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ESTROGEN-INDUCED MODIFICATION OF UTERINE RNA POLYMERASE ACTIVITY DEPENDS ON LOCALIZATION OF THE ESTROGEN RECEPTOR

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Abstract – The aim of this study was to examine the effects of estradiol (E2) on activity of RNA polymerase I and RNA polymerase II in uterine nuclei of ovariectomized (OVX) female rats. The obtained results show that estrogen-receptor (E-R) complexes in 30 min induced an increase of polymerase II activity. A second increase of polymerase II activity was observed after 3 h-incubation of nuclei with the E-R complex formed in the cytosol fraction. However, activity of polymerase I was increased 2 h after the start of incubation, with highest activity detected at 3 h in nuclei incubated with E-R complexes. On the contrary, no stimulatory effect on either polymerase I or polymerase II activity was observed in nuclei incubated with E2 alone. These results indicate that E2 stimulates the cytosolic estrogen receptor (ER), which in turn causes uterotrophic responses in OVX rats. In addition, they suggest that in order to provoke uterotrophic responses E-R complexes formed in the cytosol need to be retained in the nucleus for a longer period of time.

Key words: estradiol, RNA polymerase I, RNA polymerase II, estrogen receptor, estradiol-receptor complex, uterus.

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INTRODUCTION

Stimulation of the uterus with estradiol (E2) elicits a spectrum of biochemical responses which culminate in uterine growth (Anderson et al., 1975). The intracellular events which precede this growth have been proved to be related to and regulated by steroid hormone binding to the intracellular receptor molecule. Earlier studies suggested that the unoccupied estrogen receptor (ER) was a soluble cytoplasmic molecule which upon binding the hormone translocates to the nucleus. The previous attempts at localization of ER by biochemical fractionation led to a two-step model of steroid hormone action (Jensen et al., 1968). Binding to the cytosolic steroid hormone receptor leads to its transformation and subsequent translocation to the nucleus, where it regulates gene expression. This view was revised when the ER was shown to be ligand-independent in the nucleus (King and Greene, 1984). However, attempts to define these two biochemically distinct states of ER failed to reveal any notable difference in the intranuclear localization of hormone bound forms of cytosolic and nuclear ER (Greene and Press, 1986). The ERs are now generally thought to be found predominantly in the nucleus independent of hormonal presence (Press et al., 1989; Brink et. al., 1992). However, there are several potential compartments that receptors can occupy. The general consensus now is that these receptors are a fraction of the total cellular receptors present in the cytoplasm anchored by various heat shock proteins (Segnitz and Gehring, 1995). Upon addition of a ligand, these cytoplasmic receptors enter the nucleus. A substantial fraction of the receptor associates with the nuclear matrix, which represents the residual nuclear fraction that remains after most chromatin has been extracted. At least two forms of steroid receptor complex appear to be tightly bound to chromatin. One of these forms can be extracted with salt (0.4 M KCl) and one is resistant to salt extraction and may be associated with transcriptionally active DNA on the nuclear matrix (Alexander et al., 1987; Robyr and Wolffe, 1998; Ribarac-Stepić et al., 2005; Isenović et al., 2006a; Isenović et al., 2006b; Žakula et al., 2005). The function of either cytoplasmatic or nuclear receptor association with the matrix is still unresolved, and the physiological significance of

these cytoplasmic and nuclear estrogen binding sites remains speculative, although it is apparent from published studies that levels of nuclear binding sites in the rat uterus are increased by the hormone, i.e., after E2 administration (M a r k a v e r i c h and C l a r c, 1979; H a l a c h m i et al., 1994; H t u n et al., 1999; A n d e r s o n et al., 1975).

