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EFFECT OF ESTROGEN ON THE ELONGATION RATE AND NUMBER OF RNA CHAINS BEING SYNTHESIZED IN UTERINE NUCLEOLI

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Summary—Administration of estradiol (E_2) to ovariectomized mature rats resulted in a time-dependent increased transcriptional activity of uterine nucleoli isolated from hormone-treated animals compared to uterine nucleoli isolated from control animals. Early (4 h) E_2 stimulation of uterine nucleolar transcription, resulted from an increased rate of elongation of chain growth on preinitiated nucleolar RNA with no significant effect of E_2 on the number of nucleolar RNA chains being synthesized. Longer (24 h) treatment of animals with hormone resulted in both significant increased numbers of uterine nucleolar RNA chains in the act of synthesis and increased rate of elongation of nucleolar RNA chain growth. Salt extraction (150 mM NaCl) of uterine nucleoli isolated from 4 h E_2-treated animals decreased transcriptional activity to the level observed in nucleoli isolated from control animals. The loss in nucleolar transcriptional activity from salt extraction was due to decreased rate of elongation of nucleolar RNA synthesis with no significant effect on the number of RNA chains being synthesized. Salt extracts from nucleoli isolated from 4 h E_2-treated animals, but not control animals, contained factor(s) capable of stimulating the rate of elongation of nucleoli isolated from control animals to elongation rates observed in unextracted nucleoli isolated from 4 h E_2-treated animals. Synthesis and phosphorylation of a high molecular weight uterine nucleolar protein(s) was seen after 4 h of E_2 treatment with the nucleolar phosphoprotein(s) salt extractable.

INTRODUCTION

Administration of estrogen (E_2) to mature ovariectomized rats produce a number of physiological and biochemical changes in the uterus. One effect of E_2 administration is a rapid increase in uterine RNA synthesis. The increased synthesis of RNA has been measured in whole uteri [1–3], isolated uterine nuclei [4] and isolated uterine nucleoli [5, 6]. Most of the quantitative changes in uterine RNA synthesis are due to increased synthesis of nucleolar RNA, presumably ribosomal RNA.

Despite the fact that numerous studies have been conducted to elucidate the mechanism(s) by which E_2 stimulates uterine nucleolar RNA synthesis the precise mechanism is yet unknown. Studies have shown that the stimulation of uterine nucleolar RNA synthesis is hormone specific, time- and dose-dependent [1, 6–8]. Protein synthesis is required for the initiation of E_2 stimulation of nucleolar RNA synthesis. Administration of cycloheximide, an inhibitor of protein synthesis, prior to administration of E_2, blocked the E_2 stimulation of nucleolar RNA synthesis. Continued protein synthesis was required to maintain the early (<4 h) effect of E_2 on nucleolar RNA synthesis, but was not required for maintaining the stimulation of nucleolar RNA synthesis during later time (>8 h) of hormone action.

The reaction steps in the overall process of nucleolar RNA synthesis effected by E_2 during early and late treatment times with the homone is not known. E_2 may act by stimulating the rate of initiation of RNA synthesis, either by increasing the number of RNA polymerase molecules transcribing a given number of genes coding for ribosomal RNA or by increasing the number of genes that are active for nucleolar RNA synthesis. Alternatively, E_2 may act by increasing the rate at which the same number of RNA polymerase molecules transcribe or elongate the RNA by the same number of nucleolar RNA genes. Barry and Gorski [8] observed that a 2 h E_2 treatment of immature rats did not influence the number of RNA chains being synthesized in isolated uterine nuclei, but rather stimulated the rate of elongation of RNA synthesis. The long-termed effect of the hormone on nucleolar transcription was not investigated in this study and the RNA product being synthesized and effected by E_2 treatment was not identified but it was presumed to be ribosomal RNA.

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These studies were undertaken to determine whether estrogen activated uterine nucleolar RNA synthesis by different molecular mechanisms during the early and late stages of hormone action. Results are presented which indicate that the early action of the hormone on uterine nucleolar transcription is through a different molecular mechanism than at later times following hormone administration. Results are presented which indicate the early (4 h) stimulatory responses to the hormone at the nucleolar level are: (1) totally accounted for by an increase in the rate of elongation of previously initiated nucleolar RNA chains, and (2) dependent upon the presence of salt extractable nucleolar factors. The late (24 h) stimulatory responses to the hormone at the nucleolar level are: (1) accounted for by increased initiation of nucleolar RNA chains and increased rate of elongation of chain growth, and (2) independent of salt extractable factors. Salt extracts from uterine nucleoli isolated from 4 h E_2-treated animals contained a factor(s) that was capable of stimulating the elongation rate of uterine nucleoli isolated from control animals. Results will also show that synthesis and phosphorylation of a high molecular weight protein(s) was seen after 4 h of E_2 treatment with the phosphorylated protein being salt extractable.

MATERIALS AND METHODS

Chemicals

Unlabeled nucleoside triphosphates, and steroids were obtained from Sigma Chemical Co. (St Louis, Mo.). Radioactive isotopes were obtained from New England Nuclear, (Boston, Mass). All other chemicals were reagent grade.

