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IN VITRO STIMULATION OF NUCLEOLAR AND NUCLEOPLASMIC RNA POLYMERASES IN CALF UTERUS

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SUMMARY

Two types of RNA polymerases have been isolated from calf uterus. One of them, Nucleolar RNA polymerase, is stimulated by the 5 S oestradiol fraction. The other, Nucleoplasmic RNA polymerase, is not stimulated by the same fraction. We examined the variation in activity of purified nucleoplasmic RNA polymerase as a function of the level of phosphorylation of the acidic nuclear proteins in reconstituted chromatin from calf thymus DNA. The activity of the Nucleoplasmic RNA polymerase varies in the same way as the level of the phosphorylation of the acidic proteins used to reconstitute the chromatin. Yet neither *in vivo* nor *in vitro* was oestradiol found to increase cyclic AMP level in the uterus. Moreover, IP biosynthesis induced by oestradiol is blocked by Actinomycin D and Cordycepin. The part played by IP in the transcription process is discussed.

INTRODUCTION

It is well established that estrogen-dependent tissues contain specific oestradiol-binding macromolecules, estrogen receptors, with which the hormone interacts through a stepwise mechanism. First, oestradiol associates with the cytoplasmic 8 S (7·7 S) unit, then the hormone is transferred to the nucleus where it is recovered linked to a 5 S protein species. It has been demonstrated that this process is temperature dependent. *In vivo*, this translocation induces the biological effect [1].

In our first experiments we showed that exposure to the oestradiol–receptor complex increases RNA biosynthesis in purified uterine nuclei. This stimulation is effected only by complex in which the binding unit of the receptor has undergone oestrogen-induced conversion from the native 7·7 S to the transformed 5 S form [2]. We suggested that an important function of oestrogenic hormone is to promote transformation of the 7·7 S receptor protein, to an active form, 5 S, which can enter the nucleus and stimulate RNA synthesis. This process depends on physiological conditions i.e. temperature, 37°C, and molarity, 0·1 M KCl [2, 3].

For the first event of oestradiol action in the uterus we presented the following scheme:

$$7\text{·}7\ \text{S} \xrightarrow[37°\text{C/0·1 M KCl}]{\text{Oestradiol}} \begin{cases} 7\text{·}7\ \text{S OE} \\ \downarrow + \\ 5\ \text{S OE} \xrightarrow{\text{Nucleus}} \text{RNA biosynthesis} \end{cases}$$

[2, 3]

These conditions were precisely determined by an original analytical ultracentrifugation method: the sedimentation coefficients of labelled proteins present in low concentrations in a mixture of non-labelled macromolecules were obtained by analytical centrifugation, using a mechanical separation cell. Correction factors for hydrostatic pressure and cell sectorial shape were detailed [4]. This "Radioactivity" method was tested with bovine serum albumin and showed a good agreement with the usual method. The sedimentation coefficients of estrogen binding proteins in calf endometrium were determined by this method. The results were 7·8 ± 0·1 S and 5·1 ± 0·1 S for the inactive and active oestradiol receptors, respectively. When traces of two labelled proteins were present in the mixture, their relative percentages were determined by an extension of this method. Using this method to study the transformation of cytoplasmic receptor which occurs at KCl concentrations close to the intracellular concentration, the following results were obtained: at

Table 1.

KCl molarity (mm)	Speed (rev/min)	Time (min)	% 7·7 S	% 5·1 S
30	45,000	50	100	
60	45,000	50	100	
90	55,000	50	15	85
120	55,000	50		100
180	55,000	50		100

0°C or 37°C and 0·03 M KCl only the 7·7 S OE form was observed. At 37° and 0·09 M KCl 85% had been transformed to the 5 S OE form [3, 4]. The results are summarized in Table 1.

Subsequently, RNA polymerases were extracted and the effect of the oestradiol receptor on RNA biosynthesis was examined. In these preliminary studies two different extraction techniques were used [3, 5]:

(a) The first technique lyses the nuclear membrane and gives the "Total RNA polymerase", a mixture of nucleolar and nucleoplasmic RNA polymerases.

(b) The second technique does not lyse the nuclear membrane and gives nucleoplasmic RNA polymerase, the "Soluble RNA polymerase". 5 S oestradiol receptor was found to be effective in increasing RNA biosynthesis only by "total RNA polymerase" with calf thymus DNA [3, 5]. It was then necessary to clarify these results with more selective RNA polymerase preparations. At this time, the 5 S oestradiol receptor was thought to be playing a role similar to that of σ factor [3, 5].

