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ARTICLES

Nuclear Estrogen Receptor II (nER-II) is Involved in the Estrogen-Dependent Ribonucleoprotein Transport in the Goat Uterus I. Localization of nER-II in snRNP

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Abstract Exposure of goat uterine nuclei to estradiol in vitro results in an immediate exit of ribonucleoproteins (RNP) from the nuclei to the medium. This RNP exit appears to be mediated by an estrogen receptor localized in small nuclear ribonucleoproteins containing U1 and U2 snRNA. Available evidence indicates that the estrogen receptor involved is not the ERα, but an alternative form, which is also a 66 kDa protein. This is the nuclear estrogen receptor II (nER-II) that has no DNA-binding capacity. The transport is estrogen-specific since non-estrogenic steroids do not stimulate the transport of the RNP where the receptor is localized. J. Cell. Biochem. 84: 217–226, 2002.
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Key words: uterine snRNP; nuclear estrogen receptor-II; RNP transport

The nuclear estrogen receptor II (nER-II) and its plasma membrane counterpart, the non-activated estrogen receptor (naER) are among the newest entrants to the superfamily of nuclear hormone receptors. The naER, a 66 kDa glycoprotein and a tyrosine kinase, has the same affinity to bind estradiol as that of ERα [Anuradha et al., 1994]. It is, however, incapable of binding to DNA on its own. Within the nucleus the naER dimerizes with a DNA binding protein, estrogen receptor activation factor (E-RAF) [Thampan, 1987, 1989; Thampan and Clark, 1981]. Recent studies have shown that the naER, after entering the nucleus undergoes a permanent conformational change as a result of deglycosylation and becomes the nER-II (Jaya and Thampan, 2000). A 62 kDa nuclear protein that transforms the goat uterine non-activated estrogen receptor (naER) to nuclear estrogen receptor II (nER-II) has been isolated and purified [Jaya and Thampan, 2000]. The nER-II or the deglycosylated naER does not dimerize with E-RAF and it interacts with nuclear proteins including RNA polymerases. The role of glycosylation in giving the proper conformation to proteins has been reviewed recently [Parodi, 2000]. Studies carried out several years ago on the in vivo association of nuclear estrogen receptors with nuclear proteins in the rat uterus clearly identified that an estrogen receptor bound with high affinity to nuclear RNA polymerases [Thampan, 1989]. The biological characteristics displayed by the ER indicated that the receptor is nER-II and not ERα [Thampan, 1989].

Biochemical [Thampan, 1985, 1988], as well as, ultrastructural [Vazquez-Nin et al., 1991] studies carried out in the rat uterine system showed the association of estrogen receptor with nuclear ribonucleoproteins (RNP). It was observed that estradiol bound with high affinity to a 17S–19S RNP within the nucleus and also that this interaction resulted in an immediate exit of the RNP-E_2 complex from the nucleus, both under in vivo and in vitro conditions. The RNP-E_2 complex that moved out of the nuclei was subsequently found in association with the polysomes [Thampan, 1985].

Abbreviations used: mRNP, messenger ribonucleoprotein; snRNP, small nuclear ribonucleoprotein; naER, non-activated estrogen receptor; nER-II, nuclear estrogen receptor-II; ERα, estrogen receptor α; m^7G, 7-methyl guanosine; m_3G, trimethylguanosine.

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The data presented in this and the companion paper [Sebastian and Thampan, 2001] is a natural extension of the experimental data reported earlier [Thampan, 1985, 1988, 1989]. A clear lead has been obtained with reference to the biological functions of nER-II in view of the localization of nER-II in small nuclear ribonucleoproteins (snRNPs). An apparent possibility is hereby indicated that nER-II has a functional role in post-transcriptional regulation of estradiol-dependent gene expression. It is premature at this stage to speculate whether nER-II is involved in the regulation of the splicing event. Nevertheless, the positive role that it plays in the nucleocytoplasmic transport of RNP appears to be a reality.

MATERIALS AND METHODS

2,4,6,7-[^{3}H]Estradiol 17β (specific activity 101Ci/mmol), Sepharose 4B and DEAE-Sepharose were purchased from Amersham-Pharmacia Biotech., UK. Estradiol 17β, diethylstilbestrol, 7-methyl guanosine, phenyl methyl sulphonyl fluoride (PMSF), dithiothreitol (DTT) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) were obtained from Sigma Chemical Company, USA. SP6 RNA polymerase was purchased from New England Biolabs, UK and (α-^{32}P) cytidine triphosphate (specific activity 3000 Ci/mmol) was obtained from BRIT (Board of Radiation and Isotope Technology), Mumbai, India. All the other chemicals used were of the analytical grade and were purchased from local chemical companies. The anti-m_3G IgG (mAb H20) was a kind gift from Dr. R. Lührmann, Philipps University, Marburg, Germany. The anti-Sm IgG (mAb Y12) was a kind gift received from Dr. W. J. Van Venrooij, University of Nijmegen, Netherlands, and the U1 and U2 snRNA clones were generous gifts from Dr. Joan A Steitz, Yale University, USA.

