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EFFECT OF RIBONUCLEASE ON THE PHYSICO-CHEMICAL PROPERTIES OF ESTROGEN RECEPTOR

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Summary—Estrogen receptors (ER) from rat and rabbit uterine cytosol were examined for their sensitivity to ribonuclease (RNase). After RNase treatment, a major part of rabbit uterine ER was converted from the 7S to 3–4S form, and its binding to DNA-cellulose was increased by 40%. Similar treatment on rat uterine ER showed a shift from 7S to 4.5S, and the DNA-cellulose binding was stimulated by 20%. Measurement of endogenous RNase levels showed that lower RNase concentration in rabbit uterine cytosol coincided with larger stimulation of DNA-cellulose binding by exogenous RNase. These results indicate that a major part of 7S ER is susceptible to RNase, and cleavage of bound RNA seems to uncover additional binding sites for DNA. In contrast to the general thinking that 4S to 5S transformation is essential for nuclear binding, we have observed that RNase-treated rat uterine ER did not undergo such a transformation by warming at 25°C, while DNA-cellulose binding of the receptors increased. Thus, temperature activation could occur independent of 4S to 5S transformation.

INTRODUCTION

The mechanism of steroid receptor interaction with chromosomal sites and subsequent gene activation are topics of extensive research [1–5]. Receptor interaction with DNA is considered to be part of the gene regulation process [2–5]. However, there is little definitive information on the nature of the functional form of receptor. Recently, there has been renewed interest in the involvement of RNA in the structure and function of steroid receptors [6–9]. Two reports on glucocorticoid receptors indicate that the 8S receptor can be converted to a 4S form by treatment with RNase [9, 11]. This conversion was accompanied by an increase in DNA-cellulose binding [9]. However, Chong and Lippman[10] showed that the sedimentation profile of estrogen receptors (ER) from the MCF-7 cell line was not altered by RNase, even though the DNA-cellulose binding of these receptors was increased by RNase treatment. Feldman *et al.*[12] observed an increase in the sedimentation constant, due to the interaction of RNase with the 8S ER. Thus, it would appear from the literature that ER is different from glucocorticoid receptors in its interaction with RNA molecules. In a recent report, we have shown heterogeneity of ERs based on differences in activation mechanisms [13]. Results presented in this paper demonstrate the alteration of physico-chemical properties of ER by RNase and its possible role in receptor activation.

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EXPERIMENTAL

Chemicals

17β[2,4,6,7,-^{3}H]estradiol ([^{3}H]E_2) and ^{14}C-labeled bovine serum albumin and human γ-globulin were purchased from New England Nuclear. RNase A was from Worthington Biochemicals and ribonuclease inhibitor was obtained from Bethesda Research Laboratories. Diethylstilbestrol (DES), DNA-cellulose, and yeast tRNA were from Sigma Chemical Company. Ribosomal RNA (calf liver) was from P.L. Biochemicals.

Methods

Uterine tissue was collected from immature rats (150–200 g, Sprague–Dawley) or rabbits (4–5 lbs, New Zealand White). The tissue was minced and homogenized at 4°C in 10 vol of TED (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, and 1 mM dithiothreitol) buffer. Minced tissue was homogenized by 3 × 10 s bursts of Polytron 10ST homogenizer (Brinkman Instruments). The tissue homogenate was centrifuged at 105,000 ***g*** to obtain cytosol. The cytosolic ER was labeled by incubating with 2.5 nM [^{3}H]E_2 in the presence or absence of a 100-fold excess of DES for 2 h at 4°C. RNase solutions were made in TED buffer at a concentration of 4 mg/ml, and boiled at 100°C for 15 min to eliminate heat labile protease activities. Aliquots of 0.5 ml cytosol (3–4 mg protein/ml, determined by the Lowry procedure [14]) were treated with 1–25 μl RNase solution. After incubation at 4 or 25°C for 15–60 min, the cytosol was treated with dextran-coated charcoal (DCC) to

remove free estradiol, and analysed on sucrose density gradients [13]. Sucrose solutions were prepared in TED buffer and 10–30% gradients were made using a Buchler automatic gradient former. ^{14}C-Labeled bovine serum albumin (4.5S) and ^{14}C-labeled γ-globulin (7.1S) were used as internal markers. Cytosol samples (0.2 ml) were layered on 3.8 ml sucrose solutions and centrifuged at 257,000 *g* for 16–18 h at 2°C using an SW60Ti rotor. After centrifugation, fractions (5 drops) were collected from the bottom of the tube and radioactivity was quantitated. Deviations from this procedure are noted in the legends.

