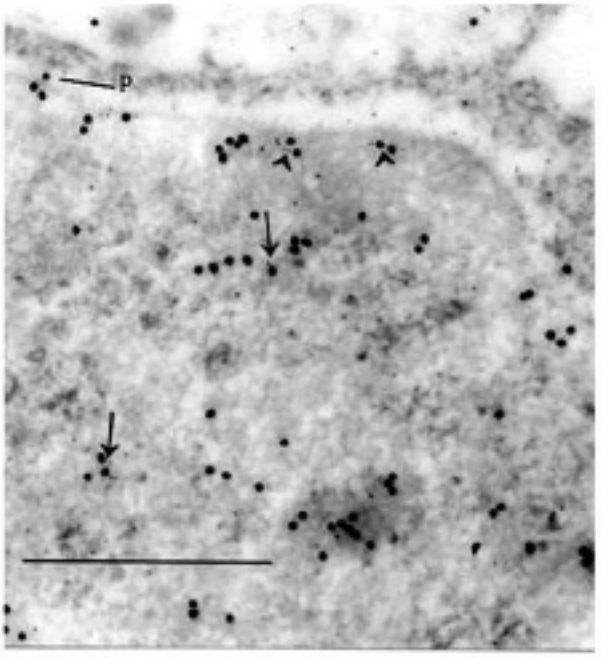


Figure 8. Epithelial endometrial cell of a normal animal. Double immunolocalization anti-SC35 (5 nm gold grains) and the anti-snRNP proteins, p32, p55 and p60 (18 nm gold grains). Colocalizations can be seen surrounding the peripheral clumps of semi-condensed chromatin (arrow heads), in the nucleoplasm (arrows). Large gold grains localizing the snRNPs p32, p55, p60 are associated with the pores of the nuclear envelope (p). Bar 0.5 µm.

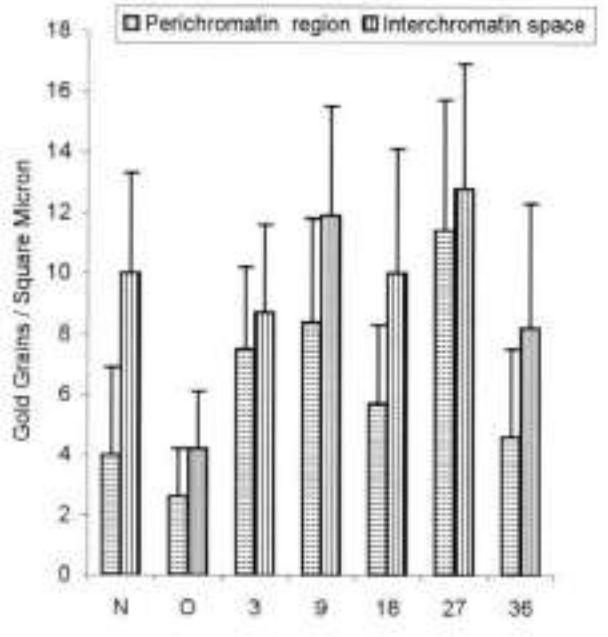


Figure 9. Changes in the density of labeling the snRNP proteins p32, p55 and p60. The bars represent standard deviation. Abscissa: N-Normal; O-Ovariectomized rats; castrated animals injected with estradiol 3, 9, 18, 27, 36 min before fixation of tissues.

oplasm corresponds to the sparse distribution of transcription sites as demonstrated by fluorescent and high resolution immunolocalization of nascent non-nucleolar RNA labeled with Br-UTP, anti-RNA polymerase II, and anti-poly(A)-polymerase (Cmarko *et al.,* 1999). A previous study on the effects of ovariectomy and estradiol restitution demonstrated important variation in chromatin structure and disposition using a preferential staining for DNA. It is interesting to note that the reduction of transcription caused by ovariectomy and the activation of transcription caused by the administration of estradiol to ovariectomized rats are accompanied by the formation of areas of semi-condensed chromatin in rat epithelial endometrial cells nuclei (Vázquez-Nin *et al.,* 1978). These areas are formed by a web of chromatin filaments, looser than compact chromatin, but denser than extended one. In the present work we show that E-RAF and the three snRNP proteins, p32, p55 and p60 are frequently found associated with this type of chromatin. These observations suggest the possibility that semi-condensed chromatin is caused by sudden changes of transcription and that it keeps the capacity of sustaining transcription to some extent.

