Biochimica et Biophysica Acta, 972 (1988) 179–191
Elsevier

BBA 12357

Estradiol regulation of reactions involved in turnover of the amino acid acceptor terminus of tRNA in the rat uterus

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(Received 6 May 1988)
(Revised manuscript received 15 August 1988)

Key words: tRNA acceptor activity; Estrogen action; CTP synthetase; tRNA turnover; (Rat uterus)

Estradiol (E_2) increases the specific amino acid acceptor activity of rat uterine tRNAs by increasing the proportion of certain tRNAs with intact and functional 3′-CCA acceptor termini. Activities of tRNA nucleotidyltransferase and 3′-exoribonuclease which synthesize and degrade this terminus, respectively, were measured and neither enzyme was modified by hormone treatment. Since cytidine triphosphate (CTP) levels are below reported K_m values for nucleotidyltransferase, changes in CTP concentrations may regulate nucleotidyltransferase activity. An E_2-induced 3-fold increase was seen in CTP synthetase activity (conversion of uridine triphosphate, UTP, into CTP). Uterine CTP levels in controls are minute (9 nmol/uterus, approx. 90 μM), and are increased 2.5-fold in E_2(12 h)-treated rats. The rate of incorporation of [^{3}H]UTP into the 3′-CCA terminus of tRNA was measured as coupled CTP synthetase-nucleotidyltransferase reactions and a 2.5-fold increase in incorporation occurred 8–12 h after E_2 treatment. Injection of azaserine, (inhibitor of CTP synthetase) reduced E_2-induced increases in CTP levels, CTP synthetase activity, and leucine acceptor activity of tRNAs. These results indicate that E_2 regulates CTP levels by modulation of CTP synthetase activity, and that regulation of synthesis and/or repair of the 3′-CCA terminus of tRNA is proportional to E_2-induced uterine cytosolic CTP levels.

Introduction

Turnover of part or all of the 3′-terminal CCA sequence of tRNA molecules appears to play a significant role in controlling protein synthesis in vivo by regulating the amino acid acceptor activity of certain tRNAs [1]. Addition of the CCA sequence to tRNAs lacking a complete 3′-terminus is catalyzed by the enzyme tRNA nucleotidyltransferase, EC 2.7.7.25 [2], and removal of the 3′-terminus of deacylated tRNAs is exercised by a 3′-exoribonuclease that recognizes the short single-stranded 3′-end of tRNA that includes the CCA terminus. Such nuclease activity has been detected in *Escherichia coli* [3], rat liver [4], and interferon-treated mouse L cells [5]. There is substantial evidence for a control function through turnover of the 3′-terminal nucleotides of tRNA in regulation of protein synthesis. The 3′-terminus is defective in some tRNA populations of stationary phase yeast [6], dormant spores of *Bacillus megatherium* [7], dry ungerminated lupin seeds [8], non-lactating mammary gland [9–11], unfertilized sea urchin eggs [12], polymyopathic hamsters [13,14] and in human placenta [15]. Most of these systems which contain a defective 3′-terminal se-

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quence in tRNA populations are under physiological or pathological conditions of relative tissue dormancy.

Several observations have indicated a role of hormones in regulating tRNA populations. tRNAs isolated from non-lactating mammary gland lack the terminal trinucleotide 3′-CCA sequence and therefore cannot accept amino acids, whereas tRNAs isolated from lactating mammary gland are readily able to accept amino acids [9–11]. Investigators examining estrogen-induced ovalbumin synthesis in chick oviducts observed that addition of tRNAs isolated from oviducts of laying hens or estrogen-stimulated chicks produced an amplification in ovalbumin synthesis in vitro by hen oviduct ribosomes relative to tRNAs taken from estrogen-deprived chicks [16]. Others have found that estrogen-induced hepatic synthesis of the serine-rich protein, phosvitin, in chickens was accompanied by an increase in relative serine acceptor activity of total hepatic tRNA; however, the rate of synthesis of hepatic $tRNA^{ser}$ relative to total tRNA was not modified [17].

Examination of specific amino acid acceptor activity for 18 amino acids by deacylated tRNA isolated from uteri of control and 17β-estradiol (E_2)-treated ovariectomized mature rats revealed that specific amino acid acceptor activity was increased for uterine tRNA from E_2(14 h)-treated rats to levels that were 1.4–4.3-times the acceptor activity of the same tRNAs from control rats [1]. When tRNAs isolated from control and E_2(14 h)-treated rats were incubated with nucleotidyltransferase, there was a larger increase in specific amino acid acceptor activity for uterine tRNA from control rats than for tRNA from E_2(14 h)-treated rats, suggesting that E_2 increased the proportion of tRNAs with complete and functional 3′-CCA termini, in addition to increasing the total amount of tRNA in the tissue. The extent of the increase seen in the amino acid acceptor activity of tRNA from control rats by nucleotidyltransferase was similar in magnitude and specificity to the in vivo E_2-induced increase. Uterine tRNAs from E_2-treated rats and tRNAs which had been repaired by incubation with nucleotidyltransferase were 2–4-times as efficient in support of protein synthesis in a tRNA-dependent translation system.

The following studies were carried out to evaluate the mechanism by which E_2 regulates the 3′-CCA sequence of selected tRNAs in the uterus of the ovariectomized mature rat. Results will indicate that the status of the 3′-CCA terminus of tRNA molecules in the rat uterus is not determined by E_2 modification of levels of nucleotidyltransferase or 3′-exoribonuclease, but instead will indicate that synthesis of the 3′-CCA terminus is regulated by changes in the CTP pool sizes which in turn regulate nucleotidyltransferase activity proportional to changes in levels of this rate-limiting substrate.