Two types of enzymes showing RNA polymerase activity were prepared simultaneously from calf uteri. One of them, nucleoplasmic RNA polymerase (previously called "soluble RNA polymerase"), was prepared from purified nuclei by a now classical process: ultracentrifugation of nuclei in a medium of high sucrose molarity. The preparation and properties of this enzyme will be discussed in the last part of this paper [8]. The other enzyme "nucleolar RNA polymerase", was recovered from nucleoli which had been obtained by sonication of isolated nuclei. More recently the enzyme was prepared according to the process described by Zalta in determining precisely the $MgCl_2$ molarity convenient for the best preservation of the nucleolus [17]. The preparations were observed after inclusion by electron microscopy. The extracted enzyme was tightly bound to the gene, was inhibited by actinomycin D, was not inhibited by α-amanitin, was stimulated by Mn^{2+} and Mg^{2+} and was strongly inhibited by KCl (a dramatic drop of activity is observed at >0·1 M KCl). Nucleolar RNA polymerase was stimulated by the cytoplasmic 5 S oestradiol receptor[6, 7]. Tables 2 and 3 show that the level of RNA biosynthesis is higher in nuclei, nucleoli and nucleolar RNA polymerase fractions extracted from tissues incubated with oestradiol than from tissues incubated without oestradiol [6, 7].

When endometrial tissues were incubated with [^{3}H]-oestradiol, part of the radioactivity measured in the nuclei was recovered in the nucleoli from which a radioactive RNA polymerase was extracted; its enzymatic activity was greatly enhanced compared to the enzyme prepared from tissue not treated with the hormone. By chromatography on phosphocellulose, the [^{3}H]-oestradiol complex accounted for all the radioactivity in the fraction excluded by the column: this fraction had no measurable polymerase activity. By increasing the $(NH_4)_2SO_4$ gradient, RNA polymerase activity was eluted in a rather narrow peak which was not radioactive; the enzymatic activity of this fraction was increased when mixed with the fraction excluded from the column or with 5 S oestradiol complex. The sedimentation constant of the protein entity bound to oestradiol was found to be 5 S, (measured by the "radioactive" UCFA method). A 5 S radioactive protein could also be liberated by KCl from nucleoli prepared from tissue incubated with [^{3}H]-oestradiol [6, 7].

These results place one of the effects of oestradiol at the level of the nucleolus. This biochemical evidence confirms the early observation made in 1966 by Irina Pollard who demonstrated by electron microscopy that oestradiol effected a profound change in the aspect of the nucleolus in the vaginal epithelium of the ovariectomized mouse [9]. Since these preliminary observations, other results obtained with oestradiol and testosterone show their rapid effect on nucleolar RNA biosynthesis [10].

In what concerns the phosphorylation of 5 S oestradiol complex, the question is still controversial. The results were not sufficient to confirm or deny the

Table 2. Effect of exogenous DNA on RNA biosynthesis measured in nuclei, nucleoli and nucleolar RNA polymerase fractions

Assays		counts/min	
		Without exogenous DNA	With exogenous DNA
Nuclei	1	1465	1716
	2	1732	2288
Nucleoli	1	1356	1499
	2	1587	1846
E	1	123	793
	2	244	1595

1 Extraction from tissue incubated without estradiol.
2 Extraction from tissue incubated with estradiol.

Table 3. Enzymatic activity of nucleolar RNA polymerase fractions

Assays	E_1	E_2
1	455	750
2	1371	1576
3	1650	1919
4	793	1595

E_1 Fraction extracted from tissue incubated without oestradiol.

E_2 Fraction extracted from tissue incubated with oestradiol.

preliminary results obtained using a crude fraction of the 5 S oestradiol receptor. Nevertheless, a rapid phosphorylation process which is induced by oestradiol was shown to be independent of cyclic AMP, contrary to what was previously found [11]. The hypothesis that cyclic AMP plays an essential role in mediating the biological action of oestradiol on the uterus was tested by determining the tissue concentration of the cyclic nucleotide after injection of this steroid into ovariectomized and adrenalectomized rats or after *in vitro* experiments. In neither system was oestradiol found to enhance the concentration of cyclic AMP in the uterine tissue. Using the same systems, it was observed that isoproterenol stimulates adenyl cyclase activity. Like other authors [12–14], we were not able to reproduce the results described in the literature which led us to a wrong interpretation [11].