Animals

Mature female albino rats (Small Animal Supply Co., Omaha, Neb.) weighing 160–180 g were ovariectomized 3–4 weeks prior to use. 17β-estradiol (2.5 μg) was given by a single tail vein injection in 0.5 ml of vehicle composed of 5% (v/v) ethanol in isotonic saline for the hours indicated. [4,5-^{3}H]leucine (40 Ci/mmol), [^{14}C(U)]leucine (300 mCi/mmol) and phosphorous-32 (40 mCi at 370 M Bq/ml) were administered by the transcervical intrauterine route in a vehicle composed of 0.3 M NaCl.

Preparation of uterine nucleoli

Groups of 5–20 uteri from identically treated rats were homogenized (2 ml/uterus) in homogenization buffer (1 M hexylene glycol; 0.05 mM Pipes (1,4-piperazinediethanesulphonic acid) pH 7.2; 2.5 mM $MgCl_2$; 5 mM 2-mercaptoethanol) in a Polytron PT20 (Brinkman Instruments Inc.) at a setting of 4 with 30 s burst with intermittent 30 s coolings repeated 3 times. Following homogenization, the samples were passed (2 passes/gauge) through a series of needles (18, 20, 21 and 22 and 23 gauge). Homogenates were filtered through 1 layer of cheese cloth and then filtered by Nitrex Cloth (Tetco Inc., Elmsford, N.Y.). The homogenates were centrifuged at 1000 ***g*** for 15 min. The crude nuclear pellet was resuspended (3 ml/uterus) in nuclear wash (0.34 M sucrose, pH 7.0) by vigorous vortexing followed by centrifugation at 1000 ***g*** for 15 min. This step was repeated 3 times. The nuclear pellets were resuspended in nuclear wash (2 ml/uterus) and layered over 0.88 M sucrose (3 ml/uterus) and centrifuged at 2000 ***g*** for 15 min. This last step was repeated until clean nuclei were obtained. The clean nuclei were resuspended in nuclear wash (0.5 ml/uterus) and then disrupted by sonication using a Heats System sonicator W375 at an output of 70 W for 10 s with intermittent coolings of 30 s. Nuclear disruptions usually required a total of 45 s of sonication and were monitored by staining a small aliquot of nuclei with 0.1% (w/v) methylene blue then viewed by light microscopy. The nucleoli were isolated from the other nuclear components by sedimentation through 0.88 M sucrose (2–3 times the volume of the nuclear suspension) at 2000 ***g*** for 20 min. Nucleoli were resuspended in nuclear wash buffer (0.5 ml/uterus) and layered over 1.2 M Sucrose (2–3 times the nuclear suspension) and centrifuged at 2000 ***g*** for 20 min. This step was repeated until clean nucleoli were obtained. Nucleoli were then resuspended in a small volume (0.25–0.5 ml/uterus) of TGMED (50 mM Tris–HCl, pH 8.0; 25% v/v glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA; 1.5 mM dithiothreitol) containing 50 mM NaCl and centrifuged at 2000 ***g*** for 15 min to remove any non-nucleolar DNA. The nucleoli were then resuspended in a small volume (0.25–0.5 ml/uterus) of TGMED and immediately assayed or frozen at −80°C for use at a later time. Freezing nucleoli has been shown to have no effect on RNA synthesis capacity.

Uterine nucleolar transcriptional assay

The RNA synthesis capacity of isolated uterine nucleoli was determined by measuring the rate of incorporation of [5, 6-^{3}H]UTP into cold trichloroacetic acid insoluble material. The reaction mixture (0.2 ml) had the following composition: 25 mM Tris–HCl, pH 8.0; 12.5% (v/v) glycerol; 5 mM $MgCl_2$; 0.05 mM EDTA; 0.75 mM dithiothreitol; 50 mM KCl; 0.4 mM ATP; 0.4 mM GTP; 0.4 mM CTP 0.05 mM unlabeled UTP; 2.5 μCi [5,6-^{3}H]UTP (46 Ci/mmol). Typical assays containing 1–5 μg of nucleolar DNA were incubated at 37°C for 15 min. The reaction was terminated by the addition of 5 ml of cold 20% (w/v) trichloroacetic acid containing 1% (w/v) pyrophosphate. Bovine serum albumin (400 μg) was added and incubated at 4°C for 1 h. The acid insoluble material was then collected on glass fiber filters, washed 4 times with 5 ml of cold 5% trichloroacetic acid and 2 times with 5 ml of ethanol. The filters were dried and solubilized of 0.5 ml of NCS Tissue Solubilizers (Amersham/Searle, Chicago, Ill.), and counted after the addition of 10 ml of a

toluene based scintillator. Zero time controls were run with each assay and radioactivity in the unincubated samples was subtracted from the radioactivity in the incubated samples for the determination of the rate of RNA synthesis (CPM of [5,6-^{3}H]UTP incorporated into RNA/μg DNA/15 min) by the isolated nucleoli.