Materials and Methods

Animals

Mature rats (Small Animal Supply Co., Omaha) weighing 160–180 g were ovariectomized 3–4 weeks prior to use. Animals received Purina rat chow and water ad libitum. At the indicated times prior to removal of the uterus, animals were injected with 5 μg E_2 in 0.5 ml of 5% ethanol in 0.15 M NaCl; zero-time controls were ovariectomized but not injected with E_2. Animal surgery and treatments were carried out under light diethyl ether anesthesia. Where employed, progesterone (2 mg) was administered in corn oil, subcutaneously. Cycloheximide (400 μg/rat) and actinomycin D (50 μg/rat) were given by transcervical intrauterine injection in a volume of 0.05 ml of 0.30 M NaCl. Actinomycin D was injected once 15 min prior to E_2, and animals were autopsied 12 h later. Cycloheximide was administered 15 min prior to E_2, 4 h after E_2, or 8 h after E_2 and the animals were autopsied 12 h after injection of E_2.

Assay for tRNA nucleotidyltransferase activity

A rat uterine fraction having tRNA nucleotidyltransferase activity was prepared as follows. At indicated time intervals after treatment, rats were killed by cervical dislocation, uteri were immediately removed, dissected free of adjacent fat and connective tissue, frozen in liquid N_2, and stored at −80 °C until assayed. All steps in the preparation of uterine cytosol were carried out at 0–4 °C. Uteri were pooled and homogenized with three 15-s bursts using a Polytron PT-20 homogenizer (Brinkman Instruments, Westbury, NY)

equipped with Teflon bearings, in homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 5 mM 2-mercaptoethanol, 0.25 M sucrose and 70 mM NaF). NaF was used to inhibit endogenous phosphatase activity [18]. The homogenate was centrifuged at $26000 \times g$ for 20 min to obtain the post-mitochondrial supernatant. The supernatant was passed through two layers of cheesecloth and centrifuged at $105000 \times g$ for 60 min to obtain a post-microsomal supernatant. Dextran-treated charcoal was added to the post-microsomal fraction to remove endogenous pools of nucleotides [19] which change with E_2-treatment.

Nucleotidyltransferase activity (incorporation of radioactivity from labeled adenosine triphosphate (ATP) and CTP into the 3′-terminus of tRNA) was measured in a 0.65 ml reaction mixture containing final concentrations of the following: 50 mM glycine-NaOH, pH 8.0, 5 mM $MgCl_2$, 100 μg tRNA, and 5 μCi [^{3}H]ATP or [^{3}H]CTP (24.3 or 52 Ci/mmol, respectively) (New England Nuclear, Boston, MA) [20]. For ATP incorporation yeast tRNA (which contains approximately 60% tRNApCpC) was used, whereas for CTP incorporation, 3′-snake venom phosphodiesterase (Sigma, St. Louis, MO) treated tRNA was utilized. To remove both adenylic and cytidylic residues from the 3′-terminus of yeast tRNA, phosphodiesterase was incubated with yeast tRNA as described elsewhere [21]. In all experiments, the nucleotidyltransferase reaction was terminated by addition of cold 10% (w/v) trichloroacetic acid and 200 μg of carrier yeast tRNA, followed by immersion of the tubes in an ice bath for 30 min. The precipitate was washed successively with cold 5% (w/v) trichloroacetic acid and ethanol on GF/C filters. Filters were air-dried and counted in a liquid scintillation cocktail which contained 10% NCS (Amersham, Arlington Heights, IL).

To be sure that activity measured in nucleotidyltransferase assays was incorporation of [^{3}H]ATP or [^{3}H]CTP into the 3′-terminus of tRNA, thin layer chromatography (TLC) was carried out to analyze samples. After the reaction was complete, an equal volume of H_2O-saturated phenol was added to each sample and tRNA was extracted and prepared as described elsewhere [1]. tRNA samples were hydrolyzed in 0.3 N KOH for 45 min at 90°C or overnight at 37°C [22]. After base hydrolysis, 2 vol. of 10% perchloric acid were added to samples to acid-extract nucleotides. The preparation was allowed to sit on ice for 20 min and then centrifuged at $13000 \times g$ for 10 min. To remove perchloric acid from extracts, the method outlined by Warner and Finamore [23] was followed. A slight excess of an alamine/chloroform solution was added and mixed thoroughly by inversion and aqueous and chloroform phases were separated by centrifugation at $15000 \times g$ for 15 min. Nucleotide monophosphates were separated on PEI-cellulose plates using TLC by the method of Randerath and Randerath [24]. Spots corresponding to 2′(3′)-CMP and adenylic acid (spots were identified by co-chromatographing appropriate standards (were cut-out, solubilized in NCS overnight and counted in a liquid scintillation counter. To further identify cytidine, adenosine, and uridine, thin layer plates were also run using the solvent isopropanol (65%)/HCl (17.2%) [25]. Results confirmed that activity being measured was due to incorporation of radioactivity into either AMP or CMP in the 3′-terminus of tRNA.

To assay for UTP incorporation into the 3′-CCA terminus of tRNA, the nucleotidyltransferase reaction mixture contained in a total of 0.5 ml: 80 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 5 μCi [^{3}H]UTP (52 Ci/mmol), 700 μg of phosphodiesterase-treated yeast tRNA, 0.62 nmol ATP, and 0.15 ml of charcoal-treated uterine post-microsomal fraction. In the indicated experiments, azaserine (Sigma) (500 μM) was used to block CTP synthetase activity [26]. The incubation was carried out at 37°C for 30 min. Following alkaline hydrolysis, tRNA samples were prepared for TLC and chromatographed as described above.

Assay of exoribonuclease activity in uterine fractions

3′-Exoribonuclease activity present in uterine cytosolic fractions obtained at different stages of purification was determined by incubation of the following components at 37°C for 30 min [27]: Each reaction mixture contained the indicated amount of uterine cytosol in 80 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 4 mM 2-mercaptoethanol and labeled yeast tRNA prepared as described below.

Post-mitochondrial and microsomal supernatants and microsomal pellets were analyzed for 3′-exoribonuclease activity with most of the nuclease activity associated with membrane fractions of microsomes. Assays containing no uterine cytosol were included to ensure that no breakdown of RNA occurred under the experimental conditions. After incubation, reactions were stopped by addition of 4 ml of cold 10% trichloroacetic acid plus 200 μg of bovine serum albumin followed by a 30 min incubation at 0°C. Residual ^{3}H-labeled tRNA was precipitated on GF/C filters as described above and counted using liquid scintillation spectrometry. Degradation of 3′-end labeled tRNA was directly related to the amount of uterine extract added.