The following sections of this paper will be devoted to a more detailed study of calf uterus nucleoplasmic RNA polymerase upon which the stimulating effect of the oestradiol complex was not observed although a dependence on this hormone surely exists [8]. The question was to know whether the hormone could control nucleoplasmic RNA polymerase activity through a modification of chromatin template activity, the nuclear acidic proteins appearing to determine the specificity of genetic information. It has been suggested that an indirect hormonal dependence might exist through the phosphorylation of nuclear acidic proteins [8, 16]. Recent works have shown that nuclear acidic proteins interact with DNA to alter transcription; this regulation is tissue-specific while the negative control by histones is not [15, 16].

The relationship between purified nucleoplasmic RNA polymerase activity and the level of phosphorylation of acidic proteins which are present in chromatin reconstituted from calf thymus DNA was studied [8].

MATERIAL AND METHODS

Calf uteri were collected from the slaughter house immediately after death and kept at 4°C until used and while the endometrium was taken.

Solutions and buffers

T_1: 0·1 M Tris–HCl (pH 8·4), 8·6 M urea, 0·01 M EDTA, 0·14 M β mercaptoethanol.

T_2: 0·01 M Tris–HCl (pH 8·0), 5 M urea.

T_3: 0·01 M Tris–HCl (pH 8·0), 0·01 M NaCl.

T_4: 0·1 M Tris–HCl (pH 8·4), 0·01 M EDTA, 0·14 M β mercaptoethanol.

T_5: Cold redistilled phenol saturated in T_4.

T_6: 250 mM sucrose, 3 mM $CaCl_2$, 0·5% (w/v) tween 80.

T_7: 10 mM tris–HCl (pH 7·4).

T_8: $(NH_4)_2SO_4$ saturated at 4°C 20 mM Tris–HCl (pH 7·5).

T_9: 50 mM tris–HCl (pH 7·9), 5 mM $MgCl_2$, 0·1 mM EDTA, 0·5 mM β mercaptoethanol, 25% glycerol.

T_{10}: 4 mM EDTA, 5 mM β mercaptoethanol (pH 7·0).

T_{11}: 1 N acetic acid.

T_{12}: 0·3 M KH_2PO_4, 0·3 M K_2HPO_4, 0·06 M EDTA (Na_2).

T_{13}: 5 mM tris–HCl, 1 mM EDTA, 5 mM β mercaptoethanol (pH 7·5).

T_{14}: 45 mM NaH_2PO_4, Na_2HPO_4 (pH 6·0).

T_{15}: 50 mM tris–HCl (pH 7·4), 0·03 M KCl, 3 mM $MgCl_2$.

T_{16}: 0·1 M tris–HCl (pH 8·4), 10 mM EDTA, 0·14 M β mercaptoethanol.

T_{17}: 0·1 M acetic acid, 0·14 M β mercaptoethanol.

T_{18}: 0·05 M acetic acid, 9 M urea, 0·14 M β mercaptoethanol.

T_{19}: 1·8 M sucrose, 1 mM $MgCl_2$.

T_{20}: 320 mM sucrose, 1 mM $MgCl_2$, 20 mM tris–HCl (pH 7·4).

Preparation of nuclei from endometrium

About 60 g of endrometrial tissue were ground in T_6 (1/2 w/v) with a "Virtis 23" homogenizer (3 min, high power). The homogenate was filtered through one, two and three layers of gauze. The filtrate was centrifuged 10 min at 800 *g* (Sorvall RC2B centrifuge, SS 34 rotor). The pellet was resuspended in the same buffer, then centrifuged 10 min at 800 *g*. This operation was repeated twice. The supernatant was discarded; the nuclear pellet was resuspended in washing buffer T_7 and centriguged 10 min at 800 *g*. The washings were repeated until the nuclei were freed of cytoplasmic contamination.

Extraction of nuclear acidic proteins

A first class of proteins was separated by 0·14 M NaCl. The nuclei were treated for 5 min by 30 vol of 0·14 M NaCl, the nuclei were then centrifuged for 10 min at 2000 *g*. This extraction was repeated 3 times.

Basic proteins were extracted by resuspending the nuclei for 10 min in 0·25 M HCl. Three such extractions were effected on the pellet recovered by 10 min centrifugation at 2000 *g*.