Determination of the number of RNA chains being synthesized in isolated uterine nucleoli

The number of RNA chains being synthesized in isolated uterine nucleoli were determined by slight modification of the method of Olszewski and Guilfoyle [9]. The reaction mixture (0.2 ml) had the following composition: 25 mM Tris–HCl, pH 8.0; 12.5% (v/v) glycerol; 5 mM $MgCl_2$; 0.05 mM EDTA; 0.75 mM dithiothreitol; 50 mM KCl; 0.4 mM GTP; 0.4 mM CTP; 0.4 mM UTP; 2.5 mM unlabeled cordycepin 5′-triphosphate and 2.5 μCi [α^{32}P]cordycepin 5′-triphosphate (3000 Ci/mmol). Reactions containing 1–5 μg uterine nucleolar DNA were incubated at 37°C for 10 min. The assay was terminated by the addition of 5 ml of cold 20% (w/v) trichloroacetic acid containing 1% (w/v) pyrophosphate. Bovine Serum albumin (400 μg) was added and the reaction was incubated at 4°C for 1 h. The acid insoluble material was then collected on glass fiber filters, washed 4 times with 5 ml of cold 5% trichloroacetic acid and 2 times with 5 ml of ethanol. The filters were dried and solubilized in 0.5 ml of NCS Tissue Solubilizers (Amersham/Searle, Chicago, Ill.), and counted after the addition of 10 ml of a toluene based scintillator. Zero time controls were run with each assay and radioactivity in the unincubated samples was subtracted from the radioactivity in the incubated samples for the determination of the number of nucleolar RNA chains (CPM of [α^{32}P]cordycepin 5′-triphosphate incorporated into RNA/μg DNA) by the isolated uterine nucleoli.

Uterine nucleolar salt extraction and reconstitution

Uterine nucleoli were reisolated by centrifugation, resuspended in TGMED containing 50 mM or 150 mM NaCl and allowed to extract 45 min with agitation every 15 min at 4°C. Following extraction uterine nucleoli were reisolated by centrifugation, salt extracted supernatants were reserved and pelleted nucleoli were resuspended in TGMED. Resuspension and reisolation of the nucleoli were repeated twice in order to remove any remaining NaCl prior to transcriptional assay or reconstitution.

The 150 mM NaCl extract was diluted 2:1 with TGMED thereby reducing the salt concentration to 50 mM. Unextracted uterine nucleoli isolated from control animals were resuspended in the diluted salt extract and reconstitution was allowed to take place for 45 min at 4°C with agitation every 15 min. Nucleoli were then assayed under standard nucleolar transcriptional conditions in the presence of the salt extracted fractions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Nucleolar proteins were separated on sodium dodecyl sulfate–polyacrylamide gels in linear gradient of 7.5–20% acrylamide according to the method of Laemmili [10]. Gels were sliced in 1.5 mm sections and solubilized in 1 ml of NCS Tissue Solubilizer (Amersham/Searle, Chicago, Ill.) overnight at 25°C and counted after the addition of 10 ml of a toluene-based scintillator.

DNA determinations

DNA was determined by the diphenylamine method of Burton [11].

Statistical analysis

The values in the figures are expressed as mean $\pm$ SE. Where multiple comparisons with control were made, Dunnett's Test was used and $P < 0.05$ was considered significant. Where appropriate (Fig. 3) Student's t-test (two-tailed) was used with $P < 0.05$ considered significant.

RESULTS

Effect of E_2 on uterine nucleolar RNA chain number and the rate of elongation of nucleolar RNA chains

Uterine nucleoli were isolated from animals receiving E_2 for 0, 4 or 24 h and assayed to determine the effect of E_2 on the nucleolar RNA synthesis capacity. E_2 treatment for 4 h results in a 200% increase in the transcriptional activity of uterine nucleoli compared to the transcriptional activity of uterine nucleoli isolated from control animals. Following 24 h of E_2 treatment uterine nucleolar transcriptional activity is increased 400% above that seen in nucleoli isolated from the uterus of control animals (Fig. 1, panel A).

The number of nucleolar RNA chains being elongated in uterine nucleoli and the effect of E_2 on the number of nucleolar RNA chain number was determined by [^{32}P]cordycepin 5′ triphosphate incorporation. Incorporation of the nucleotide analog into RNA transcripts terminate RNA chain propagation and indicates the number of transcripts in the act of synthesis. Early after E_2 treatment (4 h) there is no significant increase in the number of nucleolar RNA chains in the act of synthesis. Treatment of animals with E_2 for 24 h resulted in 100% increase in the number of uterine nucleolar RNA chains reflected by increased [^{32}P]cordycepin 5′ triphosphate incorporation. (Fig. 1, panel B).

The effect of E_2 on the rate of nucleolar RNA chain growth was determined by calculating the ratio of [^{3}H]UTP incorporation into RNA/[^{32}P]cordycepin-5′ triphosphate incorporation. Administration of E_2 for 4 and 24 h resulted in 220 and 200% increased rate of elongation of uterine nucleolar RNA chains, respectively (Fig. 1, panel C).