To label tRNA with [^{3}H]CTP, yeast tRNA was treated with phosphodiesterase, reisolated and then used as a substrate in the nucleotidyltransferase reaction by a modification of the protocol of Igarashi and McCalla [21]. Briefly, the incubation medium contained purified rat liver nucleotidyltransferase (5 mg), 80 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 4 mM 2-mercaptoethanol, yeast tRNA (5 mg) and [^{3}H]CTP (25.6 Ci/mmol) (300 μCi) in a total volume of 4 ml. Incubations were carried out at 37°C for 30 min. When tRNA was labeled with [^{3}H]ATP (44 Ci/mmol), yeast tRNA (which contains about 60% tRNApCpC when not treated with phosphodiesterase) and unlabeled CTP were added to the assay. Liver nucleotidyltransferase was purified following the protocol of Daniel and Littauer [28] with the exception that the calcium phosphate gel adsorption step was omitted.

Measurement of CTP synthetase activity in uterine fractions

The CTP synthetase assay followed the protocol of Hurlbert and Kammen [29]. Components of the standard incubation were: 0.25 μmol GTP, 0.25 μmol UTP, 2.5 μmol L-glutamine, 20 μmol $MgCl_2$, 10 μmol ATP, 26 μmol Tris-HCl, (pH 7.2), and 8 μCi [^{3}H]UTP in a total volume of 0.25 ml. 0.75 ml of the charcoal-treated uterine post-microsomal fraction was added to this solution with the final pH being 7.2. The incubation was carried out at 38°C for 30 min. In the indicated treatments, azaserine (500 μM) was added to abrogate CTP synthetase activity. After reactions were stopped by cold 10% perchloric acid, conversion of [^{3}H]UTP to cytidine nucleotides by the uterine cytosolic fraction was analyzed using two-dimensional TLC as described by Neuhard et al. [30].

HPLC of uterine samples for nucleotide quantitation

At various time intervals after E_2 injection, rat uteri were removed, trimmed and quickly frozen in liquid N_2 within 30 s of the time of killing. Uteri were homogenized in ice-cold 1 N perchloric acid using a power-driven Kontes dual all-glass tissue grinder (Kontes, Vineland, NJ). The homogenate was centrifuged at $15000 \times g$ for 20 min, and the supernatant fraction was retained. Supernatants were neutralized with 0.2 N $KHCO_3$ and centrifuged at $15000 \times g$ for 10 min at 4°C. Precipitated $KClO_4$ was removed and the supernatant was utilized for HPLC analysis.

All solutions used for HPLC analysis were prefiltered through a 0.2 μm Millipore filter. Nucleotide standards were obtained from Sigma and were prepared as 10 mM stock solutions in double-distilled water, and diluted before use. Stock solutions were maintained at −20°C. The solvent used was a 0.5 M ammonium dihydrogen phosphate buffer, pH 6.2 [31]. The pH was adjusted with 0.5 M NH_4OH or 0.5 M H_3PO_4. An Econosphere C_{18} Reverse Phase 5 micron column (Alltech, Deerfield, IL) was used with a Waters Associates Model 440 HPLC instrument for all separations. Nucleotides were eluted from the C_{18} column at 1 ml/min (1000–2000 p.s.i.). In the indicated experiments, azaserine (25 μmol) was administered by transcervical intrauterine injection to block CTP synthetase activity in vivo.

Inhibition of synthesis / repair of the 3′-CCA terminus in vivo by azaserine

Groups of 12 animals were injected with E_2 plus and minus transcervical intrauterine administration of 25 μmol of azaserine 12 h prior to harvesting uteri (control animals received the appropriate intrauterine injection vehicle). tRNAs were prepared from a $100000 \times g$ supernatant of uterine homogenates and specific amino acid acceptor activity of $tRNA^{leu}$ preparations was measured by incubating deacylated tRNA pre-

parations with [^{3}H]leucine (120 Ci/mmol) (Amersham) and tRNA-free aminoacyl tRNA synthetase following the procedure described by Lutz and Barker [1]. Results are expressed as pmol of leucine accepted/nmol of tRNA.

Results

Effect of E_2 on tRNA nucleotidyltransferase and 3′-exoribonuclease activities of uterine fractions

Effects of a single injection of E_2 on nucleotidyltransferase activity in uterine cytosolic preparations are given in Fig. 1. Incorporation of [^{3}H]CTP or [^{3}H]ATP into tRNA was linear with respect to time, substrate concentration, and amount of each uterine fraction added, and was dependent on addition of yeast tRNA (data not shown). Assays performed in the absence of yeast tRNA gave less than 2% of tRNA-dependent activity, and have been subtracted from the data presented. Results indicate that no major changes in activity of this enzyme occur during the first 24 h of the response under the assay conditions used with either [^{3}H]ATP or [^{3}H]CTP as substrate.

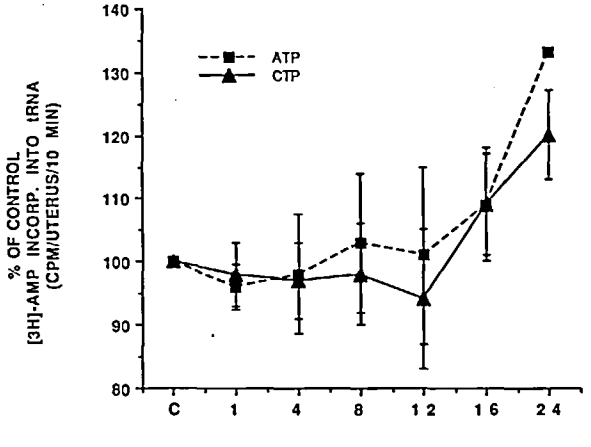

Fig. 1. Time course of estrogen treatment on tRNA nucleotidyltransferase activity. Animals (five per group) were treated with E_2 (5 μg/rat) at the indicated times prior to death; uteri from each group were pooled and incorporation of [^{3}H]ATP or [^{3}H]CTP was determined as described in Materials and Methods. Results are from three separate experiments performed on different days. Each point represents the mean of duplicate measurements on a uterine fraction obtained by pooling uteri from five animals. Values shown represent the means (and indicated S.E.) from three separate experiments and data are expressed as percent of control value in each experiment.