The lipid fractions were removed by washing three times with chloroform/methanol.

The residue was finally resuspended in 5 volumes of T_{16} and the suspension shaken for 12 h at 4°C with an equal volume of freshly distilled phenol saturated in T_{16}. The aqueous layer was recovered after 10 min. centrifugation at 800 *g* and extracted once more for 5 h with diluted phenol. The collected phenolic layers were dialysed against 200 vol. of T_{17}.

When the phenolic layer was reduced to 1/20, the dialysis tube was opened, the aqueous layer discarded, and the phenolic layer dialysed for 24 h against 200 vol. of T_{18}, then 2 h against 200 vol. of T_1. At the end of this process, the aqueous layer, which contains the acidic proteins, was dialysed to remove the urea (12 h at 4° against T_2, 2 h against T_3). The solution was clarified by 5 min. centrifugation at 800 *g*. The protein concentration was usually 7 mg/ml. The traces of remaining DNA were hydrolyzed by DNase for 10 min at 37°: 2 ml protein solution + 100 µl DNase I Sigma (500 µg/ml containing 5 mM Mg^{2+}). At the end of this incubation, the solution was added to an equal volume of phenol saturated in T_{16} which destroys DNase; the purified acidic proteins were extracted as described above [16]. Chromosomal RNA was removed by RNase: 2 ml protein solution + 400 µl pancreatic RNase (1 mg/ml containing 5 mM Mg^{2+}). After a 2 h incubation, the extraction process with diluted phenol was effected which also destroyed RNase.

Extraction and purification of a 3'5' AMP-dependent protein kinase from calf uterus

The uteri were kept at 4°C, cut into pieces and ground in T_{10} medium (2·5 l/kg of tissue). The homogenate was filtered through a nylon sieve and the filtrate centrifuged 30 min. at 10,000 *g*; the pH of the supernatant was adjusted to 5·5 by addition of T_{11}. The precipitate was removed by a 30 min centrifugation at 10,000 *g*. $(NH_4)_2SO_4$ (325 gl of supernatant) was slowly added to the neutralized supernatant (addition of 120 ml T_{12} per l. of supernatant). The precipitate was recovered by 40 min centrifugation at 10,000 *g* and dissolved in T_{13} (0·15 l./kg of uterus). The solution was dialyzed for 20 h against 3 × 50 vol. of T_{13}, then centrifuged 1 h at 80,000 *g*. 50 ml of supernatant were filtered through DEAE cellulose (25 cm × 1·5 cm) equilibrated with T_{13}. A first elution was effected with the same buffer until the absorbancy had fallen (about 200 ml), then a second elution was effected with 650 ml of a linear NaCl concentration gradient (0–0·3 M). The highest protein kinase activities were found at NaCl concentrations between 0·14 and 0·22 M (vol. 180 ml). These collected fractions were concentrated to 15 ml by ultrafiltration (Amicon cell, PM 10 Diaflo membrane, 3 bars nitrogen pressure). The 3'5' AMP-dependent and non-dependent protein kinase activities were separated by filtration on Sephadex G 200 (90 cm × 5 cm) in T_3. The 3'5' AMP-dependent fractions (150 ml following the 650th ml) were reduced to 20 ml by ultrafiltration. The protein concentration of this preparation was 5 to 6 mg/ml and its enzymatic activity was enhanced 8–12 fold by 10^{-6} M 3'5' AMP.

Ox spleen phosphoprotein phosphatase

The method of preparation was that described by N. R. Revel. The enzyme had no proteolytic activity [18].

Acidic protein phosphorylation

The solutions of acidic proteins were incubated 30 min at 37°C with 2 vol of a phosphorylation medium containing 18 mM KF, 0·9 mM EDTA, 1·3 mM ATP, 4·5 mM $Mg(AcO)_2$, 1·8 mM Theophylline, 90 mM β Na glycerophosphate, 0·11 mM 3'5' AMP, 7·5 mM γ^{32}P–ATP (224 mCi/mmol), 13 mg/ml protein kinase. The phosphorylated acidic proteins were then extracted with T_5.

Dephosphorylation of phosphorylated acidic proteins

Two volumes of phosphorylated acidic proteins were incubated at 37° with 1 vol. of phosphoprotein phosphatase (50 mM tris–HCl pH 7·4, 400 mM KCl, 3 mM $MgCl_2$, 1·5 mg/ml phosphatase). The incubation lasted from 1 to 30 min. The dephosphorylation was stopped by addition of an equal volume of T_5 which destroys phosphatase and extracts the acidic proteins. Phosphoserine and phosphothreonine were obtained and identified upon hydrolysis of ^{32}P phosphorylated acidic proteins.