Receptor binding to DNA-cellulose was measured by the centrifugation assay [15, 16]. Aliquots of [^{3}H]E_2 labeled cytosol in TED buffer were treated with different amounts of RNase for 30 min at 4°C and then mixed with DNA-cellulose containing 100 μg DNA. After incubating at 25°C for 90 min, the reaction mixtures were centrifuged. The DNA-cellulose was then washed 2 times with TED buffer and extracted with ethanol to quantitate bound radioactivity.

RESULTS

Figure 1 shows the effect of RNase A on ER from rabbit uteri. Final concentrations of RNase were 20 μg/ml and 200 μg/ml at temperatures 4°C and 25°C. When 20 μg/ml RNase was used, the receptor sedimented in the 7S and 4.5S regions. Treatment with 200 μg/ml RNase caused the major peak to shift

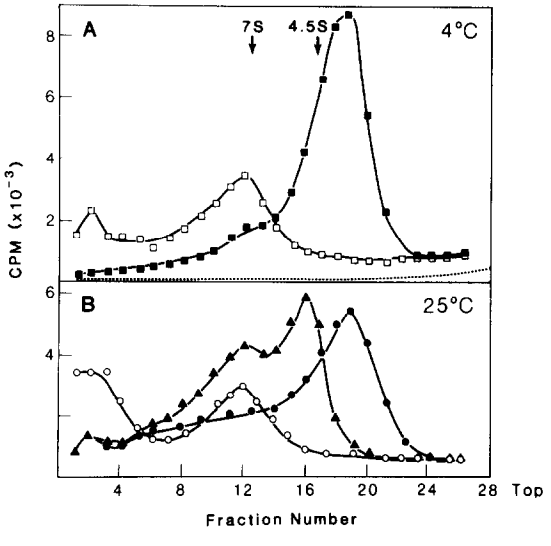

Fig. 1. Effect of RNase on rabbit uterine ER. A: Sedimentation profile of ER from control cytosol at 4°C (□——□), treated with 200 μg/ml RNase at 4°C (■——■) or nonspecific binding in the presence of excess DES (----). B: Sedimentation pattern of samples heated at 25°C (○——○), treated with 20 μg/ml RNase at 25°C (▲——▲) or with 200 μg/ml RNase at 25°C (●——●). Incubations at 4°C were for 60 min, and those at 25°C were for 15 min.

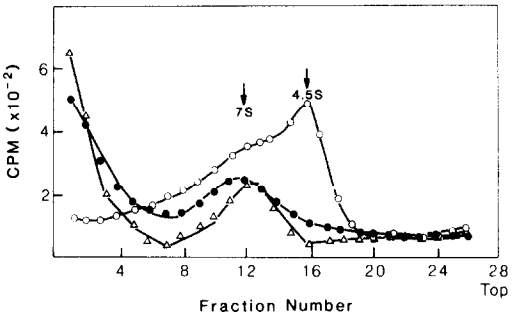

Fig. 2. Prevention of action of RNase by RNase inhibitor. Sedimentation patterns of ER samples, heated at 25°C for 15 min (△——△), heated at 25°C with 4 μg/ml RNase (○——○) and that of sample heated with 400 units of ribonuclease inhibitor and 4 μg/ml RNase (●——●).

to the 3–4S, both at 4 and 25°C. A minor portion of the ER remained at the 7S position as a shoulder of 4S peak. Incubation at 4°C appeared to minimize receptor degradation and aggregation. The possibility that these changes were due to protease action was ruled out by including ribonuclease inhibitor in the reaction mixture. One unit of inhibitor is defined as that amount which will inhibit the activity of 5 ng of RNase A by 50%. Thus, it was necessary to use the least amount of RNase for the reaction. A reaction mixture containing 1 μg RNase (5 μl in 1% BSA), 50 μl rabbit uterine cytosol and 200 μl 1% BSA showed a similar change in sedimentation pattern as to that of 20 μg/ml RNase. By adding 200 μl (400 units) of RNase inhibitor and 1 μg RNase to 50 μl of cytosol, the effect of RNase was prevented and ER was sedimented as a 7S species (Fig. 2). The aggregation of ER observed in control samples also reappeared in the presence of RNase inhibitor. The inhibitor is known to bind to RNase and thus makes it inactive. In our experiments as well as in other reports [9, 11], it has been observed that RNase prevents aggregation. Thus, inactivation of RNase by the inhibitor is accompanied by aggregation, producing a considerable amount of radioactivity at the bottom end of the gradient.