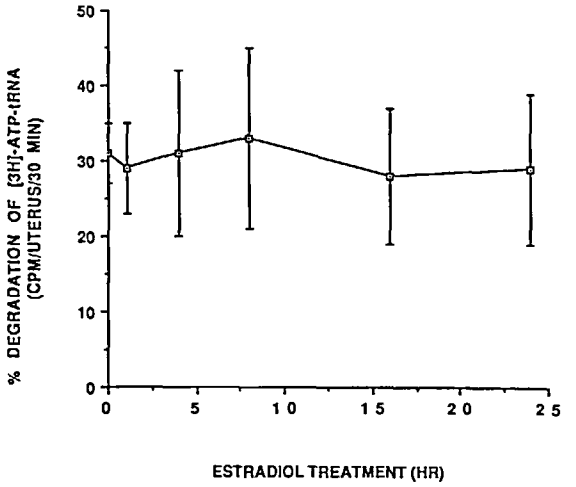

Fig. 2. Time course of estrogen treatment on 3′-exoribonuclease activity. Nuclease activity present in uterine cytosolic fractions obtained at different times after E_2 treatment was determined as described in Materials and Methods. Values shown represent means (and indicated S.E.) from four experiments, and data are expressed as percent of degradation of radiolabeled tRNA.

3′-Exoribonuclease activity was analyzed over a time course of E_2 stimulation using [^{3}H]ATP- or [^{3}H]CTP-labeled yeast tRNA as substrate. The data suggest that 3′-exoribonuclease activity does not change significantly ($P \leq 0.01$) during a period of 24 h of E_2 stimulation under the assay conditions used (Fig. 2). These results indicate that alterations in nucleotidyltransferase or 3′-exoribonuclease activity assayed under optimal conditions do not account for E_2-regulated turnover of the 3′-CCA terminus of tRNAs.

Effect of estrogen on nucleotide levels in the ovariectomized rat uterus

Fig. 3 describes the effects of in vivo hormone treatment for 1, 4, 12 and 24 h on levels of ATP, CTP, guanosine triphosphate (GTP) and UTP pools as determined by HPLC analysis. It is obvious that the predominant nucleotide of the rat uterus is ATP, with ATP alone accounting for approximately 61% of the total nucleotide, UTP 18% of total nucleotide, and GTP and CTP, 11 and 10% of the total nucleotide, respectively, in control uterus. These results agree with those of Gorski and Mueller [32] in which they demonstrated E_2-treatment modified nucleotide pools in the immature rat uterus.

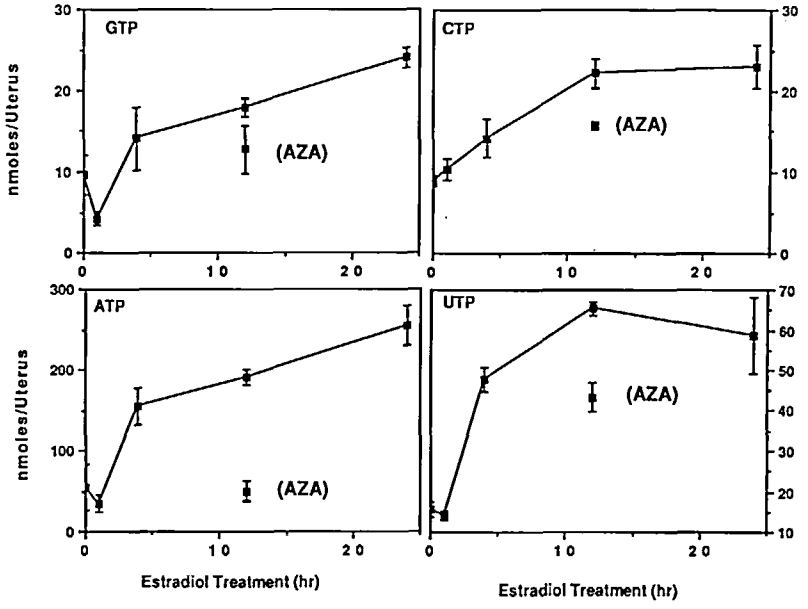

Fig. 3. Effect of E_2 treatment on free nucleotides. Animals were treated with E_2 (5 μg) at the indicated times prior to death; uteri from each group were prepared and HPLC analysis of free nucleotide levels was carried out as described under Materials and Methods. Values shown represent means (and indicated S.E.) from three separate experiments. (AZA) indicates time point at which inhibition of E_2-induced increase in nucleotides was observed with in vivo injections of azaserine.

The hormone caused a rapid reduction in concentrations of purine nucleotides, ATP and GTP, to less than 37 and 56% of control values, respectively, at 1 h. This was followed by a large increase at 4 h, and continued to increase to about 367 and 157% of control values for ATP and GTP, respectively, 24 h after hormone treatment. The early rapid reduction in pools of purine nucleotides has been observed by others [33,34] and probably relates to their use in early activation of carbohydrate metabolism [35].