Preparation of Splicing Extract

Goat uteri were obtained from a local slaughterhouse and transported in ice to the laboratory for experimentation. Uterine nuclei were isolated following the method published earlier [Thampan, 1985]. Splicing extract was prepared from goat uterine nuclei essentially by the method of Dignam et al. [1983]. The following buffers were used: (1) Buffer C: 20 mM Hepes-KOH (pH set to 7.9 at 4°C), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF; (2) Buffer D: 20 mM Hepes-KOH (pH 7.9), 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT.

Anti-m_3G Immunoaffinity Chromatography

The affinity purification of snRNPs U1 to U6 was performed using an affinity column in which monoclonal antibody H2O was bound to CNBr-activated Sepharose 4B [Bochnig et al., 1987]. Before being chromatographed over the affinity column, the splicing extract was dialysed against buffer C-5 (20 mM HEPES/KOH, pH 7.9, 200 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 5% (v/v) glycerol). The extract was subsequently chromatographed on an anti-m_3G column that was equilibrated with the same buffer. Washing of the column and specific desorption of antibody-bound snRNPs using 7-methyl guanosine were carried out following a published procedure [Hackl et al., 1994].

DEAE–Sepharose Chromatography

The m^7G eluate from the anti-m_3G affinity column was dialysed for 5 h against 80 volumes of DE buffer (20 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and 5% (v/v) glycerol) with two changes of the dialysis buffer. The resulting dialysate was chromatographed on a DEAE-Sepharose column, which was pre-equilibrated with DE buffer. snRNPs were eluted from the column using a linear 0-1M NaCl gradient in DE-buffer.

Hydroxylapatite Assay for Estradiol Binding

Estrogen binding activity associated with the RNP fractions was monitored using a hydroxylapatite (HAP) adsorption assay [Clark and Peck, 1979].

Sucrose Density Gradient Centrifugation

The snRNPs prepared as described above were layered over a linear 10–30% (vol/vol) sucrose gradient in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 300 mM NaCl, 2 mM $MgCl_2$ and 0.5 mM PMSF. The gradients were centrifuged in a Beckman SW 41 rotor at 39,000 rpm for 9 h. The fractions collected by gravity flow were subjected to HAP assay for estradiol binding.

Electron Microscopy of the snRNP Fraction

Negative staining of the sample with 1% uranyl acetate was carried out using the double carbon film method. The preparations were examined under a Philips electron microscope with an acceleration voltage of 80 kV. Electron micrographs were taken at magnifications of ×85,000–140,000.

Antibodies and Immunoblotting

Goat uterine estrogen receptor (ERα) was purified as described by Zafar and Thampan [1993]. The method developed by Karthikeyan and Thampan [1995] was followed for the purification of the nuclear estrogen receptor II. Polyclonal antibody against ERα and nER-II were raised in rabbits. Monoclonal antibody against human ERα was purchased from commercial sources. For immunological investigation, proteins pre-fractionated on high TEMED 12.5% SDS gels [Lehmeier et al., 1990] and 10% SDS-PAGE, were transferred to a nitrocellulose membrane. Specific immunocomplex formation with the cognate antigens was made visible by the alkaline phosphatase reaction.

Northern Hybridization

Isolation of RNA (phenol/chloroform/isoamylalcohol, 50:50:1, method), electrophoresis in urea–10% acrylamide gels, blotting onto Hybond N nylon membranes and finally, hybridization with U1 and U2 snRNA probes were accomplished following standard protocols [Sambrook et al., 1989].

RESULTS

Exposure of Isolated Uterine Nuclei to Estradiol Results in the Release of Estradiol-RNP Complex From the Nucleus

Isolated nuclei were exposed to estradiol in vitro for a brief interval following which the macromolecular fraction that moved out of the nuclei was analysed.