The question of whether extranuclear receptor plays a role in the interaction of E2 with target cells can not be answered with certainty at present. Published evidence (K i n g and G r e e n, 1984; W e l s h o n s et al., 1984; P r e s s et al., 1989; B r i n k et al., 1992) indicating that the majority of native ERs reside in the nuclear compartment does not preclude the possibility that this estrophilin constitutes a pool in equilibrium with extranuclear receptor that associates with the hormone as it enters the cell and upon activation of a formed estrogen-receptor complex (E-R) translocates into the nucleus, where binding to acceptor sites and subsequent access of these receptor complexes to chromatin take place. These events trigger an E2 response in the cell stimulating the expression of target genes through induction of synthesis of RNA(s), which is brought about by changes of RNA polymerase activity (R o b y r and W o l f f e, 1998). Accordingly, modification of RNA polymerase activity emerges as one of the controlling elements for gene expression under steroid action (G o v i n d and T h a m p a n, 2001). The present study is based on the assumption that cytoplasmic and/or nuclear ER represents a transcription factor that influences modification of controlling elements for gene expression such as RNA polymerases.

Each eukaryotic RNA polymerase catalyzes transcription of genes encoding different classes of RNA. RNA polymerase I, located in the nucleolus, transcribes genes encoding precursor rRNA (pre-rRNA), which is processed into 28S, 5.8S, and 18S rRNAs. RNA polymerase III transcribes genes encoding tRNAs, 5S rRNA, and an array of small, stable RNAs, including one involved in RNA splicing (U6) and the RNA component of the signal-recognition particle (SRP) involved in directing nascent proteins to the endoplasmic reticulum. RNA polymerase II transcribes all protein-coding genes; that is, it functions in production of mRNAs. RNA polymerase II also produces four of the five small nuclear RNAs that take part in RNA splicing (C r a m e r, 2002).

A relatively unexamined aspect of the function steroid receptors, namely their compartmentalization in the cell, prompted our interest in finding out whether they have similar or different effects on modification of RNA polymerase activity. Our interest in this area was stimulated by a provocative series of reports which raised the possibility that different physiological responses of the uterus to E2 might result from interaction of the hormone with cytoplasmic or nuclear receptors. The present study provides further evidence that the cytoplasmic form of receptor is an authentic cellular type of estrogen binding sites.

MATERIALS AND METHODS

Chemicals

^{14}C-UTP (2.18 GBq/mmol) was from Amersham (Amersham Plc, Buckinghamshire, UK). GTP, CTP, UTP, and ATP were from Merck KGaA (Darmstadt, Germany). E2 and all other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA).

Animals

Female Wistar rats (2-3 months old, 200-250 g b.w.) were maintained at 22 °C, under conditions of a 12/12 h light-dark regime (rat chow and water *ad libitum*) and ovariectomized (OVX) under Nesdonal anesthesia 10 days prior to the experiment. Each experiment was performed three times with a total of 26 animals. Experimental protocols were approved by the local ethical committee and were in compliance with "Good Laboratory Animal Practice".

Preparation of cytosol and nuclei

All procedures were carried out as previously described (R i b a r a c-S t e p i ć et al., 2005). The animals were killed by cervical dislocation and uteri were quickly removed, weighed, minced, and homogenized with Ultra-Turrax in 7 volumes (w:V) of buffer Tris (pH 7.5) containing 0.25 M sucrose, 1 mM ethylenediaminetetraacetate (EDTA), 0.5 mM dithiotreitol, and 10% glycerol. The homogenate was filtered through four layers of gauze and centrifuged at 800 x g for 15 min at 4 °C. To obtain the cytosol fraction, the supernatant was centrifuged at 105000 x g for 90 min. For isolation of nuclei, the crude nuclear pellet obtained after this first centrifugation was resuspended in a cold buffer (pH 7.4) containing 0.05 M Tris and 1 mM $MgCl_2$ and centrifuged at 800 x g for 10 min at 4 °C. The washed nuclear pellet was finally resuspended in the same buffer immediately before the enzyme assay.

Preparation of E-R complex

Isolated cytosol was incubated for 30 min at 25 °C with 8 μM E2. At the end of the incubation period, samples were treated with dextran-coated charcoal (3.75 % Norit A and 0.375 % dextran T-500 in the buffer for homogenization) in order to eliminate free and low affinity bound hormone (R i b a r a c-S t e p i ć et al., 2005).