The amount of UTP remained essentially constant at 1 h after hormone treatment, increased to approximately 318% of control levels at 12 h and dropped to 275% of control values at 24 h after E_2-stimulation. There was a steady increase in the amount of CTP for 12 h after E_2 injection with levels 158% of control values seen at 24 h. The E_2(12 h)-induced increases in nucleotide pool sizes were significantly decreased by intrauterine azaserine treatment; ATP levels decreased 100%, GTP 62%, CTP 49% and UTP 44%. These results indicate that E_2 has a dramatic effect on nucleoside triphosphate pools in the ovariectomized rat uterus. The source of expanded nucleotide pools in the E_2-treated uterus could involve salvage of nucleic acid catabolites and/or de novo purine and pyrimidine synthesis. Our results suggest that expansion in the nucleoside triphosphate pools arises in part from de novo synthesis, since E_2-induced increase in pools could be blocked by the inhibitor azaserine. In addition to blocking CTP synthetase (the enzyme in the de novo pathway which catalyzes the conversion of UTP into CTP), azaserine inhibits two steps in general purine biosynthesis, one step in GTP synthesis from IMP, and the first step in pyrimidine biosynthesis. Further studies were carried out in our laboratory to determine whether a possible regulation of the biosynthesis of the 3′-CCA of tRNA by coupled nucleoside diphosphate kinase-nucleotidyltransferase reactions occur in the ovariectomized rat uterus. Nucleotidyltransferase activity was accompanied by a catalytic activity that enabled nucleotidyltransferase to utilize ^{3}H-labeled nucleoside diphosphates as substrates in the presence of GTP; however, no major changes in this catalytic activity occurred during the first 12 h of the E_2-induced response with either ADP or CDP as substrate, suggesting that the enzyme, nucleo-

TABLE I

EFFECT OF AZASERINE GIVEN IN VIVO ON THE ABILITY OF E_2 TO INDUCE AN INCREASE IN THE LEUCINE ACCEPTOR ACTIVITY OF ISOLATED UTERINE tRNA

Azaserine (25 μmol/rat) or the 1.8% saline vehicle was given by intrauterine (i.u.) injections 15 min before administration of 5 μg of E_2 or 0.9% saline by tail-vein injections (i.v.). All animals were killed at 12 h, uteri were frozen and pooled (12 animals per group) and tRNA was isolated, leucine acceptor activity was assayed and tRNA nucleotidyltransferase (NTT) repair of defective 3′-CCA termini was performed as described by Lutz and Barker [1]. Numbers in parenthesis are percentages of saline treated control. [^{3}H]Leucine incorporation into leucyl tRNA was measured in triplicate.

Treatment (in vivo)		tRNA (μg)/ uterus	[^{3}H]Leucine incorporation (cpm) into 2 μg uterine tRNA		
i.v.	i.u.		−NTT	+NTT	+/−NTT
Saline	Saline	15	24162	71260	2.95
Saline	Azaserine	14	26420 (109)	72506 (102)	2.74
E_2 (12 h)	Saline	23	73067 (302)	73796 (104)	1.01
E_2 (12 h)	Azaserine	14	46331 (192)	83173 (117)	1.80

side diphosphate kinase, in the salvage pathway does not play a significant role in regulating purine and pyrimidine nucleoside triphosphate levels (data not shown). Others have demonstrated that E_2 induces the de novo synthetic but not the salvage pathway for pyrimidines [36] in the immature rat uterus.

CTP synthetase activity

The relative rate of conversion of [^{3}H]UTP into [^{3}H]CTP (CTP synthetase activity) by uterine cytosol fractions is given in Fig 4. A 3-fold increase in CTP synthesis was observed after a 12 h E_2 stimulation. When the glutamine antagonist, azaserine, was added to the in vitro assays, the E_2-induced increase in CTP synthetase activity was reduced to near control levels. These results indicate that E_2 stimulation of CTP synthetase increases the rate of conversion of UTP into CTP during the period of 4–12 h which coincides with the period of enhanced repair of the 3′-CCA terminus of uterine tRNAs [37]. Presumably E_2 has a direct or indirect effect on the amount of CTP synthetase activity in the uterus between 1 and 4 h into the response.

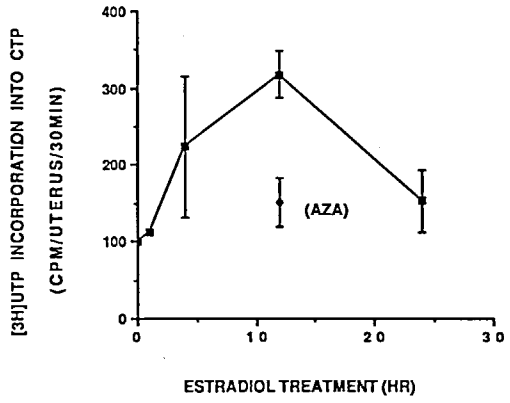

Fig. 4. Time course of CTP synthetase response to E_2. CTP synthetase activity in uterine preparations was determined as described under Materials and Methods. Results are expressed as means+S.E. of three experiments, each performed in duplicate.

Effect of azaserine on the specific amino acid acceptor activity of uterine tRNA

The leucine acceptor activity of uterine tRNAs prepared from animals treated with and without azaserine and the effect of these treatments on the proportion of tRNA that could be activated by tRNA nucleotidyltransferase were measured. The results indicated that the in vivo administration of azaserine prior to E_2-treatment significantly suppressed the ability of E_2 to induce specific leucine acceptor activity while azaserine treatment by itself was without effect (Table I). After the same tRNAs were treated with nucleotidyltransferase to repair defective 3′-CCA termini on the tRNAs, specific leucine acceptor activity was the same for tRNA from all in vivo treatment groups. These results indicate that the E_2-induced increase in leucine acceptor activity was due to repair of the 3′-CCA terminus of the tRNA and azaserine inhibited the ability of E_2 to induce the in vivo tRNA repair. A noteworthy observation is that

azaserine treatment also appeared to inhibit the ability of E_2 to increase the amount of tRNA in the uterus. Inhibition of CTP synthetase by azaserine occurs by an irreversible inactivation of CTP synthetase which is delayed by the presence of glutamine [26]. Considering that endogenous levels of glutamine exist in the rat uterus, complete inhibition of the E_2-induced increase in aminoacylation of leucyl tRNA would not be expected to occur in vivo. These results strongly suggest that azaserine blocks the E_2-induced increase in levels of CTP and the repair of the 3′-CCA terminus of uterine tRNAs by blocking CTP synthetase activity.