The nuclei, suspended in TMKC-sucrose buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$, 0.2 mM PMSF and 250 mM sucrose) were first incubated without any exogenous hormone at 37°C for 10 min. The incubated nuclear suspension was centrifuged at 800*g* for 10 min at 4°C and the nuclear pellet was resuspended in TMKC-sucrose buffer. These nuclei were reincubated at 37°C for 10 min in the presence of 20 nM estradiol. Following incubation; the nuclear suspension was centrifuged at 800*g* for 10 min. The supernatant was collected and KCl was added to the supernatant to achieve a 500 mM concentration of the salt. The supernatant was chromatographed over a 5 ml column of oligo (dT)-cellulose equilibrated with 10 mM Tris-HCl, pH 7.5 containing 500 mM KCl. The oligo (dT) cellulose was washed extensively with 10 mM Tris-HCl, pH 7.5 containing 500 mM KCl. The elution was achieved using zero salt 10 mM Tris-HCl, pH 7.5 that was pre-warmed to 37°C prior to use. The fractions collected were examined for absorbance at 260 and 280 nm. The absorbance profile observed is shown in Figure 1A.

Previous studies carried out using uterine nuclei isolated from ovariectomized rats showed that the ribonucleoprotein (RNP) export from the nuclei was exclusively dependent on the exposure of the nuclei to estradiol [Thampan, 1985, 1988]. Since the goat uteri are collected from cycling animals with an endogenous source of estradiol, mere exposure of the nuclear suspension to 37°C in vitro results in the exit of the ribonucleoprotein from the nuclei. In the present study, the goat uterine nuclei were subjected to a pre-incubation prior to the exposure to exogenous hormone in order to rule out the possible influence of endogenous estradiol in stimulating the RNP transport from the nuclei.

Time-dependent release of the ribonucleoproteins from the nuclei following exposure to estradiol was studied next. Nuclei were incubated with 20 nM estradiol at 37°C for varying time intervals. The incubated nuclei were centrifuged at 800*g* for 10 min. KCl was added to 500 mM concentration to the supernatant of the nuclear suspension following which the supernatant was chromatographed over a column of oligo (dT)-cellulose equilibrated with 10-mM Tris-HCl, pH 7.5 containing 500 mM KCl. The oligo (dT)-cellulose was washed with 10 mM Tris-HCl, pH 7.5 containing 500 mM KCl following which elution was carried out using salt-free buffer (10 mM Tris-HCl, pH 7.5) at 37°C. The fractions collected were assayed for the binding of ^{3}H-estradiol using a hydroxylapatite (HAP) adsorption assay. Duplicate aliquots of the 800*g* supernatants were directly employed in the HAP assay without subjecting them to a prior chromatography on oligo

