insertions discussed previously, may contribute significantly to the very weak binding of oxidized glutathione to trypanothione reductase, a feature that may provide a basis for subsequent structural investigations.

With the *T. congolense* trypanothione reductase structural gene cloned and sequenced, efforts to express and overproduce the enzyme in *E. coli* are under way. Large quantities of the enzyme will be necessary for both mechanistic studies and the testing of inhibitors that may select between parasite trypanothione reductase and host glutathione reductase. Furthermore, since preliminary crystals of the *T. cruzi* trypanothione reductase have been reported recently (Krauth-Siegel et al., 1987) but enzyme availability was severely limited, use of the *T. congolense* gene as a probe to facilitate *T. cruzi* gene cloning, sequencing, and enzyme overproduction is a current strategy in these laboratories.

Registry No. Trypanothione reductase, 102210-35-5; DNA (trypanothione reductase gene), 114691-51-9; trypanothione reductase (protein moiety reduced), 114718-82-0; glutathione reductase, 9001-48-3.

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Estradiol-Stimulated Nuclear Ribonucleoprotein Transport in the Rat Uterus: A Molecular Basis†

Raghava Varman Thampan

School of Life Sciences, University of Hyderabad, Hyderabad 500 134, India

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ABSTRACT: The present investigation probes the intranuclear molecular changes that serve to link the nuclear binding of estradiol with the hormone-stimulated ribonucleoprotein (RNP) transport in the rat uterus. Within 2 min of in vitro exposure of isolated uterine nuclei to 10 nM 17β-estradiol a Mg^{2+}-dependent nuclear ATPase becomes activated and reaches its peak activity. This is immediately followed by a phase of ATP resynthesis. This newly synthesized ATP serves as the substrate for the nuclear protein kinases. Cyclic AMP inhibits this ATP resynthesis and, as a consequence, prevents the estradiol-stimulated nuclear protein kinase activity and the exit of the RNP–estradiol complex from the nuclei. cGMP is stimulatory to the estradiol-mediated nuclear ribonucleoprotein transport.

17β-Estradiol (E_2) has a direct stimulatory influence upon ribonucleoprotein (RNP) transport in the rabbit and rat uteri (Vazquez-Nin et al., 1978, 1979; Thampan, 1985). Exposure of uterine nuclei either in vivo or in vitro to physiological concentrations of estradiol results in hormone binding to nuclear RNP and an immediate release of the hormone–RNP complex from the nuclei. It has been found that under in vivo conditions hormone withdrawal from the system results in retention of the RNP within the nuclei. The hormone binding to the nuclear RNP brings about the release of the RNP from the nuclei. The estradiol–RNP complexes thus released are

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found in association with the cytoplasmic polysomes (Thampan, 1985).

The present study attempts to discover the intermediate molecular reactions that take place within the nuclei between estradiol binding and RNP release. The first molecular reaction that was investigated in this context was the nuclear Mg^{2+}-activated ATPase since there is overwhelming evidence for its involvement in nuclear ribonucleoprotein transport (Schumm et al., 1973; Smuckler & Koplitz, 1974; Schumm & Webb, 1974, 1978; Agutter et al., 1976, 1977, 1979; Agutter & McCaldin, 1978; Clawson & Smuckler, 1978; Clawson et al., 1978, 1980, 1984).

Materials and Methods

17β-[2,4,6,7-^{3}H]Estradiol, specific activity 90 Ci/mmol, [2,8-^{3}H]adenosine 5′-triphosphate, specific activity 40 Ci/mmol, and adenosine 5′-[γ-^{32}P]triphosphate, specific activity 3000 Ci/mmol, were obtained from Amersham. The unlabeled hormones, the nucleotides, heparin, metrizamide, quercetin, oligomycin, ouabain, and the salts and buffers used in these studies were purchased from Sigma. Poly(ethylene imine) (PEI)–cellulose was a product of Brinkmann. Oligo(dT)–cellulose was purchased from Collaborative Research.

Isolation of Uterine Nuclei. Adult female rats of the Holtzman strain were ovariectomized and used in the studies 2 weeks after the surgery. Uterine nuclei were isolated as described earlier (Thampan, 1985). The purified nuclei were suspended in TMKC–sucrose buffer [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, containing 4 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$, and 250 mM sucrose] at a concentration of 250–300 μg DNA/mL. The nuclear preparation had a DNA:protein ratio of 0.084.

Assay for Nuclear RNP Release in Response to Exposure to Estradiol. The method employed is essentially the same as reported earlier (Thampan, 1985) except that for the sake of clarity a few additional steps have been introduced. The experimental nuclei were incubated with 10 nM 17β-[^{3}H]-estradiol ± 100 × unlabeled estradiol at 37 °C for varying intervals. At the end of the incubation the nuclear suspension (in 50 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 20 mM KCl, 4 mM $MgCl_2$, and 1 mM $CaCl_2$) was layered over 0.34 M sucrose buffer (the above-mentioned buffer containing 0.34 M sucrose) and centrifuged at 800g (4 °C) for 10 min. The pellet was extracted with ethanol and the radioactivity in the ethanol extract measured. The control nuclei were first subjected to the time-dependent incubation at 37 °C in the absence of estradiol. The incubated nuclei were resuspended in the 0.25 M sucrose buffer and exposed to 10 nM [^{3}H]estradiol ± 100 × unlabeled estradiol at 37 °C for 3 min before the nuclei were processed for the measurement of radioactivity.

In order to further confirm the assumption that estradiol binding in vitro to nuclei stimulates RNP release from the nuclei, additional steps were introduced into the experiment mentioned above. Unlabeled estradiol (10 nM) was added to the media containing the control nuclei as the nuclei were subjected to the time-dependent incubation. These nuclei were reexposed to 10 nM [^{3}H]estradiol ± 100 × unlabeled estradiol, as before, and the labeled hormone bound to the nuclei was estimated.