[^{3}H]UTP incorporation into CCA of tRNA

Since no major changes in activities of either nucleotidyltransferase or 3′-exoribonuclease occurred during the first 24 h of the E_2 response, since CTP levels in the uterus are very low and induced by E_2, and since CTP synthetase activity is induced by E_2, we considered that substrate levels for nucleotidyltransferase, particularly CTP, may be rate-limiting and the critical point for hormonal regulation of synthesis and repair of the 3′-CCA terminus of tRNAs. We considered that the overall effect of incorporation of UTP into tRNA should be a valid measure of both CTP synthetase and nucleotidyltransferase activities that were present in uterine extracts. Data in Fig. 5 indicate that E_2(12 h)-treatment induced an approximately 2-fold increase in the ability of uterine cytosol to support incorporation of UTP into the 3′-CCA terminus of tRNA. tRNAs labeled in these assays were then hydrolyzed and analyzed on TLC plates and results indicated a 2-fold increase in incorporation of [^{3}H]UTP as CMP. This 2-fold increase in activity between the 8th and 12th h of the E_2 response was inhibited 83% by addition of azaserine. These data indicate that E_2 stimulates the overall conversion of UTP to CTP and then incorporation of CTP into the tRNA by endogenous nucleotidyltransferase, at a rate that nearly parallels the increase in rate of CTP synthesis. This coupled assay was therefore used in subsequent experiments. Further evidence that CTP levels are rate-limiting in the synthesis of 3′-CCA termini of tRNAs is indicated in Fig. 6. These results indicate that increasing CTP concentrations in the uterine nucleotidyltransferase reaction from 0.01 to 0.75 mM gave a nearly linear increase in the rate of the reaction.

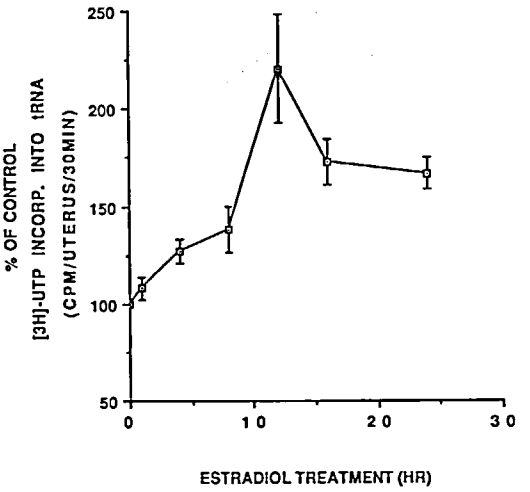

Fig. 5. Time course of UTP incorporation into 3′-CCA terminus of tRNA (CTP synthetase + nucleotidyltransferase activity). Animals (5–10 per group) were treated with E_2 (5 μg/animal) at the indicated times prior to death. Uteri from each group were pooled and incorporation of [^{3}H]CTP into tRNA was measured as described in Materials and Methods. Values represent the means (with the indicated S.E.) for three separate experiments, and data are expressed as percent of the vehicle-treated control group for each experiment.

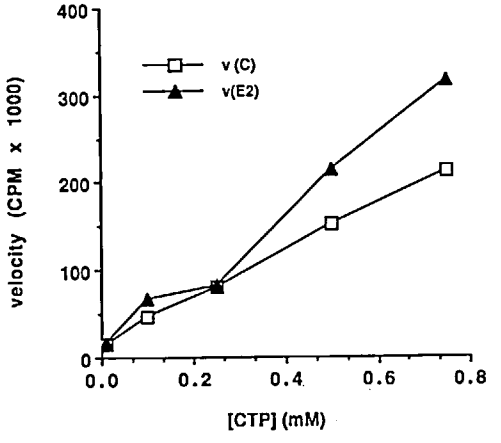

Fig. 6. Effect of increasing the concentration of CTP on the rate of the nucleotidyltransferase reaction by uterine extracts from control (□) and 24 h E_2-treated rats (▲). Nucleotidyltransferase was assayed as indicated in Fig. 1 at final CTP concentrations in the reaction mixture ranging from 0.01 to 0.75 mM.

Hormone specificity of [^{3}H]UTP incorporation into CCA of tRNA

Hormonal specificity of the E_2-induced increase in [^{3}H]UTP incorporation into CMP of tRNA was evaluated. As indicated in Fig. 7, neither progesterone, corticosterone, estrone, 17α-estradiol, testosterone or dihydrotestosterone treatment caused an increase in the coupled enzyme activities involved in this response. The active estrogens 17β-estradiol, estriol and diethylstilbestrol were all effective. These studies with different steroids indicate that increases in [^{3}H]UTP incorporation into the 3′-CCA of tRNA are specific for biologically active estrogens. The significant effect by the short-acting estrogen, estriol but not estrone, which must be converted to 17β-estradiol to be active, suggests that the estrogen effect must occur by a transient early action of active estrogens to lead to later increases in this response at 12 h.

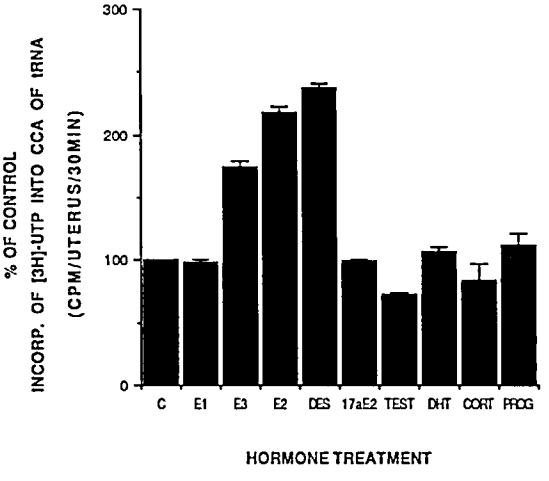

Fig. 7. Hormonal specificity of [^{3}H]UTP incorporation into the 3′-CCA terminus of tRNA. Animals (five per group) were treated with E_2 (E2) corticosterone (CORT), progesterone (PROG), 5α-dihydrotestosterone (DHT), estrone (E1), estriol (E3), 17α-estradiol (17a E2), testosterone (TEST), diethylestilbestrol (DES) or vehicle alone 12 h prior to death as a single intravenous dose (5 μg) in saline ethanol, except progesterone, which was administered as a dose of 2 mg in corn oil. Uteri from each group were pooled and enzyme activity was determined as previously described in Materials and Methods. Data are presented as percent of control (C) ± S.E. of triplicate assays.