Determination of RNA polymerases activity

Activity of RNA polymerase I and II was determined as previously described (R o e d e r and R u t t e r, 1970; G l a s s e r et al., 1972). Briefly, niclei (20 μl or 50 μg of DNA) were incubated in RNA polymerase I medium: 0.1 M Tris (pH 8.5), 3 mM $MgCl_2$, 3 mM NaF, 50 mM KCl, 20 mM β-mercaptoethanol, 1 mM each of GTP, CTP, ATP, 12 μM UTP, and 17.66 μM ^{14}C-labeled UTP; or in RNA polymerase medium: II: 0.1M Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 3 mM NaF, 50 mM $(NH_4)_2SO_4$, 20 mM β-mercaptoethanol, 1 mM each of GTP, CTP, ATP, 12 μM UTP, and 17.66 μM ^{14}C-labeled UTP. The enzyme reactions ran at 25°C and enzyme activity was measured at different time intervals after addition of the cytosol E-R complex to the incubated mixtures. Reactions were terminated by adding ice-cold 10% trichloroacetic acid (TCA). Precipitates were collected on Millipore filters (pore size 0.45 μm) and washed with cold 5% TCA containing 1 % $Na_4P_2O_7$. Filters were dried, directly introduced into 5 ml of scintillation fluid (H a r d i n et al., 1976) and counted in an LKB 1219 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland) at an efficiency of about 33% with automatic cpm/dpm calculation. Unless otherwise stated, each value represents the mean of triplicate determinations from three separate experiments.

Determination of DNA

The filters were removed from the vials, dried, and hydrolyzed in 0.5 ml of 0.3 M $HClO_4$ at 90 °C for 30 min to solubilize DNA (G l a s s e r et al., 1972). DNA was determined by the diphenylamine reaction (B u r t o n, 1956). Specific radioactivity (dpm/mg of DNA) was calculated from dpm/filter and μg of DNA on the same filter.

Statistical analysis

Values are expressed as means ± SE, with n representing the number of experiments. Statistical significance was evaluated with the ANOVA test. A value of $p<0.05$ was considered significant (compared to the control value).

RESULTS

The obtained data indicate that there is no significant alteration of low-ionic strength polymerase (I) activity in the presence of cytosolic E-R complexes over a short time interval. Moreover, this polymerase exhibited linear incorporation of nucleotides into RNA for a long time after addition of nuclei to the reactions (Fig. 1A). At 3 h, nucleotide incorporation was increased 4.1-fold at low-ionic strength compared to the control values (Fig. 1A). However, high salt (II) polymerase activity showed

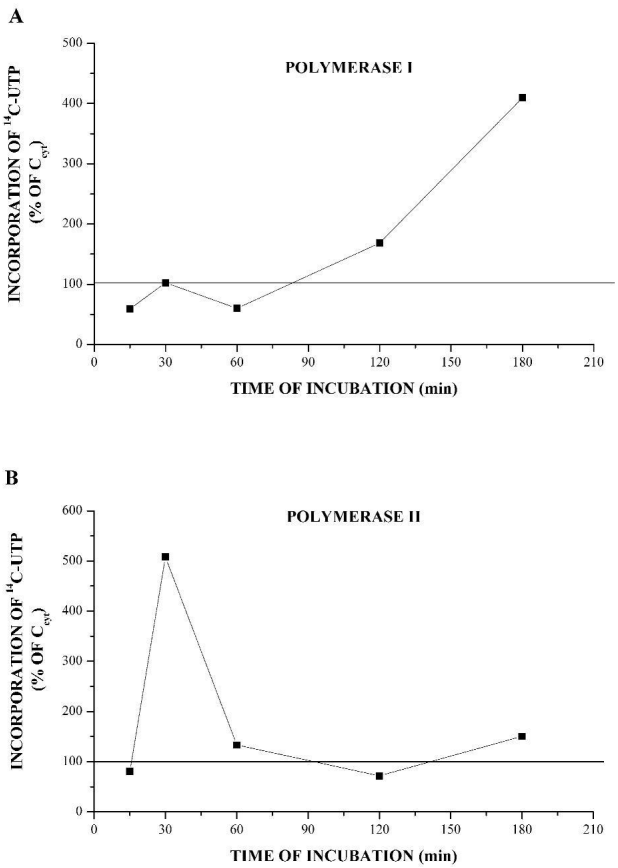

Fig. 1. Time course of nuclear RNA polymerase activity. The E-R complex and nuclei were prepared from uteri of OVX animals as described in the Methods section. At the indicated times of incubation, RNA polymerase I (A) and RNA polymerase II (B) activities were measured as incorporation of ^{14}C-UTP in RNA and determined in nuclei after incubation with the cytosolic E-R complex. The results are presented as percentages of control values obtained in nuclei incubated with cytosol.