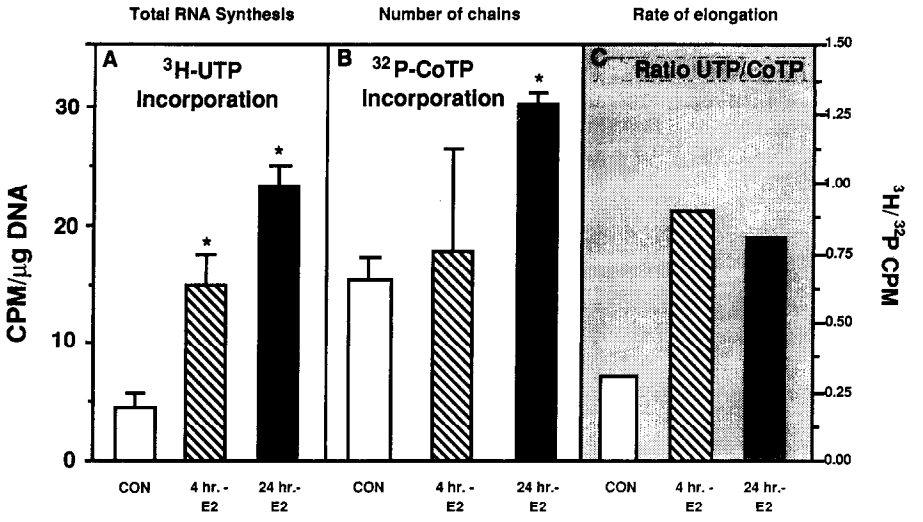

Fig. 1. The effect of E_2 on transcriptional activity, number of RNA chains and rate of elongation of the RNA chain of uterine nucleoli. Groups of 10 animals were given either 2.5 μg E_2 or saline carrier by intravenous injection. Animals were sacrificed at the indicated times and the uteri frozen at −80°C until used. Nucleoli were isolated and assayed by [^{3}H]UTP incorporation (panel A) for the determination of nucleolar transcriptional activity and [^{32}P]cordycepin triphosphate incorporation (panel B) for determination of the number of RNA chains as described in Materials and Methods. Panel C shows the rate of elongation of nucleolar RNA chains determined from the ratio of [^{3}H]UTP incorporation to [^{32}P]cordycepin triphoshate incorporation. Values are given as mean ± SEM. $^*P < 0.05$ relative to control.

Effect of salt extraction of uterine nucleoli on transcriptional activity, chain number and rate of RNA chain growth

Uterine nucleoli were isolated from control, 4 and 24 h E_2-treated animals. Nucleoli were extracted with 50 mM or 150 mM NaCl at 4°C for 45 min. Nucleoli were reisolated and assayed for transcriptional activity, RNA chain number and rate of RNA chain growth as described in Materials and Methods. The results are illustrated in Fig. 2. Extraction (50 mM NaCl) of nucleoli isolated from the uterus of control, 4 h E_2 or 24 h E_2 is without effect on transcriptional activity (panel A), RNA chain number (panel B), and rate of RNA chain growth (panel C). Transcriptional activity in nucleoli, isolated from the uterus of control, 4 h E_2- and 24 h E_2-treated animals extracted with 150 mM NaCl is decreased by 0, 70 and 20%, respectively, compared to nucleoli extracted with 50 mM NaCl (panel A′). Extraction of nucleoli with 150 mM NaCl eliminated the stimulation in transcriptional activity observed in uterine nucleoli isolated from 4 h E_2-treated animals but not in nucleoli isolated from the uterus of 24 h E_2-treated animals when compared to uterine nucleoli isolated from control animals. The number of RNA chains in the act of synthesis, determined by the incorporation of [^{32}P]cordycepin 5′-triphosphate, is unaffected by extraction of nucleoli with 50 or 150 mM NaCl (panel B and B′). The rate of elongation of nucleolar RNA chain growth is decreased 0, 75 and 20% by extraction with 150 mM NaCl of nucleoli isolated from control, 4 h and 24 h E_2-treated animals, respectively, compared to nucleoli extracted with 50 mM NaCl (panel C′). Removal of salt extractable factors in uterine nucleoli with 150 mM NaCl, therefore, eliminated the stimulation in the elongation rate of nucleolar RNA synthesis in nucleoli isolated from 4 h but not 24 h E_2-treated animals when compared to nucleoli isolated from control animals.

Reconstitution of salt extracts from uterine nucleoli isolated from control or 4 h E_2-treated animals with uterine nucleoli isolated from control animals

Salt extracts (150 mM NaCl) from uterine nucleoli isolated from control or 4 h E_2-treated animals were isolated, desalted by dilution with fresh TGMED buffer and reconstituted by incubation at 4°C for 45 min with nucleoli isolated from the uterus of control animals as described in Materials and Methods. Reconstituted nucleoli were assayed for transcriptional activity, RNA chain number, and rate of elongation of RNA chain growth (Fig. 3). Reconstitution of nucleoli isolated from control animals with 150 mM NaCl extract of nucleoli isolated from the uterus of control animals did not effect the transcriptional activity (panel A), number of RNA chains (panel B) or the rate of elongation of RNA chain growth (panel C) when compared to unextracted nucleoli isolated from control animals as seen in Fig. 1. Uterine nucleoli isolated from control animals reconstituted with 150 mM NaCl extract from nucleoli isolated for 4 h E_2-treated animals resulted in 260% increased transcriptional activity compared to nucleoli reconstituted with salt extracts