TABLE II

EFFECTS OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON [^{3}H]UTP INCORPORATION INTO THE CCA TERMINUS

For each experiment, groups of five rats were treated with E_2 (5 μg/animal) and the inhibitors as illustrated prior to death. Uteri were pooled from each group, and the incorporation of [^{3}H]UTP into trichloroacetic acid precipitates was determined as described in Materials and Methods. Values represent the means (with the indicated S.E.) for three separate experiments.

Treatment	[^{3}H]UTP incorporation into CCA 12 h after estradiol (cpm/0.15 uterus/30 min)
Control	9982 ± 56
Estrogen (E_2)	16240 ± 227
Cycloheximide	8002 ± 34
Cycloheximide 15 min prior to E_2	8153 ± 100
E_2 4 h, then cycloheximide	6092 ± 124
E_2 8 h, then cycloheximide	13280 ± 66
Actinomycin D	7574 ± 93
Actinomycin D + E_2	14311 ± 243

Effects of actinomycin D and cycloheximide on [^{3}H]UTP incorporation into CCA of tRNA

As an initial approach to evaluating the mechanism by which E_2 increases uterine incorporation of [^{3}H]UTP into the 3′-CCA of tRNA, we tested the effects of cycloheximide and actinomycin D on the induction process. For these studies, animals were treated with actinomycin D (50 μg/rat) 15 min prior to E_2 treatment, and animals were killed 12 h after hormone treatment. Actinomycin D did not inhibit the E_2-induced increase in UTP incorporation into the 3′-CCA terminus of tRNA (Table II) ($P \leq 0.01$), suggesting that an E_2-induced increase in RNA synthesis was not necessary for the response induced by E_2. The dose of 50 μg of actinomycin D/rat inhibits over 90% of RNA synthesis and this suggests that E_2-induced mRNA synthesis for CTP synthetase is probably not necessary for the E_2-regulated response. Cycloheximide (400 μg/rat) completely abolished the increase in activity if administered 15 min prior to E_2 or 4 h after E_2. If given 8 h after E_2, cycloheximide was without effect. This suggests that the critical time period for protein synthesis is between 4 and 8 h after E_2 stimulation to induce activity of the enzyme CTP synthetase. Whether

the increase is due to increased synthesis of CTP synthetase or a protein which activates pre-existing CTP synthetase remains to be determined.

Discussion

The possibilities for regulating protein synthesis by adding or removing the 3′-terminal AMP and CMP residues from selected tRNA species suggest that this mechanism may serve an important control function in protein synthesis in the rat uterus. Translational level control of protein synthesis has been observed in several hormonally stimulated tissues [9–11,15,16,38–48]. One protein that is synthesized at a greater rate in the uterus by E_2 is the enzyme glucose-6-phosphate dehydrogenase (G6PDH) [46]. Twelve hours after E_2 administration to the ovariectomized mature rat, the overall G6PDH synthesis rate is elevated 18-fold and this can be accounted for by a 7-fold increase in translatable G6PDH mRNA levels and a 2.5-fold increase in the rate of transit of uterine ribosomes for the G6PDH mRNA [46,48]. An immunoreactive M_r 42000 nascent chain of G6PDH accumulates on uterine ribosomes and E_2 appears to specifically increase the rate of elongation of the G6PDH molecule beyond the size of this nascent chain [47]. Recent sequencing data for human G6PDH reveal that the region of the molecule just downstream from the point of reduced rate of translation which causes pile-up of the peptide on ribosomes is rich in proline and glycine [49]. We have observed that the amounts of proline and glycine acceptor activities in uterine tRNA have very low basal levels and are increased more than 4-fold during the interval of time that G6PDH translation rates are enhanced while the increase for most other amino acids averages 2.6-fold. Previous results indicate that only certain species of tRNAs in the E_2-deprived rat uterus are selectively increased by the actions of E_2 [1]. The E_2-induced increase in tRNA acceptor activity was found to be due to the E_2-regulated increase in the proportion of pre-existing and newly synthesized tRNAs that contain an intact 3′-CCA amino acid acceptor terminus. Since nucleotidyltransferase is not tRNA-specific we consider that the specificity of the E_2 effect is most likely related to selectivity in the relative rate of loss of the 3′-CCA terminus from selected tRNAs in the E_2-deprived uterus. We have observed the presence of an E_2-regulated (inactivated by E_2) ribosome-associated tRNA inactivating factor which inhibits the aminoacylation of newly deacylated uterine tRNAs before they are released from the ribosome [41,55]. This factor inactivates those tRNAs on ribosomes which have been recently deacylated during protein synthesis and the deacylated tRNAs then lose their 3′-CCA terminus by being susceptible to endogenous 3′-exonucleases. From those studies we propose that tRNA specificity in the E_2-induced activation of inactive tRNAs may reflect the frequency of mRNA codon usage in the tissue which occurs in the absence of E_2. This tentative model would ultimately result in selective reduction in the rate of translation of those mRNAs which dominate the E_2-deprived tissue resulting in gradual loss of translational activity. Another system which appears to be similarly regulated is the dormant unfertilized sea urchin egg, in which no significant metabolic activity of RNA occurs; however, after fertilization, protein synthesis commences [12]. The first event to occur with respect to the RNA components is addition of the 3′-CCA terminus to an inactive pool of tRNAs that have incomplete 3′-CCA termini. In addition, their results indicated that total amounts of tRNA, mRNA and rRNA did not increase at this time after fertilization suggesting that the single initial triggering event for activation of protein synthesis was addition of the 3′-CCA amino acid terminus to tRNA. Others have indicated that in dry lupin seeds defective and nonacylated tRNA molecules are present [8]. However, upon germination tRNAs are repaired and able to accept their respective amino acids. Their results indicated that this repair was not due to alterations in activities of nucleotidyltransferase or aminoacyl tRNA synthetases but rather to increased ATP pool sizes after germination. These observations clearly suggest that regulated synthesis and/or degradation of the 3′-CCA terminus of selected tRNAs may be an important part of regulation of peptide elongation rates in a variety of systems including the ovariectomized rat uterus as it transforms from a dormant to an actively growing tissue following estrogen stimulation.