The aggregation of rabbit uterine ER and its prevention by RNase can be seen more clearly from

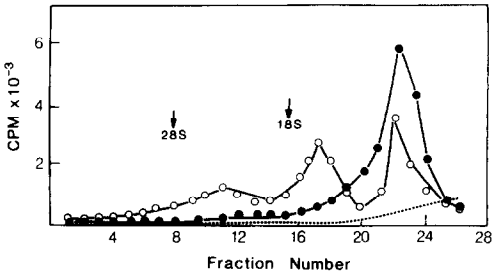

Fig. 3. Sedimentation patterns of rabbit ER samples centrifuged at 257,000 *g* for 3 h. Samples were heated at 25°C for 30 min with (●——●) and without (○——○) RNase. Ribosomal RNA, sedimented in parallel gradients and detected by A_{260}, was used as external marker.

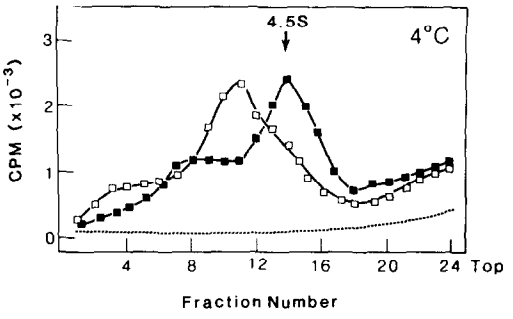

Fig. 4. Effect of RNase on rat uterine ER. Control sample without RNase (□——□), sample treated with 200 μg/ml RNase for 60 min at 4°C (■——■), and nonspecific binding (----). Similar changes in sedimentation pattern were observed in six separate experiments.

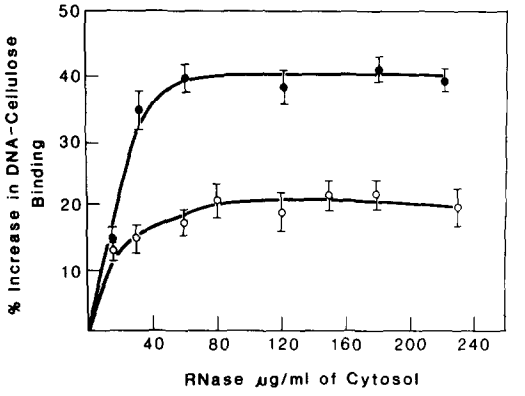

Fig. 5. Effect of RNase on the DNA-cellulose binding of rat (○——○) and rabbit (●——●) uterine ER. Each aliquot of rat uterine cytosol contained 15,000 cpm of specific binding ER, of which 36% were bound to DNA-cellulose in the absence of RNase. Each aliquot of rabbit uterine cytosol contained 24,000 cpm of specific binding ER of which 38% were bound to DNA-cellulose in the controls without RNase. Experiments were carried out in triplicate, with tubes containing excess DES for each experimental point. Nonspecific binding to DNA-cellulose was then deducted. Standard mean deviation of triplicate samples are shown.

gradients which were centrifuged for just 3 h. As shown in Fig. 3, Heating the cytosol for 30 min at 25°C caused the conversion of part of the receptor to faster sedimenting forms, approx 15S and 22S. RNase prevents this aggregation, indicating that large RNA molecules or ER–RNA complexes may be involved in the formation of these aggregates.

It is necessary to examine ER from other sources to prove that RNase-sensitive ER is present in other tissues as well. Figure 4 shows the sedimentation of rat uterine ER before and after treatment with RNase at 4°C. RNase caused a major part of the ER to shift to the 4.5S region. However, there was a distinct shoulder at 8S, indicating that part of the receptor was resistant to RNase. Variation in the amounts of RNase-sensitive and -resistant forms were observed for human breast cancer tissues as well as mouse mammary tumors (data not shown).