Following centrifugation and removal of the nuclei, the incubated medium (supernatant) was recovered and its KCl concentration adjusted to 250 mM. This was mixed with oligo(dT)–cellulose and left undisturbed at 4 °C for 2 h. The oligo(dT)–cellulose pellet, recovered after centrifugation, was extracted with 50 nM Tris-HCl, pH 7.5, containing 250 mM KCl at 4 °C; the extract was discarded. The pellet was reextracted with the same buffer containing no salt (zero-salt buffer) at 37 °C. The supernatant, which contained RNP carrying heterogeneous nuclear RNA, was analyzed for radioactivity.

Nuclear ATPase Assay. The nuclei, suspended in TMKC–sucrose buffer, were incubated either with 4 mM unlabeled ATP or with a mixture of 4 mM unlabeled ATP and 4 μCi of [^{3}H]ATP in the presence or absence of 10 nM estradiol. The total volume was 100 μL. Following incubation at 37 °C for different intervals, the reaction in the media that contained [^{3}H]ATP was terminated by the addition of carrier nucleotides (2 mM each), ATP, ADP, and AMP. The macromolecules were precipitated with 5% trichloroacetic acid (TCA). Five microliters of the acid-soluble fraction was spotted on pre-washed PEI–cellulose sheets, and the chromatograms were developed in a mixture of 1 M HCOOH and 0.5 M LiCl (Randerarth & Randerarth, 1967). The carrier nucleotides were detected under a shortwave UV lamp and the gel areas corresponding to the location of the nucleotides removed for the measurement of radioactivity.

The reaction in the media that contained unlabeled ATP was terminated by cooling the tubes in an ice bath and the subsequent addition of TCA to 5% concentration. The P_i released in the acid-soluble fraction was measured as described by Fiske and Subbarow (1925).

Nuclear Protein Kinase Assay. The nuclei, suspended in TMKC–sucrose buffer, were incubated with 4 mM ATP and 12 μCi of [γ-^{32}P]ATP in either the presence or absence of 10 nM E_2 in a total volume of 100 μL at 37 °C. The cyclic nucleotides adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate were added according to the experimental specifications. The reaction was terminated by cooling the tubes in an ice bath and acidification of the media with 5% TCA containing 4 mM sodium pyrophosphate. The macromolecular precipitate was dissolved in 0.5 M NaOH and reprecipitated with TCA. Following extensive washing with 5% TCA, alcohol, and ether, the precipitate was finally dissolved in NCS tissue solubilizer (New England Nuclear) for the measurement of radioactivity.

Metrizamide Density Gradient Centrifugation of the ^{32}P-Labeled RNP. The method described by Alberga et al. (1979) was followed. Metrizamide was dissolved in TMKC buffer (50 mM Tris-HCl, pH 7.5, containing 4 mM $MgCl_2$, 20 mM KCl, and 1 mM $CaCl_2$) without sucrose. Gradients were prepared with 750-μL layers of 20, 30, 40, 50, and 60% metrizamide, which were then equilibrated at 4 °C for 24 h. The macromolecular fractions (750 μL) were layered over the gradients, which were then centrifuged at 47 000 rpm for 4 h (4 °C) by using a Beckman SW 50.1 rotor. The fractions collected from the tubes punctured at the bottom were mixed with carrier bovine serum albumin (50 μg/tube) and acidified by the addition of 5% TCA containing 4 mM sodium pyrophosphate. The precipitate was processed for the measurement of radioactivity as described in the previous section.

Hydroxylapatite (HAP) Assay for Estradiol Binding. The procedure outlined by Peck and Clark (1977) was followed. A 60% suspension of Bio-Rad HTP in 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (250 μL), was added to the incubation medium (350 μL) at the end of the incubation of the macromolecules with labeled estradiol ± 100 × unlabeled estradiol. The suspensions were left in an ice bath for 30 min. The tubes were subjected to centrifugation, and the pellet was washed thrice with the Tris-EDTA buffer. The washed pellets were extracted with ethanol (1

mL/tube), and the radioactivity present in the ethanol extract was measured.

Miscellaneous. Protein was assayed as described by Bradford (1975) and DNA according to the method of Burton (1956). Measurement of radioactivity was carried out by use of a scintillation mixture: 5 g of 2,5-diphenyloxazole (PPO) and 500 mg of 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP)/L of toluene–Triton X-100 (67:33 v/v).

RESULTS

Estradiol-Bound Ribonucleoprotein Release from Uterine Nuclei. The release of ribonucleoproteins from the uterine nuclei upon exposure to and binding of 17β-estradiol is shown in Figure 1A. The binding of estradiol to the nuclei and to the macromolecules released into the medium was studied during the course of an incubation that lasted 8 min. There was no apparent change in the binding activity of the control nuclei that were incubated in the absence of estradiol prior to their exposure to labeled estradiol. The estradiol-binding activity associated with the nuclei exposed to labeled estradiol during the time-dependent incubation reached the maximum within 3 min. The activity registered a steep decline in the succeeding 3 min and was undetectable after 8 min of incubation. The estradiol-binding activity in the medium, at the same time, showed a progressive time-dependent increase, as the hormone-binding activity associated with the nuclei declined. The fact that the macromolecule in the medium which bound estradiol was a ribonucleoprotein containing hnRNA was indicated by the fact that this activity remained bound to the oligo(dT)–cellulose and was extracted from the matrix by zero-salt buffer at 37 °C.

The incubation of the control nuclei was further extended; this time the nuclei were exposed to 10 nM unlabeled estradiol before the incubation with 10 nM labeled estradiol. This again resulted in a time-dependent decline in the hormone-binding activity associated with the nuclei and its appearance in the medium. This was an added confirmation to the earlier observation that exposure of isolated uterine nuclei to estradiol resulted in the release of hormone-bound RNP from the nuclei.