Chromatin reconstitution

After dialysis against T_1, the acidic protein solutions were dialyzed for 12 h against T_2 + 2 M NaCl at room temperature. A solution of calf thymus DNA was prepared in this same buffer (4 mg/ml). Chromatin was reconstituted by mixing equal volumes of DNA solution and acidic protein solutions (with different levels of phosphorylation). The salt concentration was lowered by several dialysis at 4°C: 2 h against T_2 + 1 M NaCl, T_2 + 0·8 M NaCl, T_2 + 0·6 M NaCl, 12 h against T_2 + 0·4 M NaCl. The urea was removed by 2 h dialysis against T_3.

Extraction and purification of nucleoplasmic RNA polymerase

About 80 g of calf endometrium were ground in 120 ml of T_{19}, and the homogenate successively filtered through 1, 2 and 3 layers of gauze. Samples of filtrate (4 × 25 ml) were layered on T_6 (4 × 10 ml) and centrifuged 70 min. at 48,000 *g*. The nuclear pellets were resuspended in 40 ml of freshly prepared T_{20}. The suspension was kept at 0°C for 30 min and centrifuged 20 min at 800 *g*, then 20 min at 40,000 *g*. The addition of an equal volume of T_8 to the supernatant gave rise to a protein precipitate (10 min at 0°C) which was recovered by 10 min. centifugation at 40,000 *g*, dissolved in 3–4 ml of T_9 and dialyzed for 1 h against this buffer. The enzyme was purified by chromatography on a Whatman DE 52 column (1·5 cm × 15 cm equilibrated in buffer T_9, 3 ml fractions). The elution was effected with T_9 until the absorbancy had fallen, then with 200 ml of a linear $(NH_4)_2SO_4$ concentration gradient (0–0·6 M). Nucleoplasmic RNA Polymerase was eluted in the range of 0·35–0·40 M $(NH_4)_2SO_4$. The protein concentration was about 0·4 mg/ml.

Nucleoplasmic RNA polymerase activity

Nucleoplasmic RNA polymerase activity was measured by the incorporation of [^{3}H]-UMP into RNA as previously described [3]. However, the template was not calf thymus DNA but reconstituted chromatin (100 μg/ml), the proteins of which were more or less phosphorylated.

Effect of cordycepin on uterine oestradiol-induced protein

Experiments were carried out on 19–20 day-old immature rats weighing 40–45 g. The animals (3 for each group) were first injected an hour before being killed with 0·2 ml physiological serum, actinomycin D or cordycepine; they were then injected 30 min later with physiological serum or oestradiol.

The animals were killed by cervical dislocation and the uteri dissected and freed from fat tissue.

Each group of uteri was incubated in 1 ml of Eagle's medium containing either 100 μl of [^{14}C]-leucine (50 mCi/mmol specific activity, 0·2 mCi/ml concentration) or 50 μl of [^{2}H]-leucine (2 Ci/mmol specific activity, 1 mCi/ml concentration). (For details see Table 7.)

At the end of the incubations which lasted 1 h or 1 h 1/2 at 37°, the uteri of groups 1 and 2, 3 and 4, and 5 and 6 were pooled and washed three times with 50 ml of 0·05% EDTA-disodium salt. They were then ground with sand in 0·5 ml EDTA. The homogenates were centrifuged at 15,000 *g* for 30 min and the supernatants frozen in liquid nitrogen.

Acrylamide gels were prepared by successively mixing:

5 ml of a solution containing 30% acrylamide and 0·8% NN′ methylene bis acrylamide
5 ml of a 1·6% β dimethylamino-propionitrile solution in TBE buffer (266 mM Tris–HCl pH 8·6, 80 mM H_3BO_3, 12 mM Na_2–EDTA)
5 ml 0·03% potassium ferricyanure
5 ml 0·48% ammonium persulfate

The mixture was introduced into electrophoresis tubes (12·5 × 0·6 cm) and polymerized for 10 h at room temperature. Aliquots (100 μl) of the samples were mixed with 20 μl of 13% Ficoll containing bromophenol blue. The electrophoresis was carried out at room temperature in four-fold diluted TBE buffer (1–1·5 mA current for 1 h.; 2–3 mA current for 5 h.). At the end of the electrophoresis, the gels were frozen on dry ice and cut into 2·0 mm slices. Each slice was put into a counting vial and dissolved in 1 ml of soluene 350 for 15 h at 60°C; 10 ml "Instagel Packard" were added and the radioactivities measured in a Packard Tri-carb liquid-scintillation spectrometer.