The possibility that DNA binding sites of ER are masked by RNA was examined by DNA-cellulose binding assay. The results are illustrated in Fig. 5. Rabbit uterine ER showed about 40 ± 3% increase in DNA binding over the controls in which no RNase was added ($n = 4$). However, the effect of RNase was less significant for rat uterine ER (20 ± 5%). In these experiments, activation of ER was allowed to occur at 25°C during its binding to DNA-cellulose. Thus, the increase in DNA binding indicates that some of the receptors were not activated by warming, but were capable of binding after RNase treatment.

To understand the basis of variation in the increase in DNA binding, we examined the endogenous RNase concentration of cytosol preparations. RNase was measured using yeast RNA substrate (Fig. 6). One unit was defined as the amount of RNase which caused an increase of 0.005 A_{260} units per minute at 25°C. Rat uteri had 6–12 units of RNase/mg of cytosol protein, while rabbit uteri showed only 1–2 units ($n = 4$). Thus, a lower endogenous RNase concentration was associated with a higher proportion of RNase-sensitive form and a larger increase of DNA-cellulose binding due to further addition of RNase.

The relationship between RNase and activation (increase in DNA-cellulose binding) raises the possibility that activation may involve degradation of RNA from ER–RNA complexes. However, as illustrated in Table 1, RNase treatment at 4°C did not alter the receptor binding to DNA-cellulose significantly. Combination of temperature activation and RNase treatment produced maximal increase in the binding.

According to the two-step model for the action of ER [17], the 4S receptor is converted to 5S form before binding to nuclei or DNA-cellulose. Hence, it

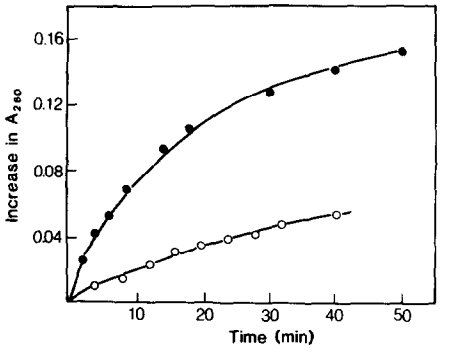

Fig. 6. Assay of RNase concentration in cytosols. Yeast tRNA (1.3 $A_{260/ml}$) was taken in a cuvette and 50 μl cytosol was added. The sample was mixed quickly, and the first reading taken within 15–20 s was considered 0 min point to adjust for the absorbance of cytosol. The increase in absorbance was recorded continuously for 1 h. The tangent on initial velocity was used to determine $A_{260/min}$. Corrections for cytosol dilution during the assay (1:20) and cytosol protein concentrations were made to obtain the values reported in the text. Curves represent measurements of RNase of rabbit (○——○) and rat (●——●) uterine cytosol samples.

Table 1. Effect of RNase and temperature activation on the DNA-cellulose binding of ER

Source of ER		ER Bound to DNA-cellulose (cpm × 10^{-3}) Control 4°C	RNase 4°C	25°C	RNase 25°C
Rat uteri	Expt. I	3.94 ± 0.15	4.29 ± 0.12	5.48 ± 0.38	6.53 ± 0.21
	Expt. II	3.30 ± 0.22	3.01 ± 0.35	4.23 ± 0.23	4.94 ± 0.24
Rabbit uteri		3.35 ± 0.30	3.70 ± 0.18	4.65 ± 0.16	6.40 ± 0.38

Aliquots of cytosol labeled with [^{3}H]E_2 were incubated with DNA-cellulose at 4°C for 90 min. RNase (200 μg/ml) treatment was at 4°C for 30 min and temperature activation was at 25°C, 30 min. Incubation with DNA-cellulose was at 4°C for 90 min. Non-specific binding determined by including 100-fold excess DES was deducted in each case. Standard mean deviation in a triplicate experiment is shown.

is interesting to examine whether the 4S ER, formed during RNAse treatment, undergoes an increase in sedimentation constant by warming at 25°C for 30 min. RNase-treated ER sedimented at 4S, similar to the salt dissociated form, in gradients containing 0.5 M KCl. Figure 7 illustrates a comparison of the effect of warming the cytosol on control and RNase-treated rat uterine ER samples. In the absence of RNase, the ER sample warmed at 25°C for 30 min, sedimented at 5S consistent with the evidence in the literature [17,18]. After RNase treatment, ER sedimented at 4S even after warming the sample. Analysis of RNase-treated ER bound to DNA-cellulose, by extraction with TED buffer containing 0.5 M KCl and subsequent gradient centrifugation, confirmed that these 4S receptors did not undergo transformation before or during their binding to DNA-cellulose (not shown).