Estradiol Stimulates Nuclear Mg^{2+}-ATPase Activity. The nuclei (about 250–300 μg of protein/100 μL) were incubated at 37 °C with 4 mM Mg^{2+}-ATP either in the presence or absence of 10 nM unlabeled estradiol. Trichloroacetic acid was added, at intervals, to the media in order to terminate the reaction, and the P_i released in the acid-soluble fraction was measured. In the presence of estradiol the Mg^{2+}ATPase reached its peak activity within 2 min of incubation at 37 °C. Within the next minute the activity registered a steep decline, and practically no ATPase activity was displayed by the nuclei incubated for 4 min with the hormone (Figure 1B). The ATPase activity appeared within 2 min of exposure of the nuclei to the hormone, irrespective of whether the hormone was added to the medium at the start of incubation or 8 min after the start of the incubation. The 8-min addition of estradiol was carried out in order to make an effective comparison with the hormone-binding study carried out (Figure 1A). Presence of an ATP-regenerating system (4 μmol of creatine phosphate and 40 μg/mL creatine phosphokinase) did not make any significant difference in the characteristics of the reaction (data not shown). The 2-min peak in ATPase activity failed to appear in the estradiol-free reaction media. In order to find out whether this 3-min decline was due to the exhaustion of the substrate in the medium, a parallel study was carried out. Half the original (protein) concentration of the incubated nuclei was introduced into the reaction media and the incubation continued. A similar, but proportionally

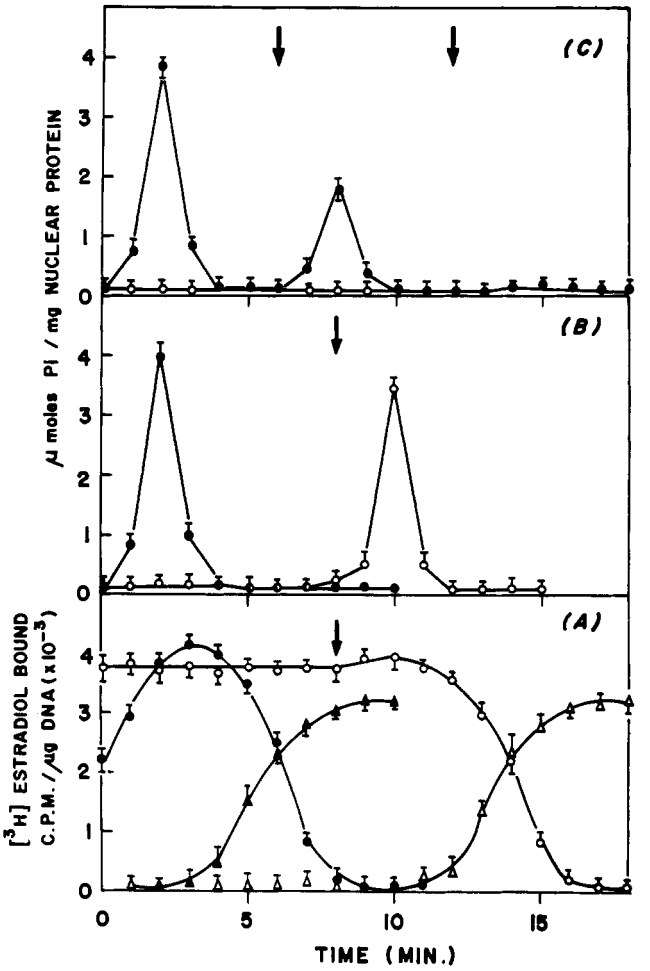

FIGURE 1: (A) Release of estradiol–ribonucleoprotein complex from the nuclei upon exposure of the nuclei in vitro to [^{3}H]estradiol. The nuclei, suspended in 250 mM sucrose buffer, were incubated with 10 nM [^{3}H]estradiol ± 100 × unlabeled estradiol at 37 °C for different intervals. At the end of the incubation, the sample was layered over 340 mM sucrose buffer and centrifuged at 800*g* for 10 min. The pellet (●) was extracted with ethanol and the radioactivity in the ethanol extract measured. The medium (supernatant) from the above experiment (▲) was mixed with oligo(dT)–cellulose after the KCl concentration of the medium was adjusted to 250 mM. Following centrifugation, the oligo(dT)–cellulose pellet was extracted once with 250 mM KCl buffer at 4 °C. The extract was discarded. The pellet was subsequently extracted with zero-salt buffer at 37 °C. The radioactivity present in the extract was measured. The control values represent the activity associated with the nuclei (○) and the medium (△) in the in vitro setup where initial time-dependent incubation was carried out in the absence of estradiol. Subsequent exposure to [^{3}H]estradiol was carried out at 37 °C for 4 min. The arrow indicates the time at which 10 nM unlabeled estradiol was added to the control media during the course of the time-dependent incubation. Subsequent to this, the labeled hormone bound to the nuclei (○) and to the RNP in the media (△) was measured. (B, C) Estradiol-stimulated Mg^{2+}ATPase activity in the uterine nuclei. Incubation of the nuclei in either the presence or absence of estradiol was carried out at 37 °C. At regular intervals the reaction was terminated following the addition of trichloroacetic acid into the media. The P_i released in the acid-soluble fraction was estimated. (B) Effect of addition of estradiol to the media on the nuclear ATPase activity: (○) control; (●) 10 nM estradiol. The arrow indicates the time of addition of 10 nM estradiol into the control media. The subsequent effect of this addition is displayed by the peak of enhanced ATPase activity. (C) Decline in ATPase is not due to the exhaustion of the substrate within the system. The first arrow shows the time at which half the original concentration of the nuclei was added to the estradiol-containing media. The second arrow indicates the time of addition of additional 4 mM ATP into the incubation media. The open circles (○) indicate the control data where incubation was carried out in the absence of estradiol. The data are the mean ± SE of six independent determinations.