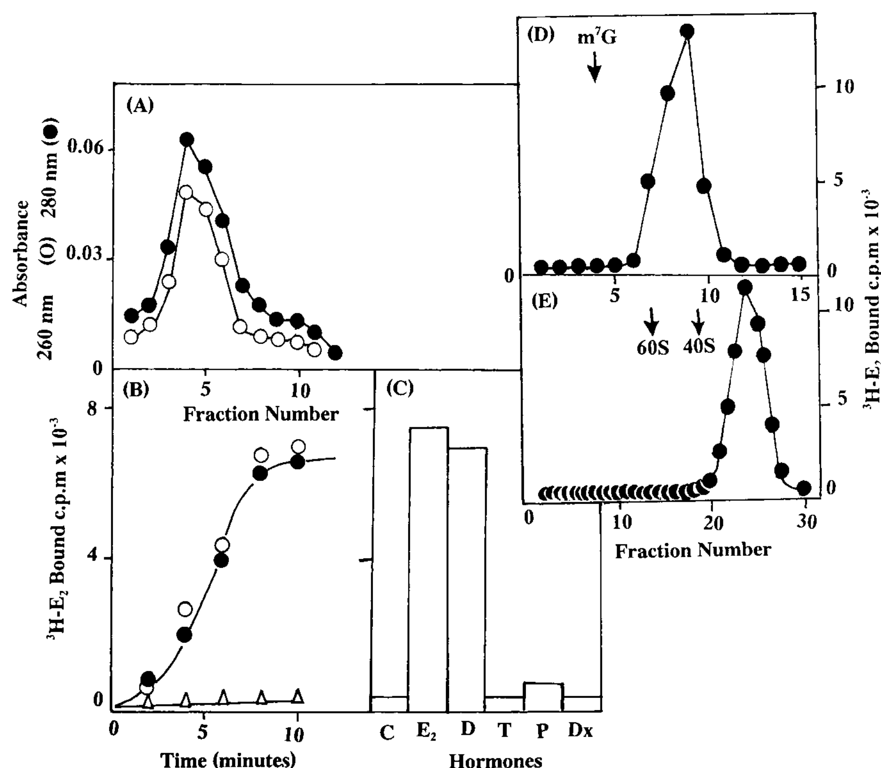

Fig. 1. Isolation and partial characterization of nuclear ribonucleoproteins that bind estradiol. **A:** Nuclei suspended in TMKC-sucrose buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$, 0.2 mM PMSF and 250 mM sucrose), were incubated with 20 nM estradiol at 37°C for 10 min. Following centrifugation of the suspension at 800*g* for 10 min, the supernatant was collected and subjected to chromatography on a column of oligo(dT)-cellulose in the presence of 0.5 M KCl. The column was washed extensively with this high salt buffer following which elution was achieved using zero salt buffer at 37°C. One milliliter fractions were collected and analysed for absorbance at 280 (●) and 260 nm (○). **B:** Time-dependent release of RNP from nuclei following exposure to estradiol. Nuclei, suspended in TMKC-sucrose buffer were exposed to 20 nM estradiol at 37°C. The reaction was stopped at intervals and the nuclear suspensions were centrifuged at 800*g* for 10 min. The supernatant collected was subjected to oligo(dT)-cellulose chromatography. ^3H-E_2 binding to the oligo(dT)-bound fractions was measured using a HAP assay. Prior to chromatography on oligo(dT)-cellulose part of the supernatant recovered was subjected to HAP assay directly. (Δ) Nuclei incubated in the absence of estradiol; (●)-nuclei incubated with estradiol and the supernatant subjected to oligo(dT)-cellulose chromatography; (○) nuclei incubated with estradiol and the medium subjected to HAP assay for estradiol binding. The results indicate that the estradiol-binding component released from the nuclei to the medium was associated with the mRNP. **C:** Hormone specificity of the RNP release. Nuclei were incubated with a hormonal agent under investigation at 37°C for 10 min following which the medium recovered was subjected to oligo(dT)-cellulose chromatography. The oligo-(dT)-bound fraction was subjected to HAP assay for estradiol binding. C, control (without any hormone); E_2, estradiol; D, diethylstilbestrol; T, testosterone; P, progesterone; Dx, dexamethasone. **D:** Chromatography of the oligo(dT)-bound fraction on anti-m_3G-Sepharose. The oligo(dT)-bound fraction from the 800*g* supernatant (nuclei incubated with 20 nM E_2 at 37°C for 10 min) was subjected to chromatography on anti-m_3G-Sepharose. The column was washed with the buffer and elution was achieved using 15 mM m^7G in the buffer. The m^7G eluted fraction was subjected to HAP assay for estradiol binding. **E:** Sucrose density gradient analysis of the anti-m_3G-Sepharose bound fractions. The m^7G eluted fractions from the anti-m_3G-Sepharose column, which displayed maximum ^3H-E_2 binding activity were pooled, concentrated and subjected to 10–30% sucrose density gradient centrifugation in a Beckman Ultracentrifuge (XL-90) using a SW-41 rotor at 39,000 rpm for 9 h. The fractions collected at the end of the centrifugation were assayed for ^3H-E_2 binding activity.

(dT)-cellulose. Parallel sets of nuclei were incubated at 37°C for varying time intervals in the absence of estradiol, which served as a control for the hormone dependent release of the macromolecular complex from the nuclei. The hormone binding properties of the supernatants recovered from the nuclear suspension that was exposed to estradiol at varying time intervals showed the presence of an mRNP fraction that bound estradiol (Fig. 1B). The appearance of estradiol-bound RNP in the medium increased along with the increase in exposure time of the nuclei to the hormone. What was apparent in the results of these studies was that

the estrogen-binding activity detected in the incubated medium was exclusively associated with the RNP complex.

The hormone specificity involved in the release of the RNP from the nuclei was studied. The nuclei were incubated with 20 nM concentration of progesterone, testosterone, dexamethasone, diethylstilbesterol or estradiol at 37°C for 10 min following which the nuclear suspension was subjected to centrifugation at 800*g* for 10 min. The supernatant was recovered and chromatographed over a column of oligo (dT)-cellulose. The washing of the column and the specific desorption of the RNP from the column were carried out as described above. The zero salt buffer (37°C) eluted fractions were examined for ^{3}H-estradiol binding using a HAP adsorption assay. It was observed that while estradiol and the synthetic estrogen, diethylstilbesterol stimulated the release of the RNP fraction from the nucleus, the non-estrogenic steroids failed to affect the RNP release from the nucleus (Fig. 1C).