RESULTS

Determination of cyclic AMP level in uterine tissue [14]

1. *In vivo experiments.* Adult female rats of the same age (2 or 3 months depending on experiments) were used after ovariectomy. Each group of 4 animals was treated as indicated in Table 4. Determination of the level of 3′5′ AMP extracted from uteri was carried out at least 4 times (observed differences = 10%). Oestradiol had no effect on 3′5′ AMP levels in the uteri. In the same system isoproterenal gives a 144% increase in the level of cyclic AMP.

2. *In vitro experiments.* (a) In vitro experiments were carried out on subcellular fractions from immature rat uteri. A stimulation of adenyl cyclase activity could be observed with NaF, but under the same conditions oestradiol was ineffective.

(b) Endometrial plasma membranes were isolated from uteri of immature lambs (3–4 months old) by differential centrifugations (to eliminate nuclei and mitochondria), followed by discontinuous sucrose density gradient. The membrane fractions were identified by enzymatic markers (5′ Nucleotidase, Na^+K^+ ATPase—Absence of succinate dehydrogenase and acid phosphatase) and by electron microscopy. Adenyl cyclase activity was measured under appropriate conditions of protein concentrations and reaction time. The level of synthesized 3′5′ AMP was measured by

Table 4. Level of cyclic AMP following *in vivo* treatment with oestradiol and isoproterenol

i.v. injection Exp no	Control	Oestradiol 1 μg/100 g	Isoproterenol 20 μg/100 g
1	8·8	7·5 (85% control)	
2	9·9	7·3 (74% control)	
3*	13·7	13·0 (95% control)	33·4 (244% control)

Uteri were obtained 5 or 10 min after i.v. injection of products.
Results are expressed in pmol 3'5' AMP/mg protein.
* Animals which were adrenalectomized 18 h before experiment.

Table 5. Adenyl cyclase activity in lamb membrane preparation

Basal	NaF (10^{-2} M)	Isoproterenol (10^{-5} M)	Oestradiol (10^{-4} M)	Oestradiol (10^{-6} M)
1·02*	8·3	1·60	1·05	0·99

* Specific activities in nmol of 3'5' AMP/h./mg protein.

Table 6. Phosphorylation of acidic nuclear proteins in reconstituted chromatin and nucleoplasmic RNA polymerase activity

Reconstituted chromatin	Times (min) of dephosphorylation	Residual phosphorylation ^{32}P (counts/min)	UMP Incorporation ^{3}H (counts/min)
No 1	0	1800	3900
No 2	1	1400	2600
No 3	5	850	2200
No 4	10	600	1950
No 5	30	200	1800
DNA	—	—	1800
Native chromatin	—	—	2200
Acidic proteins alone	—	—	40

stimulation of a 3'5' AMP dependent protein-kinase. The effects of NaF 10^{-2} M, isoproterenol 10^{-5} M and oestradiol 10^{-4}–10^{-6} M on adenyl cyclase activity were tested. Specific activities (nmol of 3'5' AMP/h./mg protein) in the absence and presence of effectors are indicated in Table 5. Oestradiol had no effect, but NaF gave a large increase (× 8) and isoproterenol a small increase (× 1·6) in the level of adenyl cyclase when compared with the basal level.

Role of Mn^{2+} *and* Mg^{2+} *on nucleoplasmic RNA polymerase activity*

The DE-52 purified enzyme was tested in an incubation medium containing various concentrations of Mn^{2+} and Mg^{2+}. 3 mM Mn^{2+} was found to be the optimal concentration for the nucleoplasmic polymerase activity, which was, on the contrary, not very sensitive to Mg^{2+} ion concentration.

Effects of ionic strength and NH_4^+ *ions on nucleoplasmic RNA polymerase*

Unlike the nucleolar RNA polymerase activity, nucleoplasmic RNA polymerase was not inhibited by high ionic strength. Maximal activity was attained at 250 mM $(NH_4)_2SO_4$.