Table I: Characterization of Estradiol-Stimulated Nuclear ATPase[a]

(a) Effect of Nuclear Protein Concentration (5)[b]

nuclear protein (mg/medium)	activity +E_2	activity −E_2
0.10	1.31 ± 0.08	ND
0.21	2.47 ± 0.18	ND
0.31	3.78 ± 0.21	ND
0.42	3.88 ± 0.20	ND
0.52	3.88 ± 0.20	ND

(b) Effect of Substrate Concentration (6)[b]

ATP (mM)	E_2 (2 min)	E_2 (3 min)
0.55	0.85 ± 0.02	0.20 ± 0.006
1.03	2.10 ● 0.15	0.51 ± 0.01
1.82+	3.33 ± 0.20	0.59 ± 0.01
2.22	3.89 ± 0.20	0.84 ± 0.02
3.01	3.88 ± 0.21	0.85 ± 0.02

(c) Effect of Estradiol Concentration (6)[b]

estradiol (nM)	activity	estradiol (nM)	activity
1.0	0.10 ± 0.02	6.0	2.31 ± 0.19
2.0	0.20 ± 0.006	8.0	3.88 ± 0.22
4.0	0.60 ± 0.02	10.0	3.89 ± 0.25
5.0	0.80 ± 0.02		

(d) Effect of Hormonal Agents (5)[b]

hormonal agent (10^{-6} M)	% activity with ref to 10 nM 17β-estradiol
17β-estradiol	100
diethylstilbestrol	52.0 ± 1.0
17α-estradiol	10.1 ± 1.5
5α-dihydrotestosterone	8.9 ± 1.0
testosterone	8.2 ± 0.5
progesterone	6.8 ± 0.7
cortisol	7.8 ± 0.2

(e) Effect of ATPase Inhibitors (4)[b]

inhibitor	concn	% activity
none		100
ouabain	1 mM	99.0 ± 1.1
ouabain	2 mM	97.5 ± 1.2
oligomycin	4 μg/mL	92.0 ± 2.1
oligomycin	8 μg/mL	72.0 ± 2.0
quercetin	2 μg/mL	80.0 ± 2.0
quercetin	4 μg/mL	60.1 ± 3.1
quercetin	12 μg/mL	10.1 ± 1.0
quercetin	20 μg/mL	ND

(f) Effect of Bivalent Cations (4)[b]

bivalent cation (4 mM)	% activity	bivalent cation (4 mM)	% activity
$MgCl_2$	4.1 ± 0.2	$CoCl_2$	1.9 ± 0.1
$MgSO_4$	4.2 ± 0.3	$ZnSO_4$	0.4 ± 0.01
$MnCl_2$	2.7 ± 0.2	$BeCl_2$	ND
$MnSO_4$	2.6 ± 0.2	$LiCl_2$	ND
$CaCl_2$	2.9 ± 0.3		

[a] ATPase activity is expressed as μmol of P_i/mg of nuclear protein. The ATP concentration in all except (b) was 4 mM, and estradiol, when added [except in (c)], had a concentration of 10 nM. The nuclear protein concentration in all except (a) ranged between 250 and 300 μg/medium. [b] ND = not detectable. The data represent the mean ± SE of between four and six independent determinations as indicated in parentheses.

smaller, peak of ATPase activity appeared within the succeeding 2 min, only to decline to the zero level as was the case before (Figure 1C). This clearly indicated that the 3-min decline in ATPase activity was not due to the exhaustion of the substrate in the reaction media. In order to reconfirm this assumption, fresh ATP (4 mM) was added to the medium 7 min after the introduction of additional uterine nuclei into the media. There was no further increase in ATPase activity detected.

Characterization of the Estradiol-Stimulated ATPase. The results of the various studies carried out are given in Table I. The ATPase activity was dependent upon the concentration of nuclear protein in the reaction medium. Irrespective of the amount of nuclear protein added, the ATPase activity failed to appear in the hormone-free systems. The effect of substrate concentration on the estradiol-stimulated ATPase was studied under two reaction conditions. The nuclei were exposed to estradiol either for 2 min or for 3 min. The maximal activity continued to appear in the nuclei exposed to the hormone for 2 min. The Mg^{2+}ATPase was also influenced by the concentration of estradiol in the medium. The activity increased in a sigmoidal fashion until the hormone concentration in the medium reached 8 nM. The activity plateaued upon further increase in the hormone concentration. An absolute correlation between the nuclear binding of the hormone and the stimulation of ATPase activity was noticed in this as well as in the hormone specificity study that followed. The enzyme activity stimulated by 1 μM diethylstilbestrol was only 50% that stimulated by 10 nM estradiol. The other hormones, which included testosterone, dihydrotestosterone, progesterone, cortisol, and 17α-estradiol, failed to stimulate the nuclear ATPase activity, a result that perfectly fell in line with the hormone-binding studies reported earlier (Thampan, 1985). The activity displayed by the nuclei in the presence of 1 μM estradiol was no different from that detected in the presence of 10 nM estradiol.

The effects of various ATPase inhibitors and bivalent cations on the estradiol-stimulated ATPase activity were studied. Of the three inhibitors examined, only quercetin was able to effect a total inhibition in this hormone-dependent phenomenon. Quercetin, at a concentration of 10 μg/mL, brought about 90% inhibition in the nuclear ATPase activity. The most ideally suited bivalent cation in this reaction was Mg^{2+}.

ATP Resynthesis and Stimulation of Nuclear Protein Kinases. The estradiol-stimulated nuclear ATPase was subjected to additional scrutiny, this time by using [^{3}H]ATP as the substrate for the enzyme. The [^{3}H]ADP formed was measured following thin-layer chromatographic separation of the nucleotides extracted from the media. The data presented in Figure 2A show a [^{3}H]ADP peak 2 min after exposure of the nuclei to estradiol. The reason for the rapid decline in P_i and [^{3}H]ADP from the media thus appeared to be the ATP resynthesis. This phase of ATP resynthesis was also short-lived. The ATP levels declined, followed by an equivalent increase in the formation of ADP. It was evident from these data that this secondary increase in the formation of ADP was not due to any ATPase activity since, under these conditions, there was no corresponding increase in the formation of P_i.

Additional experiments were carried out to examine whether this secondary increase in [^{3}H]ADP formation was due to stimulation of a nuclear protein kinase activity. The nuclei were incubated with 4 mM ATP and [γ-^{32}P]ATP either in the presence or in the absence of 10 nM estradiol, and the nuclear incorporation of ^{32}P was observed. There was a very clear enhancement in nuclear protein kinase activity in the presence of estradiol (Figure 2B). This phase of increase in protein kinase activity followed a pattern identical with the one displayed by the secondary increase in the formation of [^{3}H]ADP. Introduction of an ATP-regenerating system into the reaction medium (4 μmol of creatine phosphate and 40 μg/mL creatine phosphokinase) failed to make any significant change in the reaction characteristics of either the ATPase or the protein kinase (Figure 2).