Earlier reports [Thampan, 1985] had shown that the estradiol binding activity was associated with a rat uterine RNP fraction that moved out of the nuclei and sedimented in the 17S–19S range in linear sucrose gradients. This was an indication to the possibility that the RNP that displayed estradiol binding activity was an snRNP. This assumption needed confirmation.

The fractions eluted from the oligo (dT)-cellulose column, which displayed estradiol-binding activity, were pooled and chromatographed over a column of anti-m_3G IgG–Sepharose, which was equilibrated with buffer C-5 developed by Bochnig et al. [1987]. Monoclonal antibody (mAb H20), which is specific for the trimethyl guanosine cap structure of snRNAs, was coupled to CNBr-activated Sepharose 4B and this was used as an affinity matrix for the purification of snRNPs. The column was washed with the C-5 buffer and elution was achieved using buffer C-5 containing 15 mM 7-methyl guanosine (m^7G). The fractions collected were assayed for ^{3}H-estradiol binding activity using the HAP adsorption method. There was a clear association of estrogen binding activity with the macromolecular peak that was released by m^7G from the anti-m_3G-Sepharose column (Fig. 1D).

The fractions eluted from anti-m_3G-Sepharose, which displayed maximum ^{3}H-estradiol binding activity, were pooled and concentrated through ultrafiltration. The concentrated sample was subjected to sucrose density gradient centrifugation. Rat liver 60S and 40S ribosomal subunits were used as sedimentation markers. The fractions collected at the end of the centrifugation were assayed for ^{3}H-estradiol binding activity. The estradiol binding activity was found to be associated with a macromolecular fraction that sedimented at 17S–19S (Fig. 1E).

Confirmation of the Projected snRNP Nature of the Macromolecular Complex That Bound Estradiol

Characterization of the snRNP in the macromolecular assembly that displayed estrogen-binding activity was carried out through additional experimentation. For this purpose a splicing extract was prepared from goat uterine nuclei essentially following the method of Dignam et al. (1983).

Major snRNPs U1–U6 were purified from goat uterine splicing extracts using anti-m_3G immunoaffinity chromatography. Before being chromatographed over the affinity column, the splicing extract was dialyzed against buffer C-5. This was subsequently chromatographed on an anti-m_3G column that was equilibrated with the same buffer. The column was washed and specific desorption of the antibody-bound snRNPs was carried out using 7-methyl guanosine (m^7G) in buffer C-5. The snRNPs eluted from the anti-m_3G-Sepharose column were further subjected to ion-exchange chromatography on DEAE-Sepharose.

The m^7G eluate from the anti-m_3G affinity column was dialysed for 5 h against 80 volumes of DE buffer with a change of dialysis buffer after 2.5 h of dialysis. The dialysed extract was chromatographed over a column of DEAE-Sepharose, which was pre-equilibrated with DE buffer. The DEAE-Sepharose-bound macromolecules were eluted using a linear 0–1M NaCl gradient in DE-buffer. The fractions were examined for absorbance at 260 and 280 nm and also for estrogen binding activity using a hydroxylapatite adsorption assay. It was observed that the hormone binding activity was associated with a fraction that contained RNA as well as proteins (Fig. 2A). The identity of the proteins and the RNA required confirmation with regard to their functional characteristics. The RNA extracted from the individual fractions was subjected to electrophoresis on polyacrylamide gels and, following northern

transfer, were examined for their capacity to hybridize with U1 and U2 snRNA probes. The peak fractions showed positive signs for the presence of both U1 and U2 snRNAs (Fig. 2B). We are not in a position to exclude the presence of other snRNA species since we have not carried out studies involving probes for these snRNAs. Exposure of the macromolecular fraction eluted from DEAE-Sepharose to polyclonal antibody against estrogen receptor (ERα) resulted in the highlighting of a 66 kDa band in the peak hormone binding fractions (Fig. 2B).