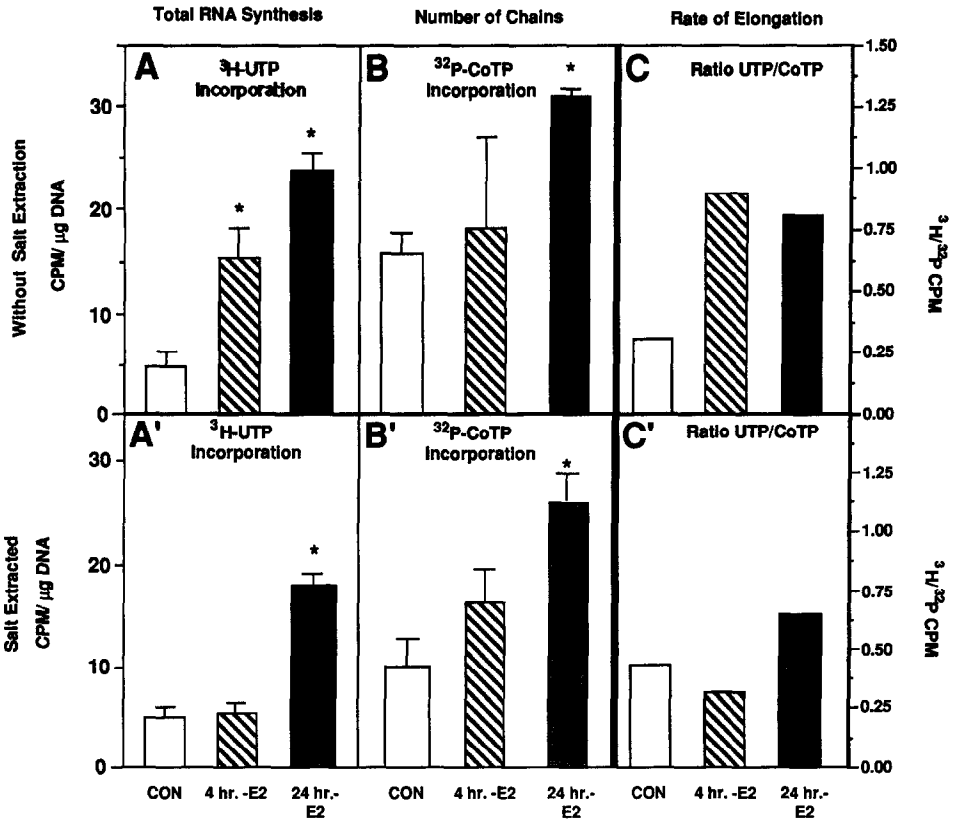

Fig. 2. The effect of 150 mM NaCl extraction on the transcriptional activity, number of RNA chains and rate of elongation of the RNA chain of isolated uterine nucleoli. Groups of 10 animals were given 2.5 μg E_2 or carrier by intravenous injection. The animals were sacrificed at the indicated times and the uteri frozen at −80°C until used. Nucleoli were isolated, extracted with either 50 or 150 mM NaCl and assayed by [^{3}H]UTP incorporation (panel A—50 mM NaCl, panel A′—150 mM NaCl) for the determination of nucleolar transcriptional activity and [^{32}P]cordycepin triphosphate incorporation (panel B—50 mM NaCl, panel B′—150 mM NaCl) for the determination of the number of RNA chains as described in Materials and Methods. Panel C—50 mM NaCl and panel C′—150 mM NaCl show the ratio of [^{3}H]UTP incorporation to [^{32}P]cordycepin triphosphate incorporation illustrating the elongation rate of nucleolar RNA chain growth. Values are given as mean ± SEM. *$P < 0.05$ relative to control.

(150 mM NaCl) from nucleoli isolated from control animals (panel A). The number of RNA chains being synthesized ([^{32}P]cordycepin 5′-triphosphate) in control nucleoli reconstituted with salt extract (150 mM NaCl) from nucleoli isolated from 4 h E_2-treated animals was similar to that seen if reconstituted with extracts from nucleoli isolated from control animals (panel B). The rate of elongation of nucleolar RNA chain growth in control nucleoli was increased 400% in the presence of 150 mM NaCl extracts isolated from uterine nucleoli of 4 h E_2-treated animals compared with 150 mM extracts from nucleoli isolated from control animals (panel C).