Cyclic Nucleotide Involvement in the Estradiol-Stimulated Nuclear Protein Kinase Activity. Studies were carried out to see whether the estradiol-stimulated nuclear protein kinase

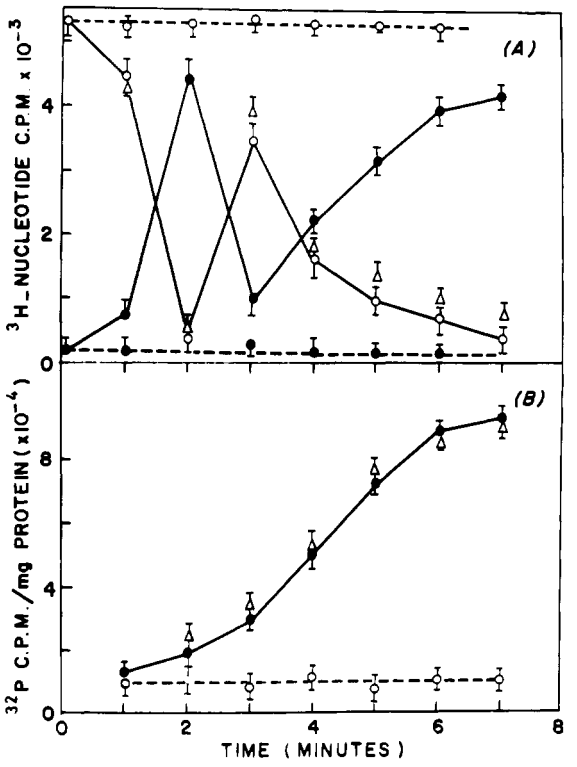

FIGURE 2: (A) Estradiol-stimulated ATPase activity. The uterine nuclei were incubated with 10 nM estradiol, 4 mM Mg^{2+}ATP, and 4 μCi of [^{3}H]ATP for varying intervals. The acid-soluble fractions from the incubated media were subjected to thin-layer chromatography on PEI–cellulose. The radioactivity associated with the carrier ADP (●) and ATP (○) spots was measured. The broken lines indicate the activity associated with the control nuclei, where the incubation was carried out in the absence of estradiol. The open triangles (△) indicate the ATPase activity associated with the nuclei in the media supplemented with an ATP-regenerating system (4 μmol of creatine phosphate and 40 μg/mL creatine phosphokinase. (B) Nuclear protein kinase activity stimulated by estradiol. The nuclei were incubated with 4 mM Mg^{2+}ATP and 12 μCi of [γ-^{32}P]ATP in either the presence (●) or absence (○) of 10 nM estradiol. The ^{32}P incorporated into the nuclei was measured. The open triangles (△) show the protein kinase activity associated with the incubation media supplemented with an ATP-regenerating system. The data are the mean ± SE of four independent determinations.

activity was cyclic nucleotide dependent. cAMP- and cGMP-dependent protein kinase activities were assayed both in the presence of estradiol and in its absence. The results (Figure 3) show that full manifestation of the cGMP-dependent enzyme activity required exposure of the nuclei to estradiol; it was virtually inactive in the absence of the hormone. On the other hand, cAMP inhibited the protein kinase activity that was under the stimulatory influence of estradiol. There was an increase in the nuclear cAMP-dependent protein kinase activity in the presence of estradiol over the activity in the control nuclei. This increase was, however, statistically insignificant. It was evident that the nuclear protein kinase activity stimulated by cAMP was different from that stimulated by estradiol.

Effect of P_i on the Nuclear Protein Kinases. It was of interest to see if any intermediate stimulatory factor existed in the estradiol-mediated enhancement in nuclear protein kinase activity. The P_i appeared a logical factor in this regard since there was a large-scale liberation of P_i within the nuclei as a result of the hormonal stimulation of nuclear ATPase activity. ADP had no effect, either stimulatory or inhibitory, upon the nuclear protein kinases (data not shown). The uterine nuclei were incubated in the protein kinase assay media containing varying concentrations of P_i, either in the presence of

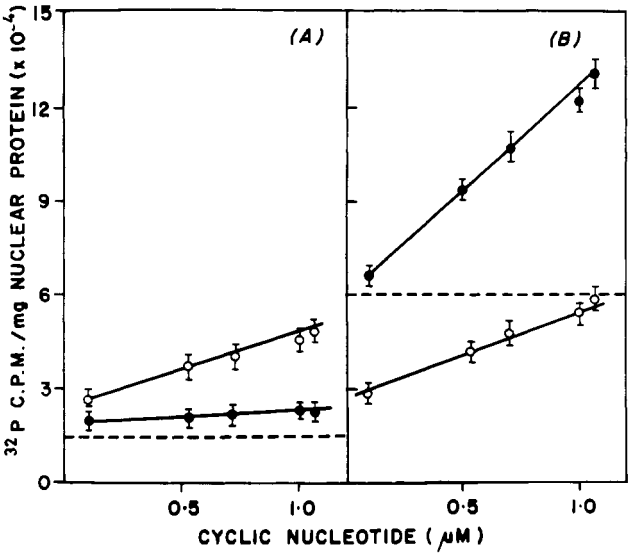

FIGURE 3: Effect of cyclic nucleotides on nuclear protein kinase activity. The horizontal broken line shows the average activity associated with the nuclei incubated either in the presence (B) or in the absence (A) of 10 nM estradiol in the absence of exogenous cyclic nucleotides. The cyclic AMP (○) and cyclic GMP (●) dependent activities were measured both in the presence (B) and in the absence (A) of 10 nM estradiol. The nuclei were incubated with [γ-^{32}P]ATP (± estradiol) at 37 °C for 10 min. The data are the mean ± SE of four independent determinations.