Attempts to reverse the 4S form to the 7S form using yeast tRNA was not successful. Incubation of 50 μg ribosomal RNA at 4°C for 1 h with a sample (0.25 ml) of rabbit uterine ER treated with RNase (0.5 μg, 30 min, 25°C) caused a partial reversal, yielding a mixture of 7S and 4S forms. The basis of the difference observed between ribosomal RNA and tRNA is being explored.

It is possible that RNA–receptor interactions observed in hypotonic buffers may not represent the state of the receptor in the intact cell. However, sedimentation of rabbit ER in buffer containing 150 mM KCl showed a peak at 5.5S with a shoulder at 7S. RNase treatment caused the 7S and 5.5S forms to shift to 4.5S form (Fig. 8). This observation indicates that although some of the receptor–RNA interactions are unstable to physiological salt concentrations, others are stable and may be more important *in vivo*.

DISCUSSION

RNA is known to compete for DNA binding sites of androgen, estrogen and dexamethasone receptors [6, 19]. However, the possibility that 7S ER is complexed with RNA has not been examined adequately. The reports of Chong and Lippman[10] and Feldman and co-workers[12] are particularly intriguing since they did not observe a reduction in sedimentation constant of ER in spite of comparable buffer conditions. Most importantly, the tissue-dependent variation of endogenous RNase and the presence of the RNase-resistant 7S form under certain conditions [12, 20] accounts for the fact that the RNase-sensitive 7S form was not detected earlier. Methods of homogenization and tissue:buffer ratio are two other factors that could affect RNA and RNase levels of cytosol. It may be noted that Feldman *et al.* had chosen a milder homogenization method (3 × 3s bursts of Polytron versus 3 × 10s) and a lower tissue:buffer ratio (1:2 versus 1:10) compared to the present study.

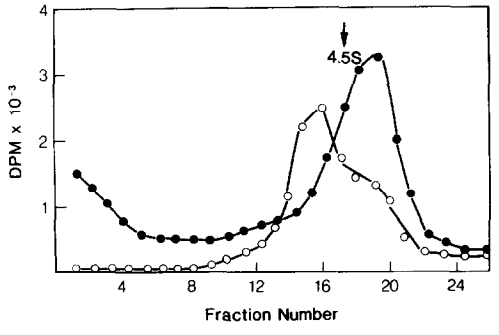

Fig. 7. Sedimentation patterns of heat-activated (25°C, 30 min) rat uterine ER samples in sucrose gradients containing TED buffer and 0.5 M KCl. Symbols represent samples without RNase (○——○) and those treated with RNase (200 μg/ml, 4°C for 30 min) prior to heat activation (●——●).

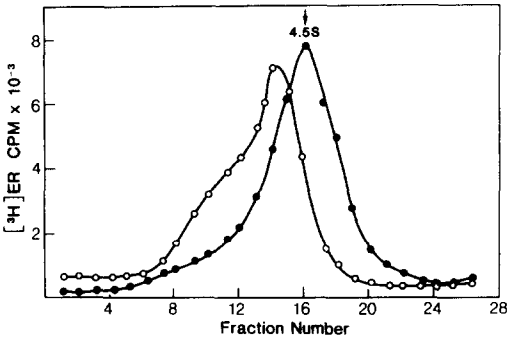

Fig. 8. Sedimentation pattern of rabbit uterine ER in TED buffer containing 150 mM KCl. Symbols represent control sample (○——○) and that treated with RNase (200 μg/ml) at 4°C for 1 h (●——●).

The effect of RNase in reducing the size of 7S ER molecules and enhancing their DNA binding suggest that DNA binding sites of some of the receptor molecules are masked by RNA, and its removal facilitates binding to DNA. Although earlier studies on the effects of RNase showed an increase in sedimentation constant of ER caused by the binding of RNase with the receptor, DNA binding was shown to increase [10, 12]. In these cases, an increase in DNA binding could be due to the destruction of free cytosolic RNA which competes for DNA sites. It is possible that while DNA and RNA would compete for common cationic sites on the receptor, base specific interaction may be involved in the formation of RNase-sensitive form. Thus, activation of RNase-sensitive form may involve dissolution of the linkage between receptor units causing the reduction in sedimentation constant, while that of RNase-resistant form may represent the destruction of free and loosely-associated RNA. RNase may also induce a conformational transition in the RNase-resistant ER leading to the formation of ER with a higher sedimentation constant. Since considerable amount of RNase are present in uterine and human breast cancer tissues, the increase in DNA-cellulose binding caused by heating may be partly due to the activation of endogenous RNase. However, the addition of RNase at 4°C does not elevate the DNA-cellulose binding of ER to the level of activated ER. Thus, in addition to the removal of RNA, other changes are also necessary for optimal binding of ER to DNA.