Stimulation of synthesis of uterine nucleolar protein(s) by E_2

Synthesis of a short lived protein(s) is apparently required for maintenance of the E_2 stimulation on nucleolar RNA synthesis during early (4 h) E_2 action [6, 8]. We therefore investigated the effect of E_2 on the synthesis of nucleolar protein and whether E_2-induced proteins are salt extractable. Results presented in Fig. 4 demonstrated that early after E_2 treatment (4 h), a nucleolar protein(s) with an apparent molecular weight of 100,000 Da exhibited a higher rate of synthesis when compared to nucleolar proteins synthesized in control animals. This is reflected in an increased ratio of [^{14}C]leucine/[^{3}H]leucine incorporation. No nucleolar protein was found to contain exclusively [^{14}C]leucine or [^{3}H]leucine suggesting no new protein is present in uterine nucleoli isolated from control or 4 h E_2-treated animals. Ratio of [^{3}H]leucine to [^{14}C]leucine did not differ from 1.0, suggesting there was no preferential loss of nucleolar proteins due to E_2 treatment. Salt extracts (150 mM NaCl) of nucleoli isolated from animals pulsed labeled with leucine did not contain the newly synthesized protein(s) (data not shown).

Stimulation of phosphorylation of uterine nucleolar protein(s) by E_2 and salt extractability of phosphorylated uterine nucleolar protein(s)

Phosphorylation mechanisms have been implicated in gene regulation, we therefore have investigated the

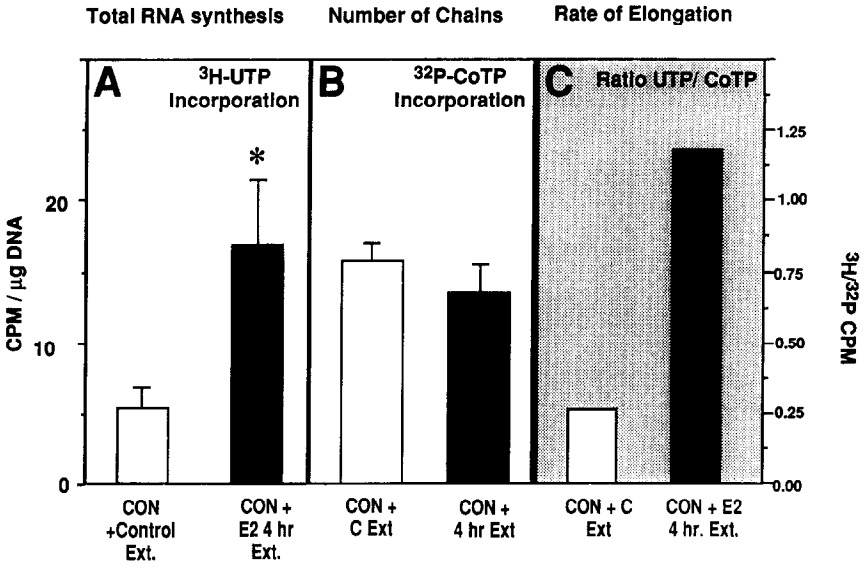

Fig. 3. Effect of addition of the salt extract from nucleoli isolated from control and 4 h E_2-treated animals on transcriptional activity, number of nucleolar RNA chains and rate of elongation of nucleolar RNA chain length in nucleoli isolated from control animals. Groups of 15 (20 for controls) animals were given 2.5 μg E_2 or carrier by intravenous injection. The animals were sacrificed after 4 h and the uteri frozen at −80°C until used. Nucleoli were isolated, split into groups and extracted with 150 mM NaCl. The extracts were isolated and the salt concentration adjusted to 50 mM NaCl with fresh buffer, nucleoli from control animals were preincubated with the diluted salt extracts for 45 minutes at 4°C and assayed by [^{3}H]UTP incorporation for nucleolar transcriptional activity (panel A) and [^{32}P]cordycepin triphosphate incorporation for the determination of the number of RNA chains (panel B) as described in Materials and Methods. Panel C shows the ratio of [^{3}H]UTP incorporation to [^{32}P]cordycepin triphosphate incorporation representing the rate of elongation of nucleolar RNA chains. Values are given as mean ± SEM. $*P < 0.05$ relative to control by Student's t-test.

effect of E_2 on phosphorylation of nucleolar proteins and the presence of E_2-induced phosphorylation on nucleolar salt extracts. Administration of E_2 for 4 h resulted in increased phosphorylation of nucleolar protein(s) with molecular weight around 100,000 Da as reflected by an increase in the ratio of [^{32}P] incorporated into protein present in nucleoli isolated from uteri of 4 h E_2-treated animals compared to [^{32}P] incorporated into nucleolar protein from uteri of controls (Fig. 5). Uterine nucleoli isolated from either control or 4 h E_2-treated animals, labeled *in vivo* with [^{32}P]orthophosphate, were extracted with 150 mM NaCl. Salt extracted nucleolar proteins and residual nucleolar proteins (unextracted with 150 mM NaCl) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. E_2 stimulated phosphorylated nucleolar protein(s) are extracted under conditions that remove the E_2-stimulated factor(s) regulating the rate of elongation of nucleolar RNA synthesis (Fig. 5).