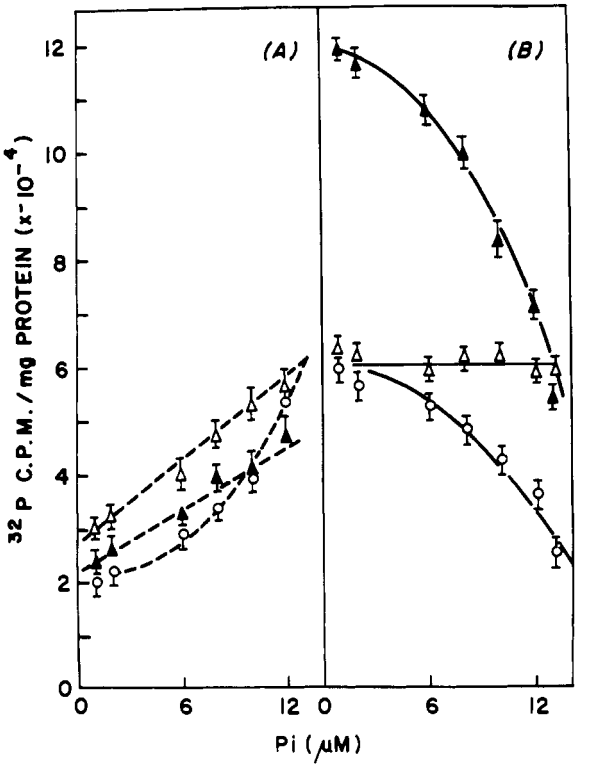

FIGURE 4: Effect of exogenous P_i on the nuclear protein kinase activity: (A) P_i-influenced nuclear protein kinase activity in the absence of estradiol; (B) activity in the presence of 10 nM estradiol. The control in each case (○) refers to the nuclear protein kinase activity measured in the absence of exogenous cyclic nucleotides. The closed triangles (▲) indicate cGMP-dependent activity, and the open triangles (△) indicate cAMP-dependent activity. The data are the mean ± SE of six independent determinations. It may be pointed out that the apparent inhibition in the protein kinase activity that is under estradiol influence is due to the dilution of the internal ^{32}P pool by the externally added P_i.

estradiol or in its absence. P_i showed a distinct dose-dependent stimulatory influence upon the nuclear protein kinases in the

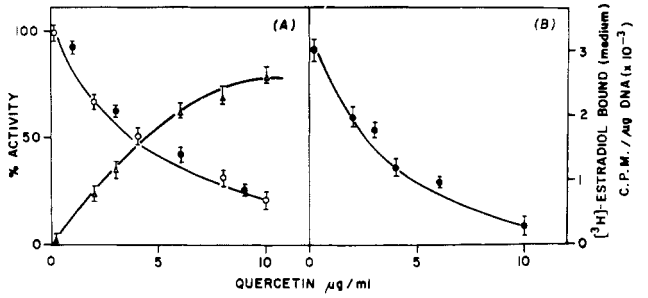

FIGURE 5: (A) Effect of quercetin on the retention, within the nuclei, of estradiol–RNP complex (△), estradiol-stimulated nuclear Mg^{2+}-ATPase (●), and estradiol-stimulated nuclear cGMP-dependent protein kinase (○). For the assay of ATPase the incubation was carried out for a period of 2 min, and for the assays of protein kinase and estradiol binding the total length of the incubation time was 8 min. (B) Assay of estradiol-binding activity associated with the oligo-(dT)-bound RNP in the media where the nuclei were incubated with varying concentrations of quercetin. The data show that quercetin inhibits the release of estradiol-bound RNP from the nuclei.

absence of estradiol (Figure 4A). The effect was more pronounced in the case of the cAMP-dependent activity than in the case of the cGMP-dependent activity. The P_i-enhanced activity in the absence of estradiol and added cyclic nucleotides was essentially the same as that observed in the presence of cGMP in the estradiol-free medium.

Addition of estradiol to the P_i-containing media brought about a totally different pattern in the activities of the nuclear protein kinases (Figure 4B). Externally added P_i was found to decrease the incorporation of ^{32}P into nuclei incubated with estradiol both in the presence of added cGMP and in its absence. The cAMP-dependent activity showed little change under these conditions.

A closer examination of these data indicated that the "inhibitory effect" exerted by external P_i on the estradiol-stimulated nuclear protein kinase was only illusory. The external P_i diluted the ^{32}P pool formed after the hydrolysis of [γ-^{32}P]ATP by the ATPase. The resynthesized ATP functioned as the substrate for the protein kinase. As the concentration of the externally added P_i increased, the specific activity of the internal ^{32}P pool reduced in a proportionate manner. This reduced specific activity of the resynthesized [γ-^{32}P]ATP was then reflected in the protein kinase activity.

Possible Interrelationship between Estradiol-Stimulated Nuclear Enzymatic Changes and the RNP Release. Quercetin, by virtue of its inhibitory effect upon the estradiol-stimulated nuclear ATPase, was chosen as the molecular tool to probe into the biochemical basis of the hormone-mediated RNP transport. This chemical inhibited all three activities that were stimulated by estradiol: Mg^{2+}ATPase, cGMP-dependent protein kinase, and eventually the RNP release from the nuclei (Figure 5). Along with a decline in the nuclear enzymatic activities, there was proportional increase in the retention of the hormone-binding activity within the nuclei when the incubation was carried out in the presence of quercetin. Quercetin, in the absence of estradiol, did not have any influence upon the nuclear protein kinases, irrespective of whether the activity was dependent upon cAMP or cGMP (data not shown). Also, quercetin did not inhibit estradiol binding to nuclear RNP. The data presented in Figure 5B show that the increase in estradiol-binding activity found associated with the nuclei, in the presence of quercetin, was not due to any stabilization of the estradiol-binding activity by the inhibitor. Instead, it was due to the total suppression of the release of the hormone-bound RNP from the nuclei, as there is a progressive decrease in the estradiol-binding