a significant 5.1-fold increase after 30 min (Fig. 1B). The initial increase of polymerase II activity was transient, and this activity fell to a value close to the unstimulated control after 1 h (Fig. 1B), whereas polymerase I activity continued to rise during the same interval (Fig. 1A). This decline of activity of polymerase II is followed by a low second elevation in activity of polymerase II at 3 h (Fig. 1B). The elevation of polymerase I activity stimulated by cytosolic E-R complex in isolated nuclei at 3 h was much higher than those of polymerase II activity (Fig. 1). Activity of polymerase I was already increased 2 h after the beginning of reactions (Fig. 1A).

Figures 2 and 3 present results comparing the effects of cytosolic and nuclear E-R complexes on regulation of RNA I and RNA II polymerase activity. The increase in polymerase I activity stimulated by cytosolic E-R complexes was observed much later (Fig. 2A) than for activity of polymerase II (Fig. 3A), whereas no increase of enzyme activities was detected in E2-treated nuclei (Figs. 2 and 3). The E-R complex formed in uterine cytosol of OVX rats stimulated RNA polymerase II activity, with maximum activity at 30 min of incubation (Fig. 3A). E2 had no effect at this early time (Fig. 3A) but caused significant ($p<0.01$) decrease of RNA polymerase II activity at 3 h (Fig. 3B). Incubation of nuclei with E2 for 2 h resulted in decrease of RNA polymerase I activity (Fig. 2A), followed by decline of activity to values remaining

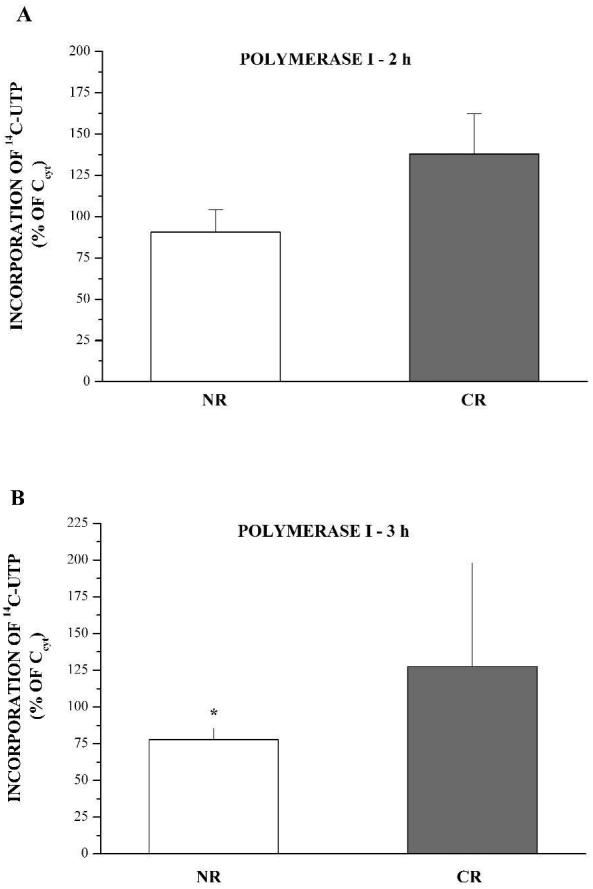

Fig. 2. Effects of cytosolic and nuclear E-R complexes on RNA polymerase I activity. This figure depicts the effect of cytosolic and nuclear E-R complexes on endogenous RNA polymerase I activity of uterine nuclei assayed at 2 h and 3 h after the start of incubation, as described in the Methods section. The results are presented as percentages of control values obtained in nuclei incubated with cytosol. Each point represents the mean ± SE of three experiments. NR-indicates nuclear ER; CR-indicates cytosolic ER. * $p<0.05$.