DISCUSSION

Previous results from this laboratory and others have shown that E_2 administration to ovariectomized mature rats resulted in an increased synthesis of uterine RNA [5–8]. Most of the quantitative changes in uterine transcription in E_2-treated animals are attributed to the synthesis of nucleolar RNA which is presumably ribosomal RNA [6]. Hormone specific, time- and dose-dependent increased nucleolar RNA synthesis is observed following E_2 administration. Increased transcriptional activity of isolated uterine nucleoli is elevated above control levels at both early (4 h) and late (24 h) time after E_2 administration. Even though the mechanisms by which E_2 stimulates uterine nucleolar RNA synthesis are not clearly established, certain requirements for the E_2-induced increase in transcriptional activity are apparent. Administration of cycloheximide, an inhibitor of protein synthesis, prior to the administration of the hormone or during the early phase of E_2 action (< 4 h) blocked the E_2-induced increase in nucleolar transcription. If the initiation phase of E_2 action was allowed to proceed to 8 h before administration of cycloheximide, inhibition of protein synthesis was without effect on the E_2-induced increase in nucleolar transcription [6].

Barry and Gorski [8] investigating the mechanism of E_2 action on nuclear RNA synthesis, had shown that 2 h after E_2 treatment of immature rats there was an increased rate of elongation of RNA synthesis with no effect on the number of RNA chains being synthesized in isolated uterine nuclei. The type of RNA being synthesized and effected by E_2 treatment was not identified. In the study reported here, the reaction steps involved in nucleolar RNA synthesis and the steps where E_2 exerted stimulatory effects at both early and late times have been investigated.

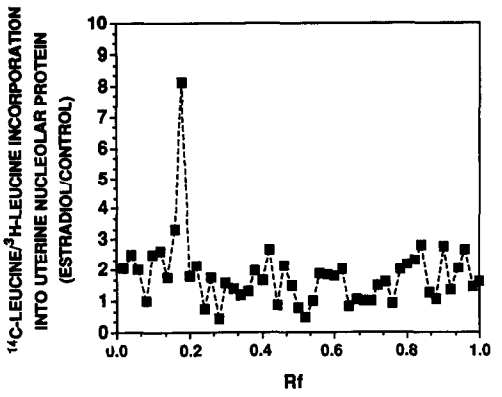

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of uterine nucleolar proteins synthesized *in vivo* in control and 4 h E_2-treated animals. Groups of 10 identically treated animals were administrated E_2 (2.5 μg) or hormone carrier by the intravenous route for 4 h. 1 h prior to sacrifice control animals received 25 μCi [4,5-^{3}H]leucine/animal and E_2-treated animals received 10 μCi [^{14}C(U)]leucine/animal by the transcervical intrauterine route. Animals were sacrificed following treatment and uteri were pooled. Uterine nucleoli were isolated from the pooled samples, solubilized and separated by electrophoresis on a 7.5–20% sodium dodecyl sulfate–polyacrylamide gel. The radioactivity present in 1.5 mm slices was determined, corrected for background incorporation and spillover of [^{14}C(U)]leucine into [4,5-^{3}H]leucine]. The results are expressed as a ratio of incorporated [^{14}C(U)]leucine to incorporated [4,5-^{3}H]leucine into uterine nucleolar proteins. Peak corresponds to a mol. wt of approximately 110,000 Da. Molecular weight standards and corresponding R_f values are: Myosin, 200,000 Da, R_f 0.5; β-galactosidase, 116,250 Da, R_f 0.2; Phosphorylase B 97,000 Da, R_f 0.27; Bovine serum albumin, 42,699 Da, R_f 0.33; Soybean trypsin inhibitor, 21,500 daltons, R_f 0.75.

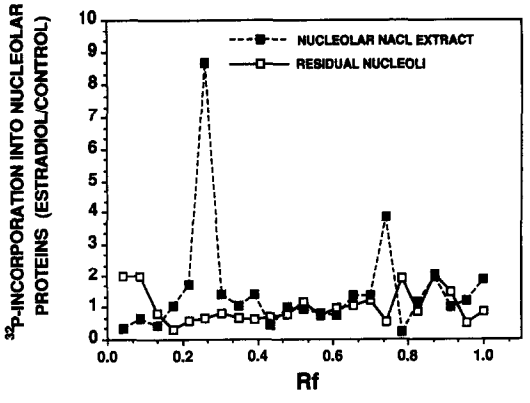

Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of uterine nucleolar proteins phosphorylated *in vivo* in control and 4 h E_2-treated animals. Groups of 10 identically treated animals were administrated E_2 (2.5 μg) or hormone carrier by intravenous route for 4 h. 1 h prior to sacrifice the animals were administrated 75 μCi phosphorous-32/animal by the transcervical intrauterine route. Animals were sacrificed 4 h after treatment with hormone or hormone carrier. Uterine nucleoli were isolated and nucleoli equivalent to 100 μg DNA was salt extracted (150 mM NaCl). Salt extracts and residual nucleoli were separated by centrifugation, solubilized and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in the legend of Fig. 4. The radioactivity present in 1.5 mm slices was determined and corrected for background incorporation. The results are expressed as a ratio of ^{32}P incorporated in nucleolar proteins from E_2-treated animals/^{32}P incorporated in nucleolar proteins from control animals. Peak corresponds to a mol. wt of approximately 110,000 Da. Molecular weight standards and corresponding R_f values are: Myosin, 200,000 Da, R_f 0.5; β-galactosidase, 116,250 Da, R_f 0.2; Phosphorylase B 97,000 Da, R_f 0.27; Bovine serum albumin, 42,699 Da, R_f 0.33; Soybean trypsin inhibitor, 21,500 Da, R_f 0.75.