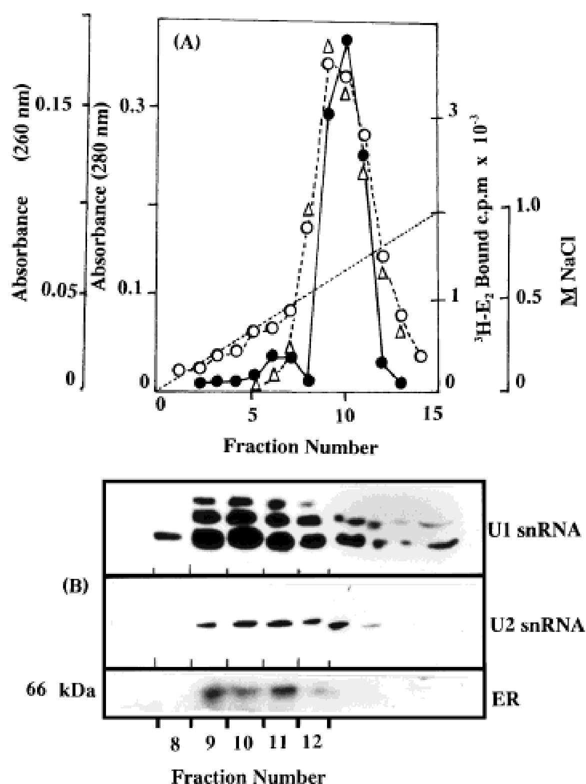

Fig. 2. Confirmation of the snRNP nature of the macromolecular complex that bound estradiol. **A:** DEAE-Sepharose chromatography. The snRNP preparation (anti-m$_3$G-Sepharose-bound fraction) was chromatographed over a DEAE-Sepharose column equilibrated with DE buffer. The column was washed with DE buffer and the snRNP complexes were eluted using 0–1M NaCl gradient in DE buffer. Fractions collected were subjected to HAP assay for estradiol binding in order to identify the receptor. The fractions were examined for absorbance at 260 nm and 280 nm. The peak activity fractions were used for further analysis. **B:** (I) and (II) Northern hybridization of the snRNA extracted from the various fractions obtained from the DEAE-Sepharose column with U1 and U2 snRNA probes. (III) Immunological detection of estrogen receptor. The snRNP preparation represented by the active fractions obtained through DEAE-Sepharose chromatography was subjected to 10% SDS-PAGE. The proteins, which were transferred to nitrocellulose filters, were exposed to anti-estrogen receptor (ERα) polyclonal antibody. Following additional exposure to alkaline phosphatase-coupled anti-rabbit IgG, the blots were stained with BCIP/NBT.

Estrogen Receptor Involved in Interaction With snRNP is not ERα

To investigate whether the estrogen receptor associated with the snRNP is the classical estrogen receptor (ERα) or an alternative form, a series of western blotting analyses were carried out using both polyclonal and monoclonal antibodies raised against human ERα. Commercially available antibodies (Santa Cruz Biotechnologies) were used for this purpose. Polyclonal antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide located at the carboxy terminus of the human ERα. The monoclonal antibody used was a mouse monoclonal IgG raised against a protein corresponding to amino acids 120–170 mapping within the amino terminal domain of the human ERα. Goat uterine estrogen receptor-II (nER-II) was purified as described by Karthikeyan and Thampan [1995] and polyclonal antibody against the receptor was raised in rabbits.

The purified snRNPs were subjected to electrophoresis on a 10% SDS gel and transferred to nitrocellulose membrane. The membrane was probed with both polyclonal and monoclonal antibody against ERα and also against polyclonal antibody against nER-II. Polyclonal antibodies against both ERα and nER-II recognised a 66 kDa protein on the nitrocellulose strips, which contained the total proteins from the affinity purified snRNPs (Fig. 3A). ERα monoclonal antibody failed to detect any protein in the blot (Fig. 3A, lane 4). That the ERα and nER-II displayed only partial immunological relationship was further demonstrated by the following experiments. Purified ERα and nER-II were subjected to electrophoresis on a 10% SDS gel and subsequently transferred to a nitrocellulose membrane. Specific immunocomplex formation with the cognate antigens was carried out using both polyclonal and monoclonal antibodies against ERα and polyclonal antibody against nER-II. As shown by the Western blots (Fig. 3A lanes 5–8), ERα polyclonal antibody reacted specifically with both ERα and nER-II. Conversely, the ERα monoclonal antibody recognized only ERα; it failed to react with the nER-II and the estrogen receptor associated with the snRNP. These data were supportive of a tentative