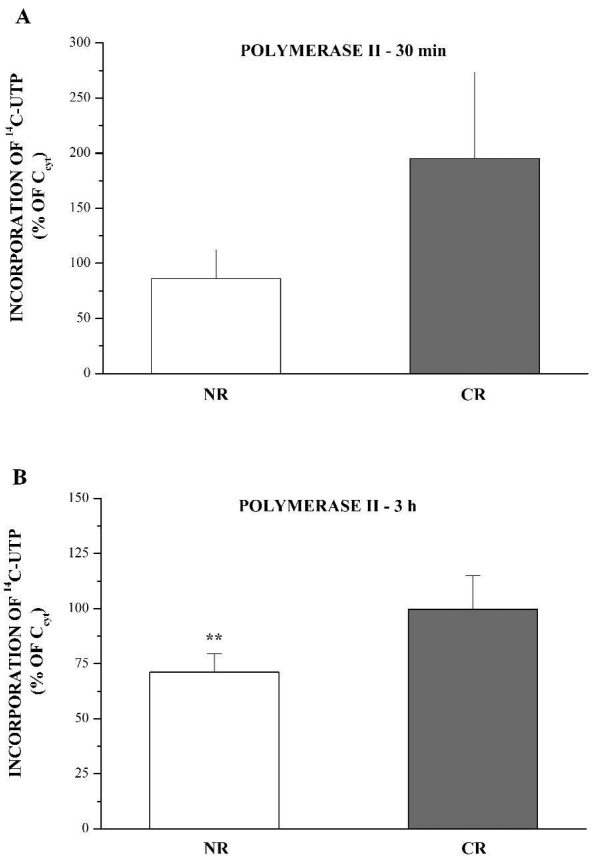

Fig. 3. Effects of cytosolic and nuclear E-R complexes on RNA polymerase II activity. The E-R complex and nuclei were prepared from uteri of OVX animals as described in the Methods section. At the indicated times, RNA polymerase II activity was determined in the nuclei incubated with E2 or the cytosolic E-R complex. The results are presented as percentages of control values obtained in nuclei incubated with cytosol. Each point represents the mean ± SE of three experiments. NR-indicates nuclear ER; CR-indicates cytosolic ER. ** $p<0.01$.

significantly ($p<0.05$) under those of the control at 3 h after the start of incubation (Fig. 2B). The E-R complex formed in the cytosol caused elevation of RNA polymerase I activity at 3 h to a level as high as those induced by cytosolic E-R complex at 2 h (Fig. 2).

DISCUSSION

A number of hormones stimulate the expression of target genes by inducing the synthesis of RNA(s) after changes of RNA polymerase activity (G l a s s e r et al., 1972; R i b a r a c-S t e p i ć et al., 1973; G o o d l a d and C l a r k, 1988; for a review, see R o b y r and W o l f f e, 1998). In order to examine the relationship between intracellular residence of ERs and uterine response to hormone action, the activities of RNA polymerase I and II were measured in isolated uterine nuclei of mature OVX rats in the presence of E2 alone or the E-R complex prepared in the cytosol of the same uterine tissue. RNA polymerase was differentially stimulated by divalent metal ions in the presence and absence of ammonium sulfate (R o e d e r and R u t t e r, 1970; G l a s s e r, 1972; G o o d l a d and C l a r k, 1988). RNA polymerase activities were measured in isolated uterine nuclei using low-ionic media containing Mg^{2+} for stimulation of RNA polymerase I and high-ionic media with Mn^{2+} for stimulation of RNA polymerase II. In order to compare the levels of RNA polymerase activities present in isolated uterine nuclei under influence of E2 action it was necessary to demonstrate that the same initial conditions of velocity kinetics existed for the assay with respect the ER activity and to time of incubation of nuclei added to the reactions. Both RNA polymerase I and II activities were measured in isolated nuclei in the presence of the E-R complex prepared in the corresponding cytosol. The responses of uterine nuclear RNA polymerases are presented in Fig. 1.