Early in E_2 (4 h) action increased rate of elongation of preinitiated RNA chains was observed with no apparent increase in the number of RNA molecules being synthesized in uterine nucleoli, similar to that seen in the Barry and Gorski study [8]. Longer termed (24 h) treatment of animals, with E_2, which was not investigated in the Barry and Gorski study, resulted in a significant increased initiation of nucleolar RNA synthesis reflected by an increased number of RNA molecules synthesized in isolated uterine nucleoli and also an increased elongation rate of nucleolar RNA chain growth. Whether nucleolar RNA chains initiated prior to E_2 treatment and/or nucleolar RNA chains initiated during E_2 action are equally effected by E_2 in the stimulation of the elongation rate is not known.

Nonhistone chromosomal proteins have been shown to play a major role in regulation of gene expression. Initially increased synthesis of nonhistone proteins occurs in cells exhibiting increased gene expression and treatment of these cells with cycloheximide eliminates the stimulation in transcription [12–17]. Chromatin reconstituted with nonhistone proteins from stimulated cells have higher template activity than chromatin reconstituted with non-histone proteins from unstimulated cells [18]. Newly synthesized nonhistone proteins are loosely associated with chromatin, the proteins are easily removed by a low concentration of salt and may be responsible for increased template activity (extraction of chromatin with low concentration of salts eliminates the increase in template activity [19]). Chromatin preparation in these studies were obtained from unfractionated nuclei. No attempt was made to determine the nuclear (nucleolar or non-nucleolar) location of the chromosomal proteins. The steps of RNA synthesis (initiation and/or elongation) effected by the nonhistone proteins was also not addressed in these studies. Data reported here confirms the importance of loosely bound proteins in the regulation of gene activity, specifically nucleolar RNA synthesis. Salt extraction of uterine nucleoli isolated from 4 h E_2-treated animals resulted in decreased nucleolar transcriptional activity to the level of transcription observed in nucleoli isolated from the uterus of control animals. The salt extractable factors are involved in E_2 regulation of RNA chain elongation. The stimulation in the rate of elongation of nucleolar RNA chain growth is eliminated, no effect on the number of RNA chains being synthesized is seen, and

no effect on the basal elongation rate of nucleolar RNA chain growth in uterine nucleoli isolated from control animals is observed in salt extracted nucleoli compared to their unextracted counterpart. Elimination of the stimulation in transcriptional activity might result from inactivation of factors associated with extracted nucleoli and/or in the salt extract. Reconstitution experiments reported in this study have eliminated inactivation of stimulatory factors as the cause for decreased nucleolar transcriptional activity. Uterine nucleoli isolated from control animals reconstituted with factors salt extracted from nucleoli isolated from 4 h E_2-treated animals stimulated the reconstituted nucleolar transcriptional activity to the transcriptional level observed in unextracted nucleoli isolated from uterus of 4 h E_2-treated animals. The increased transcriptional activity resulted from an increased rate of elongation of RNA chain growth; there was no effect on the number of RNA chains being synthesized. Experimental manipulation of the nucleoli was not responsible for the effect observed since salt extraction of nucleoli isolated from control animals had no significant effect on the RNA synthesis capacity of the nucleoli, effecting neither the rate of RNA chain growth or the number of RNA chains. Salt extracts from uterine nucleoli isolated from control animals were ineffective in stimulating or inhibiting nucleolar transcriptional activity in reconstituted systems. The salt extractable E_2-stimulated factor(s) or the role played in regulating elongation rate of nucleolar RNA chain growth have not been identified. The factor(s) does not appear to be a newly synthesized nucleolar protein(s), at least, not synthesized during the time interval required for maintenance of the early E_2 (<4 h) stimulation of elongation rate of nucleolar RNA chain growth. Salt extracts of nucleoli isolated from 4 h E_2-treated animals contain E_2-induced phosphorylated nucleolar protein(s) whether these phosphoprotein(s) are involved in regulating uterine nucleolar RNA synthesis is not known. Uterine protein kinase(s) activity is regulated by E_2 [20] and uterine protein kinase(s) may be the short-lived protein required for the maintenance of E_2 stimulation in nucleolar RNA synthesis. Regulation of elongation rates might be through: (1) activation of RNA polymerase I (subunits of RNA Polymerase I are known to be phosphorylated and activity of RNA Polymerase I is effected by phosphorylation [21]); (2) activation of Topoisomerase I which is concentrated in nucleoli, involved in nucleolar RNA synthesis and is regulated by phosphorylation [22–26]; and (3) alteration in nucleolar chromatin structure via a phosphorylation event resulting in faster movement of preinitiated RNA Polymerase I. Any or all of these mechanisms might effect the elongation rate. Resolution of this question awaits future experiments on the purification of the E_2-induced-nucleolar salt extractable factor(s).

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