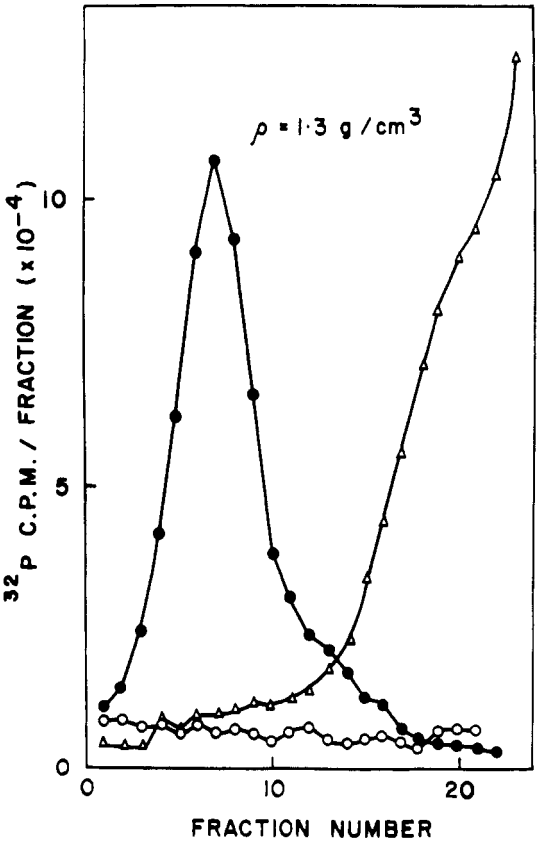

FIGURE 6: Metrizamide density gradient analysis of the phosphorylated RNP released from the nuclei upon exposure to 10 nM estradiol, 4 mM Mg^{2+}ATP, and 12 μCi of [γ-^{32}P]ATP. After centrifugation, the fractions collected were mixed with carrier bovine serum albumin (50 μg/tube) and acidified with TCA. The acid-insoluble fraction was processed as described under Materials and Methods (●). Part of the RNP sample (10 A_{260} units) was exposed to ribonucleases A and T_2 (5 μg and 5 units, respectively) at 30 °C for 30 min before it was subjected to metrizamide density gradient centrifugation (△). The open circle (○) shows the activity in the control media that did not contain estradiol.

activity of the media in response to the presence of increasing concentrations of quercetin.

RNPs Released from the Nuclei upon Exposure to Estradiol Are Phosphorylated. The uterine nuclei were incubated with 10 nM estradiol, 4 mM Mg^{2+}ATP, and 12 μCi of [γ-^{32}P]ATP for 10 min at 37 °C and centrifuged at 800g (4 °C) for 10 min. The supernatant was concentrated by Lyphogel treatment and subjected to metrizamide density gradient centrifugation. A distinct peak of activity at a density of 1.3 g/cm^3 was observed; it disappeared upon exposure of the postincubation supernatant to ribonucleases A and T_2 (5 μg and 5 units, respectively) prior to the centrifugation (Figure 6).

cAMP Inhibits ATP Resynthesis and Exit of the Estradiol–RNP Complex from the Nuclei. The virtual absence of any noticable change in the nuclear cAMP-dependent protein kinase activity in response to the altered P_i concentrations in the estradiol-containing media (see Figure 4B) indicated that the externally added P_i was not being incorporated into the nuclei during the formation of ATP. It appeared that cAMP inhibited the resynthesis of ATP after the initial ATP hydrolysis. In order to verify this assumption, the studies presented in Figures 1A (nuclear binding of estradiol) and 2A (ATPase) were repeated using incubation media containing either 1 μM cAMP or 1 μM cGMP. In the presence of estradiol, cGMP enhanced the ATP resynthesis as well as the release of RNP–estradiol complex from the nuclei. On the

Table II: Effect of Cyclic Nucleotides on the Estradiol-Stimulated Ribonucleoprotein Transport and the ATP Resynthesis[a]

	[^{3}H]estradiol bound to the nuclei at the end of a 10-min incubation (10^{-3} cpm)	[^{3}H]ATP resynthesis obsd after 3-min incubation following initial ATP hydrolysis (10^{-3} cpm)
control (no estradiol)	3.5 ± 0.1	*b*
estradiol (10 nM)	0.1 ● 0.0	3.2 ± 0.15
estradiol (10 nM) + cAMP (1 μM)	3.2 ± 0.1	0.8 ± 0.05
estradiol (10 nM) + cGMP (1 μM)	0.1 ± 0.01	4.2 ± 0.16

[a] The experimental designs are the same as given in Figures 1A and 6A. The data are the mean ± SE of six independent determinations. [b] Not applicable since no ATP hydrolysis was detected in the absence of estradiol.

other hand, cAMP inhibited the ATP resynthesis and blocked the release of the RNP–estradiol complex (Table II).

Discussion

Whether the nuclear ATPase stimulated by estradiol is the ATPase associated with the nuclear membrane and the pore complexes, already recognized for its role in the nucleocytoplasmic RNA transport, cannot be ascertained at this stage. Also, the basic mechanism by which estradiol activates the nuclear ATPase is not understood. From the data available it is possible to derive a correlation between the hormone-stimulated ATPase and the nuclear protein kinase activity enhanced by the exposure of the nuclei to estradiol. The stimulatory signal for the protein kinase may have come from the P_i liberated as a result of the ATP hydrolysis. In the absence of confirmatory reports from other laboratories on the occurrence of P_i-activated protein kinases, this conclusion can only be approached with extreme caution. Nevertheless, the experimental evidence presented in this report indicate that the nuclei do contain a protein kinase activity that is stimulated upon exposure to P_i, even in the absence of exogenous cyclic nucleotides. Additional experimentation is required in order to arrive at a conclusion regarding the specificity of this P_i-mediated reaction.