The earliest detectable response to cytosolic E-R complexes in isolated uterine nuclei of OVX rats was an increase of RNA polymerase II activity, which reached a peak at 30 min and then decreased to control values (by 1-2 h) before displaying a second increase over control activity at 2 h after the start of incubation. The next response to cytosolic E-R complexes was an increase (2-3 h) of polymerase I activity. The presented results indicate that cytosolic receptor complexes elicit uterotrophic responses that were stimulated by E2. The ability of E2 to stimulate uterine response appears to be related to the time of retention of the receptor complex in the nucleus. To be specific, in order to produce uterotrophic responses, the E-R complex formed in the cytosol must be retained in the nucleus for a longer period of time.

In this study, we examine further the relationship between cellular localization of ERs and the uterine response by investigating the effects of receptors from the cytosol and nuclei on the activity of RNA polymerase I and II in mature OVX rat uteri. Because steroid receptors are now generally thought to be less cytosolic than nuclear protein (K i n g and G r e e n e, 1984; S a r et al., 1990), we saught to establish whether they have similar or different effects on activation of gene expression. It was deemed particularly interesting to learn whether receptors display differential ability to activate transcription depending on their cellular localization. Numerous studies have shown that steroid hormones act by promoting RNA and protein synthesis (O' M a l l e y et al., 1977). We previously reported effects of glucocorticoids on liver cells (R i b a r a c – S t e p i ć et al., 1973) that are similar to those observed on cells of other tissues (W h e l l y, 1985). If this was associated with synthesis of RNA(s), one might expect to find concurrent changes in activity of RNA polymerases (K a n a z i r et al., 1978; T r a j k o v i ć et al., 1979). The present study is based on the assumption that cytoplasmic and/or nuclear steroid receptors represent transcription factors that utilize coactivators and corepressors in transcriptional control (T s a i and O' M a l l e y, 1994; J e n s e n, 1996; P r a t t and T o f t, 1997) that these receptors influence modifications of controlling elements for gene expression such as RNA polymerases (T a m r a z i et al., 2005).

The results presented in this study show that early stimulation of RNA synthesis in rat uteri by E2 is mediated by activation of RNA polymerase II, the enzyme that synthesizes mRNA. This early rise in polymerase II activity observed within 30 min after the startof incubation could be connected with the effects of E2 on induction of the synthesis of mRNA *in vivo* in rat uteri, as previously reported (G l a s s e r et al., 1972). The factors behind the biphasic response of polymerase II activity to E2 treatment *in vivo* are at present obscure. One possibility is that the first peak of activity observed in this study (Fig. 1B) represents synthesis of RNA, which in turn induces the later responses of cells to the hormone, e.g., the second increase of polymerase II activity.

Activity of polymerase I (which synthesizes rRNA) appears to be stimulated in subsequent periods (Fig. 1A, B). The later rise in polymerase I activity is in accordance

with results reported from other laboratories (O ' M a l l e y et al., 1977; R o b y r and W o l f f e, 1998; P e c k et al., 1979).

Together with previously published data (G l a s s e r et al., 1972) the presented results indicate that stimulation of RNA synthesis in rat uteri appears to involve two temporally separate events: (1) early stimulation of DNA-dependent RNA polymerase II and (2) a second rise in both RNA polymerase I and II activities. Numerous studies have demonstrated that modification of polymerase activity emerges as one of the controlling elements for gene expression under steroid action, although the molecular basis of general increase in accessibility of chromatin domains to RNA polymerase is still not understood (G o v i n d and T h a m p a n, 2001; C h e u n g et al., 2003).