The 9–10S ER is generally conceived as tetramer of 4S monomer, while the 5–7S form is considered as dimer. This concept, discussed often since the first purification of estrogen receptor, is based on the measurements of sedimentation constant, stokes radius, molecular weight and frictional ratio of different molecular forms of steroid receptors [21–23]. Recently, analysis of various steroid receptors in the presence of sodium molybdate has helped to strengthen this concept of oligomeric protein structure consisting of 4 monomeric units, for all mammalian steroid receptors [24–27]. In this context, the RNase sensitivity of part of the receptors implies that these receptor units may be linked by RNA. The RNase-resistant form may either represent an inaccessible RNA linker or receptor units linked by other cytoplasmic components. However, association of hormone binding and non-binding units through the RNA molecule is also possible [28]. Part of the ER–RNA complexes were stable to sedimentation in gradients containing 150 mM KCl, indicating that high affinity binding is involved rather than non-specific electrostatic interactions.

Although activation and 4S to 5S transformation of rat uterine ER was initially thought to occur concurrently [17,18], recent results from different investigators have demonstrated that activation precedes transformation [29–31]. In addition, rabbit uterine ER sedimented at 4S in high salt gradients before and after temperature activation [13]. Since 4S RNase-treated rat uterine ER could be extracted from DNA-cellulose, it is clear that the 4S to 5S shift is not necessary for binding. Thus, activation of rat uterine ER could be similar to that of other steroid receptors in which an increase in sedimentation constant was not involved [30, 32].

The sedimentation constant of the smaller form of ER formed by RNase treatment varied from 4.5S to 3S in different experiments with rat and rabbit uteri. Experimental error of the gradients was less than 0.25S units since one or two ^{14}C-labeled internal markers were present in all gradients. This variation may be related to protease activities of cytosol, but requires more experimental clarification. It should also be noted that there was variation in the amounts of ER detected in sucrose gradients after treatment with RNase. However, the DCC assay did not detect any difference between control and RNase-treated samples. Studies on dissociation rate and stability of different forms of receptor are being undertaken to understand this difference.

It is interesting to note that results on RNase sensitivity of glucocorticoid receptor [9] are similar to those described for ER in this report. Tymoczko and Phillips observed RNase-sensitive and -resistant forms whose ratio was variable. Modifications of glucocorticoid receptor from mouse mammary tumor by RNase caused changes in molecular weight, sedimentation coefficient and axial ratio [11]. RNA-induced reversal of activation of glucocorticoid receptor from goat mammary tissue [36] also indicates the association of RNA with steroid receptors under certain conditions. Since receptor binding to DNA sequence is proving to be an important step in gene regulation [2–5], the modulation of receptor–DNA interaction by RNase may be of functional significance. Several investigators have reported a heat-stable "cytoplasmic factor" that enhances the activation of steroid receptor [23, 33–35]. It is possible that this factor could be RNase. Although the involvement of RNA in cellular processes needs to be established by further research, it is conceivable that receptor–RNA interaction is involved in a feedback mechanism. Receptor–RNA binding may also be involved in mRNA stabilization [38], polysome disassembly [39], and tRNA utilization [40]. It can be argued that since steroid receptors are known to be DNA binding proteins, their association with RNA may be a consequence of their general affinity for nucleic acids. However, there is evidence to suggest that some of the ribopolymers have a higher affinity for receptor than the corresponding deoxyribopolymers [7]. It has been shown that Poly G, Poly UG, and Poly I are more effective than other polynucleotides in inhibiting receptor–DNA interactions [6, 41]. Although it is impossible to rule out artifactual association of RNA with the receptor, this evidence indicates that sequence-specific interaction between receptor and RNA may

play an important role in post-transcriptional control. Whatever be the role of ER–RNA interaction, *in vitro* studies should now be carefully re-evaluated, considering the fact that often ER is bound to RNA, and the endogenous RNase level may alter its properties.

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