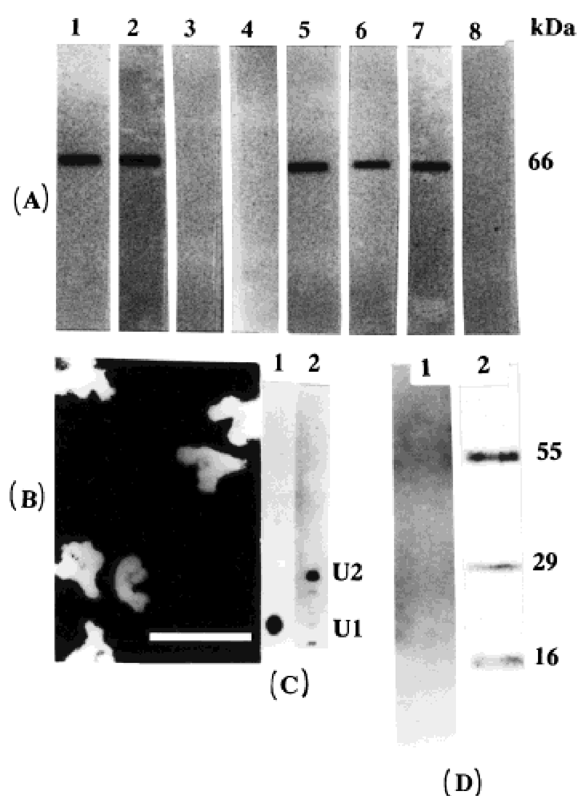

Fig. 3. Display of a possibility that the estrogen receptor involved in interaction with snRNP is nuclear estrogen receptor-II (nER-II). **A**: Immunoblot analysis of the snRNP released from the nuclei and the two isolated estrogen receptor species. Polyclonal and monoclonal antibodies raised against ERα and polyclonal antibodies against nER-II were used in these studies. 1. snRNP with anti-ERα polyclonal IgG; 2. snRNP with anti-nER-II polyclonal. IgG; 3. snRNP with anti-nER-II polyclonal IgG + 10 fold concentration of purified nER-II; 4. snRNP with anti-ERα monoclonal IgG; 5. Purified ERα with polyclonal anti-ERα IgG; 6. Purified nER-II with polyclonal anti-ERα IgG; 7. Purified ERα with monoclonal anti-ERα IgG; 8. Purified nER-II with monoclonal anti-ERα IgG. **B:** Electronmicrograph of snRNP that binds estradiol (of Fig. 1E). The bar indicates 20 nm. **C:** snRNA extracted from the purified snRNP that binds estradiol, hybridized with (1) U1 and (2) U2 snRNA probes. **D:** Immunological detection of Sm proteins in snRNP that binds estradiol. The snRNP preparation obtained through DEAE-Sepharose chromatography was subjected to a 12.5% high TEMED SDS-PAGE. The proteins, which were transferred to nitrocellulose filters, were exposed to (1) pre-immune serum (2) anti-Sm (mAb Y12) monoclonal antibody. Following additional exposure to alkaline phosphatase-coupled anti-mouse IgG, the blots were stained with BCIP/NBT.

conclusion that the estrogen receptor associated with the snRNP is the nuclear estrogen receptor II and not the classical ERα.

Electronmicroscopic data of the DE-52 fraction that displayed estradiol-binding activity revealed the presence of structures in the 10–20 nm range, very closely similar in morphology to those reported by other investigators for U1 and U2 snRNPs [Lührmann et al., 1990; Lewin, 1994]. Magnified images of some selected snRNPs are shown in Figure 3B.

Northern hybridization of the RNA extracted from the DEAE-Sepharose fractions with U1 and U2 snRNA probes was attempted once again. U1 and U2 snRNA antisense clones were transcribed in vitro and used as probes in Northern hybridization. The results clearly indicated the presence of both U1 and U2 snRNAs (Fig. 3C).

It was felt that identification of Sm proteins in the DEAE fractions would give added credibility to the assumption that the hormone binding function is associated with snRNPs. The so-called common proteins, denoted B′, B, D1, D2, D3, E, F, and G, are shared by the snRNP particles U1, U2, U5, and U4/U6 [Lührmann et al., 1990]. Since they all react with the anti-Sm autoantibodies from patients with systemic lupus erythematosus, the common proteins are also termed Sm proteins [Lerner et al., 1981]. For immunological investigation, proteins in the fractions belonging to the DEAE-Sepharose peak that bound estradiol were pre-fractionated on a 12.5% high TEMED SDS gel as described by Lehmeier et al. [1990]. After the electrophoresis, proteins were transferred to a nitrocellulose membrane. Exposure of the proteins to anti-Sm IgG (mAb Y12) showed the presence of three proteins, of molecular mass 55, 29 and 16 kDa (Fig. 3D). Twenty nine kDa and 16 kDa proteins highlighted in the blot represent the B′ and D1 Sm proteins, whereas the 55 kDa is a new protein recognized by the mAb Y12 in our system. Similar observations had earlier been reported in the snRNP literature [Wu et al., 1991]. Pre-immune serum was used as a control for the Western blotting analysis with anti-Sm antibodies. The control IgG was unable to recognize any snRNP proteins in the blot (Fig. 3D).