The primary function of E-R complexes is to induce early uterine responses that in turn stimulate the subsequent synthetic events responsible for growth (A n d e r s o n et al., 1975). This implies that cytosolic and nuclear receptors share equal potencies with respect to uterine responses. Elevation of RNA polymerase I activity (Fig. 2) and polymerase II activity (Fig. 3) was observed only in nuclei isolated from uteri of OVX rats that were incubated with cytosolic E-R complexes. These results indicate that uterine responses require accumulation and retention of E-R complexes in the nucleus. In addition, they suggest that the time required for stimulation of nuclear events by E-R complexes resulting in a long-term uterine response is at least 6 h after *in vivo* administration of estrogen (A n d e r s o n et al., 1975). However, E2 added to nuclei is not a stimulant in this case (Figs. 2 and 3) because there was no E2 after OVX, which has to promote long-term retention of the receptor within the nuclear compartment. The presented results suggest that the low uterotrophic activity of E2 in isolated nuclei is probably due to the labile nature of the nuclear receptor. Based on this assumption, it could be predicted that E2 in nonoperated animals has a role in preventing the rapid decline of nuclear E-R complexes and should lead to marked enhancement of E2 uterotrophic activity.

There are at least two possible ways to explain why nuclear ER of OVX uterine cells is not able to induce uterotrophic responses, whereas the presence of E-R complexes from the cytosol (Figs. 2 and 3) results in increase of polymerase activity over a longer period of time. The first is that the E-R complex must be present in the nucleus at the one critical time after E2 administration in order to elicit the nuclear events required for uterine processes. Alternatively, the extent of the uterotrophic response may depend on the time of ER retention in the nucleus of uterine cells. These explanations are in accordance with results obtained by other authors (A n d e r s o n et al., 1975; H a r d i n et al., 1976) indicating that ER must remain hormone-bound in the nucleus for approximately 6 h in order to stimulate true uterine responses. The results of this study have shown that the inability of E2 alone to cause elevation of polymerase activities in isolated nuclei after a longer period following OVX (10 days) could be correlated with short term nuclear retention of ER.

However, there is no clear explanation for the observed differential uterine responsiveness depending on cytosolic or nuclear residence of ER. To our knowledge, there is no information in the literature as to whether the receptor complexes formed in the cytosol or nuclei show the same state of activation or intranuclear localization as salt-resistant sites and the nuclear matrix (S e v a l j e v i ć et al., 1998; R i b a r a c-S t e p i ć et al., 2005). One possible explanation is that within uterine cells there are multiple genomic sites required for tissue response and stimulation (H a l a c h m i et al., 1994; R o b y r and W o l f f e, 1998). The ligand-receptor complex can interact with some or all of these sites depending on the state of activation of the ligand-bound receptor complex. The cytosolic receptor complexes would have properties needed for formation of complexes which interact with the specific genomic sites involved with uterotrophic stimulation resulting in changes of polymerase activities. On the other hand, E2 binding to the nuclear receptor may produce a receptor complex which can only interact with a fraction of these sites. Thus, the obtained results suggest that, at least in part, uterine responsiveness to E2 may be related to an altered ratio between cytosolic and nuclear ER, but the precise nature of the relationship between ER localization and the uterotrophic response to E2 needs to be further investigated.

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ЕСТРОГЕНОМ ИНДУКОВАНЕ ПРОМЕНЕ УТЕРУСНИХ РНК ПОЛИМЕРАЗА ЗАВИСНЕ ОД ЛОКАЛИЗАЦИЈЕ РЕЦЕПТОРА ЗА ЕСТРОГЕН

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Циљ овог рада је био да се испитају ефекти естрадиола на активност РНК полимеразе I и РНК полимеразе II у једрима утеруса оваријектомисаних женки пацова. Резултати показују да цитосолни комплекси естрадиол-рецептор индукују повећање активности полимеразе II након 30 мин. Друго повећање активности РНК полимеразе II је запажено након трочасовне инкубације једара са комплексима естрадиол-рецептор формираним у цитосолу. Међутим, активност полимеразе I је била повећана тек 2 сата након почетка инкубације, достижући највећу вредност након 3 сата инкубације једара са комплексима естрадиол-рецептор. Насупрот томе, није запажен стимулаторни ефекат једара инкубираних са самим естрадиолом на активност било које од полимераза. Добијени резултати указују да естрадиол стимулише цитосолни естрогени рецептор који затим изазива утеротрофни одговор код оваријектомисаних пацова. Поред тога, ови резултати указују да је за изазивање утеротрофног одговора неопходно да комплекс естрадиол-рецептор борави у једру дуже време.