The estimation of the concentration of epitopes in various cell compartments has been previously achieved by quantitative analysis of immunolabeling densities. The numerical density of the gold grains was found to be a reliable parameter to estimate the distribution of the epitope inside the cell and the changes of the density caused by experimental manipulations (Vázquez-Nin *et al.*; 1991: Vázquez-Nin *et al.,* 1992; Vázquez-Nin *et al.,* 1993; Echeverría *et al.,* 1994; Echeverría *et al.,* 1999). The quantitative modifications of the abundance of E-RAF in the nucleus caused by changes in the concentration of estradiol support the observations that the decrease of estradiol concentration weakens the interaction of E-RAF with the endoplasmic reticulum letting it free to enter the nucleus (Govind *et al.,* 2003 a, b).

Perichromatin fibrils are the morphological expressions of the transcription of pre-mRNA and of the cotranscriptional splicing (Petrov and Bernhard, 1971; Nash *et al,*. 1975; Bachellerie *et al.,* 1975; Fakan and Bernhard, 1971; Fakan *et al.,* 1976; Fakan *et al.,* 1984; Puvion *et al.,* 1984; Spector, 1984; Fakan *et al.,* 1986; Cmarko *et al.,* 1999; Fakan, 2004). Our results demonstrate that the complex of snRNPs containing and nER II binding snRNPs p32, p55 and p60 is located at perichromatin fibrils and thus that these proteins may be related to active spliceosomes. As these proteins bind nER-II is possibly that this form estradiol receptor is related to transcription and splicing. The relationships between the estrogen receptor and mRNA-containing nuclear structures were shown by means of high resolution immunolocal-

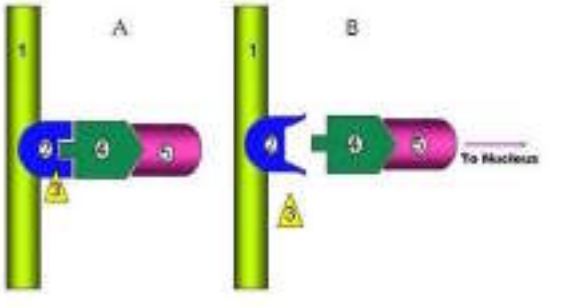


Figure 10. A model displaying the intracellular distribution of E-RAF in the presence of estradiol (A) and also in the absence of the hormone (B). The endoplasmic reticulum carries a 55 kDa high affinity estrogen binding protein, the anchor protein 55 (ap55). Estradiol binding to ap 55 gives ap 55 a special conformation which facilitates the anchoring of tp 66 and the associated E-RAF to the anchor protein. The critical concentration of estradiol that favors this anchoring function has been shown to be around 7 nM. A decline in the concentration of estradiol leads to a change in ap55 conformation and the consequent release of the tp66- E-RAF complex from its hold. tp66 apparently recognizes the nuclear localization signals in the E-RAF and transports the latter to the nucleus. 1- endoplasmic reticulum membrane; 2- ap55; 3- estradiol; 4- tp66; 5- E-RAF.

ization in rat endometrial epithelium (Vázquez-Nin *et al.,* 1991). High resolution immunocytochemical procedures showed that snRNP but not hnRNP are localized in the clusters of interchromatin granules (Fakan *et al.,* 1986), indicating that they represent the sites for storing or assembly of components of spliceosomes (Puvion and Puvion-Dutilleul, 1996; Cmarko *et al.,* 1999). The presence in these clusters of interchromatin granules of the proteins p32, p55, and p60 indicates the relationship of these proteins with the spliceosome storage place. The colocalization of SC35 with the complex of snRNP proteins p32, p55 and p60 at perichromatin fibrils and interchromatin granules indicates that different molecules that deal with the splicing process are closely associated in functional sites, as well as at the places for storing.

The association of the three snRNP proteins that bind nER-II with the nuclear pores has been previously demonstrated by Sebastian and Thampan (2002 b). It is possible to speculate that the presence of the three proteins of the snRNP, p32, p55, p60 in the nuclear pores not only provides evidence of the migration of these proteins from the nucleus to the cytoplasm but also indicates their association with the proteins of the pore during the process of mRNA export to the cytoplasm.

The reduction in the nuclear concentration of the proteins p32, p55 and p60 after ovariectomy is probably related to the decrease of the export of spliced mRNA to the cytoplasm. The rapid increase of the labeling with anti-p32-p55-p60 within 3 minutes of exposure to estradiol corresponds precisely with the timing of the early augmentation of the transport of mRNA to the cytoplasm in these experimental conditions (Vázquez-Nin et al., 1979; Thampan, 1985, 1988).

The evidences presented here, taken as a whole, strongly support previous biochemical data suggesting that nER-II is involved with transcriptional and post-transcriptional regulation of gene expression. The studies also underlie the paramount role played by E-RAF and naER (nER II) in the molecular mechanisms that lead to estrogen action. However, it is important further analyze the relationships between E-RAF, the proteins p32, p55 and p60 and the sites of transcription using high resolution cytochemical methods (Trentani *et al.,* 2003).

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