DISCUSSION

The new observation that is highlighted in the present study is that within the mRNP network, the ER remains in association with snRNPs. This is possibly the first report that deals with a functional significance of the interaction between nuclear estrogen receptor-II and snRNPs. Data on the primary structure of nER-II is still awaited. Therefore it is not possible to go deeper into the structural

comparison between ERα and nER-II at this stage. From the studies that have been reported earlier [Thampan, 1985, 1988], it appears that estradiol has a functional role to play in mRNP transport. In the absence of estradiol the mRNPs are retained within the nucleus while exposure of the nuclei to estradiol results an immediate export of the mRNP from the nucleus. The results point towards a possibility that the mRNP transport in the uterine nucleus is the result of a protein–protein interaction between the estrogen receptor-II on the one hand and snRNP associated protein(s) on the other.

It is known that steroid-receptor complexes show a preferential binding to synthetic polyribonucelotides containing U and G, under cell-free conditions [Liao et al., 1980; Tymoczko et al., 1982]. Rossini et al. [1989] had reported earlier regarding the in vitro association of glucocorticoid receptor (GR) with UsnRNAs. Webb et al. [1986] have also described the association of small RNAs with glucocorticoid receptor complexes. They identified a predominant RNA of 100–110 nucleotides, together with minor bands of longer RNAs, which were considered to be associated with purified untransformed GR. The association of receptor complexes with low molecular-weight RNAs does not appear to be confined to GR alone, as it has been shown that estrogen receptor is also associated with small RNAs [Millar et al., 1985]. The physiological role for the preferential association of estrogen receptor-II (nER-II) with snRNAs in the post-transcriptional regulation of gene expression remains to be ascertained. On the basis of the assumption that the major nucleoplasmic snRNPs, U1, U2, U4/U6 and U5 act as essential *trans* acting factors in nuclear pre-mRNA splicing [Lührmann et al., 1990], it may be suggested that post-transcriptional effects of estrogens may in part be mediated through interaction of estrogen receptor-II with one or more of the snRNP proteins in the spliceosome. The current knowledge about the tissue specific snRNP proteins is in its infancy [Lührmann et al., 1990; Matunis et al., 1993] and it will be an interesting task to investigate the consequences of the association of estrogen receptor-II with snRNPs. There is a possibility that the nucleocytoplasmic transport of mRNP might require an inter-protein interaction involving at least one protein which recognizes the nuclear export signal [Fischer et al., 1995; Fukuda et al., 1996]. Possibly, hormone binding to the nER-II might bring the nER-II close to interacting with such a protein, eventually activating the transport process. Data presented in the companion paper [Sebastian and Thampan, 2001] provides evidence for the involvement of NES on nER-II in the estradiol mediated RNP transport. Three proteins of the snRNP, which recognize the NES in nER-II, have been identified of which two of them, along with nER-II bind to the snRNA. Earlier studies had reported on the activation of a nuclear Mg^{2+}-dependent ATPase prior to the RNP exit from the nuclei following exposure to estradiol. The nature of the protein complex involved in this ATPase activity has also been recognized in the companion paper [Sebastian and Thampan, 2001].

NER II is the deglycosylated form of the plasma membrane-localized non-activated estrogen receptor (naER) which gets internalized following hormone binding [Karthikeyan and Thampan, 1996]. The 62 kDa nuclear transforming factor has recently been isolated and purified [Jaya and Thampan, 2000]. There have been reports of plasma membrane localized estrogen receptors that are recognized as related to ERα [Norfleet et al., 1999; Norfleet et al., 2000]. In this instance itself it is possible to distinguish naER/nER II from the alternative estrogen receptor reported by this group.

Studies have been carried out to learn about the fate of ERα within the uterine nucleus. Recent reports [Nirmala and Thampan, 1995; Nawaz et al., 1999] have shown that ERα undergoes ubiquitination within the nucleus in an estrogen-dependent manner and gets degraded in a proteasome-based pathway. This is an indication of the absence of an additional functional role for ERα after its stimulation of the transcriptional process. On the other hand the nER II maintains its functional integrity during its involvement in post transcriptional regulatory mechanisms.

The experimental results presented in this communication highlight the following: (1) mRNP transport from goat uterine nuclei takes place following exposure to estradiol. Only estrogens activate this process. (2) Estrogen receptor appears to be localized in an snRNP associated with the mRNP. (3) The estrogen receptor involved appears to be nER-II, the non-DNA binding estrogen receptor, and not the ERα.

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