Our studies suggest that nucleotidyltransferase activity, measured in uterine extracts with [^{3}H]ATP or [^{3}H]CTP and yeast tRNA as substrates, does not itself increase when rats have been stimulated with E_2. These results are in agreement with those of others [50,51], in which activities of aminoacyl-tRNA synthetases in *Rana pipiens* and RNA polymerases in *Xenopus laevis* increased dramatically during growth and differentiation, whereas nucleotidyltransferase did not. Deutscher et al. [52] also have shown that in *E. coli* mutants, nucleotidyltransferase activity levels only 15% of wild type are sufficient to support normal growth. They suggest that nucleotidyltransferase is simply a scavenger of incomplete tRNA molecules (both partially degraded and partially synthesized tRNA) and is present in large excess in cells, ready to repair defective tRNA when substrate is available. We therefore anticipated that control of the turnover process would be at the level of nucleotide removal. A 3′-exoribonuclease specific for the single-stranded 3′-CCA acceptor terminus of tRNA has been demonstrated in several other systems [3,5,53,54]. These studies indicated that this enzyme initiated attack at the free 3′-terminus of deacylated tRNAs. The presence of an amino acid attached to the terminal adenosine protects tRNAs against 3′-exoribonuclease degradation. Out studies revealed no apparent differences in 3′-exoribonuclease activity in uterine fractions for the substrates tRNA-C-C-[^{3}H]A or tRNA-[^{3}H]C in control animals versus E_2-stimulated rats suggesting that changes in 3′-exoribonuclease activity do not account for E_2-regulated turnover of the CCA sequence.

Since neither the synthetic nor the degradative enzyme is altered in amount, these results suggested that either substrate levels of CTP or ATP regulate the rate of synthesis of the 3′-CCA terminus or that the relative amount of time that the tRNA remains devoid of an amino acid immediately following its use for protein synthesis may regulate the rate of degradation of tRNAs. In another study, we have observed the presence of a protein on the ribosomes of E_2-deprived uteri which reacts with newly deacylated tRNAs to render them 'temporarily' unable to bind to their cognate aminoacyl-tRNA synthetase, thus leading to a theoretical increase in the amount of time tRNAs remain deacylated under in vivo conditions [55]. This ribosome-associated factor appears to be responsible for placement of tRNAs into an inactive pool that is deficient in their 3′-CCA termini, but it does not account for the E_2-regulated recruitment of tRNAs from the inactive pool.

The levels and/or synthesis of CTP in the cytosol of uterine cells appears to be the rate-limiting factor in synthesis of the 3′-CCA terminus of tRNAs. Mammalian cells have two distinctive pathways to maintain their pyrimidine nucleotide pools: a de novo pathway and a salvage pathway that utilizes preformed nucleosides or bases [56]. The conversion of UMP, UDP, and UTP to cytosine nucleosides constitute reactions in the de novo biosynthesis of pyrimidines. The enzyme CTP synthetase catalyzes the conversion of UTP to CTP in the presence of L-glutamine, ATP, Mg^{2+}, and a sulfhydryl-containing compound [57]. Others have demonstrated that CTP synthetase is the rate-limiting enzyme in hepatic CTP biosynthesis [58]. We have provided evidence in this study that E_2 simultaneously induces CTP synthetase activity and CTP pools. These results agree with other studies in which CTP synthetase activity increased in rapidly growing hepatomas [58] and studies in which E_2 induced changes in pool sizes of acid-soluble nucleotides in ovariectomized mice, immature female rats and ovariectomized rabbit vagina [32,33,59,60]. Our results suggest that the rate-limiting substrate is CTP, since the levels of CTP in the rat uterus are approximately 90 μM (assuming a water volume of 0.1 ml per uterus) which is well below the apparent K_m for CTP for rat liver nucleotidyltransferase, which is reported to be 0.18 mM [28]. We have observed apparent K_m values for rat uterine nucleotidyltransferase for CTP to be approximately 0.50 mM in both control and E_2-treated rats and as indicated in Fig. 6, the rate of the nucleotidyltransferase reaction is substrate-dependent over the range of 0.01–0.75 mM which encompasses the range seen in uterus from control and E_2-treated rats. ATP levels are also increased in response to E_2, and the levels in the uterus are below the K_m for this substrate by nucleotidyltransferase. However, the ordered addition of CMP to tRNA prior to AMP makes ATP a less likely candidate as a rate-limiting substrate.

In summary, our data suggest that turnover of

the 3′-CCA terminus of tRNAs in ovariectomized mature rat uterus is not correlated to changes in levels of either nucleotidyltransferase or 3′-exoribonuclease, but instead is regulated by substrate, particularly CTP levels and its net effect on nucleotidyltransferase activity. E_2 increases the rate of conversion of UTP into CTP in uterine cytosol suggesting that the E_2-regulated event is modulation of the enzyme, CTP synthetase. Experiments are currently being carried out in our laboratory to determine the mechanism of E_2 action on the enzyme, CTP synthetase, and to further substantiate whether CTP synthesis is the rate-limiting step in the E_2-induced repair of the 3′-CCA terminus of select tRNAs.

Acknowledgements

We thank Robin Shaw, Deborah Alberts, and Melissa Neill for technical assistance. This work was supported by research (HD 16236) and training (HD 07271) grants from the National Institutes of Health.

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