The immediate resynthesis of ATP after the initial ATP hydrolysis is probably a reflection of the increased requirement for the substrate by the activated protein kinases. This observation of ATP resynthesis also requires a cautious consideration. The experimental results provided by Smuckler and colleagues (Smuckler & Koplitz, 1974; Clawson & Smuckler, 1978; Clawson et al., 1978, 1980, 1984) bear some resemblance to the data presented here. Clawson and Smuckler (1978) demonstrated that at higher concentrations ADP stimulated RNA transport to the same extent as ATP. They found that when labeled ADP was added to the incubation medium, a significant proportion of it reappeared as ATP, which was a clear indication of the ADP phosphorylation. This dramatic redistribution took place during the initial 2.5 min of incubation when >17% of the labeled nucleotide appeared as ATP. This observation almost paralleled the experimental result presented in this paper (see Figure 2). Smuckler and colleagues envisage that the mechanism observed may be the result of an exchange reaction between two ADP molecules:

$$ADP + ADP \rightleftharpoons ATP + AMP$$

There are two major points in which the estradiol-influenced system differs from the one reported by Smuckler: (1) The ATP hydrolysis, as a result of which liberation of ADP and P_i occurred, declined to undetectable levels within 4 min of incubation of the nuclei in the presence of estradiol. The pattern of decline of P_i was identical with that of ADP. Smuckler, on the other hand, observed a constant rate of ATP hydrolysis between 2.5 and 20 min in addition to the rapid resynthesis of ATP that occurred within 2.5 min of incubation. Therefore, it appears reasonable here to speculate that the ATP resynthesis observed in the presence of estradiol utilized both ADP and P_i liberated as a result of the ATP hydrolysis. Had it been the result of an ADP–ADP exchange, the P_i level within the medium should have remained unchanged while the ADP titer declined. (2) AMP is one of the products of the ADP–ADP exchange reaction. No labeled AMP was detected in the present experiment where [^{3}H]ATP was used as the substrate.

Schumm and Webb (1978) showed that both cAMP and cGMP stimulated RNP transport from isolated nuclei in a reconstituted in vitro system. The present study also shows the presence of a cAMP-dependent protein kinase activity in the isolated uterine nuclei. The emphasis lies in that this activity is distinct from that of the protein kinase which is under the stimulatory influence of estradiol. In addition, in the presence of cAMP, this estradiol-dependent activity remains suppressed. There is already a large body of evidence that shows an inverse relationship which exists between the biological actions of estradiol and those of cAMP (Hanschin et al., 1983; Kapoor et al., 1983). The estradiol antagonistic action of cAMP in this specific instance may also come from the inhibition of the estradiol-dependent ATP resynthesis by the cyclic nucleotide and the resultant depletion of the substrate pool required to be utilized by the activated protein kinases. The interrelationship between this phosphorylation and the RNP release, however, remains an enigma.

The major emphasis in our present-day understanding of steroid hormone action is on the regulation of the transcriptional machinery of the target cell by the hormone–receptor complex. What lies ahead for the receptor–hormone complex after its stimulation of transcription is a topic that has not received the attention that it deserves. Additional experiments that shall involve utilization of antireceptor (estradiol) IgG should prove whether the hormone binding component associated with the uterine nuclear RNP is a modified form of the nuclear estrogen receptor. If that is the case, the observations presented here should form a basis for the exploration of the mechanisms involved in the posttranscriptional regulation of gene expression by steroid hormones.

Acknowledgments

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Registry No. ATPase, 9000-83-3; ATP, 56-65-5; cAMP, 60-92-4; cGMP, 7665-99-8; estradiol, 50-28-2; protein kinase, 9026-43-1; diethylstilbestrol, 56-53-1.

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Astacus Protease, a Zinc Metalloenzyme†

Walter Stöcker, Russell L. Wolz, and Robert Zwilling*
Department of Physiology, Institute of Zoology, University of Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, FRG
Daniel J. Strydom and David S. Auld
Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, 250 Longwood Avenue, Boston, Massachusetts 02115
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ABSTRACT: *Astacus* protease, an endoprotease of molecular weight 22 614, from the freshwater crayfish *Astacus fluviatilis* contains 0.97 ± 0.03 mol of zinc/mol of protein, as measured by atomic absorption spectroscopy. The metal chelating agents ethylenediaminetetraacetate (EDTA), 1,10-phenanthroline, dipicolinic acid, 8-hydroxyquinoline-5-sulfonic acid, and 2,2′-bipyridyl inactivate the enzyme reversibly in a time-dependent manner. Inactivation by 1,10-phenanthroline occurs within a few minutes whereas EDTA requires days. The inactivation data are consistent with a proposed model in which a transient ternary enzyme–metal–chelator complex is formed that subsequently dissociates to yield apoenzyme plus a chelator–metal complex. The half-life for metal dissociation in the absence of chelator is estimated to be 40 days, much slower than for carboxypeptidase A (28.3 min) or angiotensin converting enzyme (92.4 s) though much faster than carbonic anhydrase (5.4 years). Dialysis against 1,10-phenanthroline results in inactive apoenzyme which can be reactivated by the addition of stoichiometric amounts of zinc, copper, or cobalt to 100%, 70%, or 50% of native activity, respectively, indicating that the metal is required for catalysis. Cobalt-*Astacus* protease exhibits an absorption spectrum with a maximum at 514 nm (ϵ_{514} = 76.5 M^{-1} cm^{-1}) and shoulders at 505 and 550 nm, indicative of a distorted tetrahedral-like geometry about the cobalt ion. This spectrum is similar to that seen for metalloneutral proteases such as thermolysin. On the basis of similarities of sequences for thermolysin about residues 142–148 (HELTHAV) and residues 92–98 for *Astacus* protease (HELMHAI), histidyl residues 92 and 96 may be ligands to the zinc, and Glu-93 may play a role in catalysis.

Proteolytic enzymes have become the focus of much attention because of their importance in many diverse physiological systems such as complement activation, hormone production, blood coagulation, and digestion (Neurath & Walsh, 1976). The freshwater crayfish *Astacus fluviatilis*, an invertebrate species that represents a taxonomic point distant from both microorganisms and chordates, has been useful in examining the evolutionary aspects of proteases (Zwilling & Neurath, 1981).

As was first described by Pfleiderer et al. (1967), the stomachlike cardia of this crayfish contains an endoprotease, *Astacus* protease, which has been considered unique by virtue of its cleavage specificity. It use as an aid in the structural analysis of tubulin revealed that *Astacus* protease prefers a

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* Address correspondence to this author.

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