Effects of K^+ *ions*

Nucleoplasmic RNA polymerase was still active when the concentration of K^+ was 500 mM. There was a maximum activity at 50 mM and 150 mM, concentrations which are consistent with the physiological medium.

Effect of α-amanitin

[^{3}H]-UMP incorporation was strongly reduced by the addition of α-amanitin (0·25 μg/ml) to the incubation mixture. The extent of inhibition was 86% within 1 min.

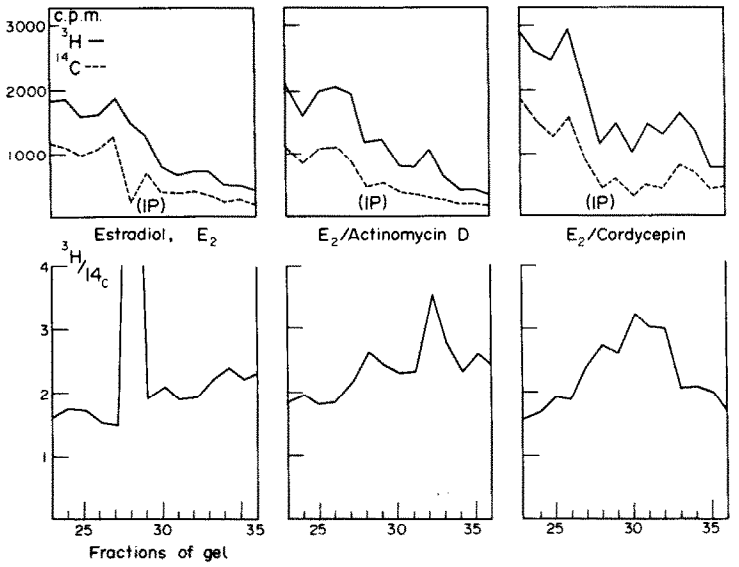

Fig. 1. Acrylamide gel electrophoresis of uterine soluble proteins synthesized *in vitro*. The upper figures show the radioactivity profiles (counts/min). The lower figures show the ratio of radioactivities.

Influence of the level of phosphorylation of acidic proteins on nucleoplasmic RNA polymerase activity

The purified solution of acidic proteins was divided into two parts:

One of them was phosphorylated, then dephosphorylated by phosphoprotein phosphatase for 1–30 min; the different levels of phosphorylation of the acidic proteins were measured by the residual ^{32}P radioactivity.

The other fraction was treated by the phosphorylation medium deprived of protein kinase; this fraction was called native acidic proteins.

The results which are summarized in Table 6 demonstrated that the nucleoplasmic RNA polymerase activity increases with the level of phosphorylation of the acidic proteins in the reconstituted chromatin. It can be noticed that the enzyme activity is the same with calf Thymus DNA and dephosphorylated chromatin (containing only 200 CPM of residual ^{32}P radioactivity). Furthermore, native acidic proteins stimulate RNA synthesis to the same extent as partially phosphorylated acidic proteins (850 counts/min of residual ^{32}P radioactivity).

Effect on uterine oestradiol-induced protein (Fig. 1 and Table 7)

(a) *Effect of oestradiol.* The results are similar to those reported by Gorski *et al.*[21] and Beaulieu *et al.*[22]: a specific protein fraction is synthesized by uteri from oestradiol-injected animals. This protein fraction is characterized by the enhancement of the $^3H/^{14}C$ ratio of the corresponding acrylamide gel slices, while this ratio is constant for all other fractions.

Under the conditions used, the migration of this induced fraction (about 5 cm fraction no. 20) was higher than that of bovine serum albumin.

Table 7. Animal treatment

No group	1st injection	2nd injection	Isotope incubation
1	NaCl*	NaCl	^{14}C
2	NaCl	Oestradiol§	^3H
3	Actinomycin D†	NaCl	^{14}C
4	Actinomycin D	Oestradiol	^3H
5	Cordycepin‡	NaCl	^{14}C
6	Cordycepin	Oestradiol	^3H

* 0·154 M.
† 2·5 mg/ml.
‡ 5 mg/ml.
§ 10^{-4} M.

(b) *Effect of oestradiol on actinomycin D-treated animals.* Our results are in agreement with previous works [21, 22]. The synthesis of oestradiol-induced protein is suppressed when the rats have been injected with Actinomycin D.

(c) *Effect of oestradiol on cordycepin-treated animals.* As actinomycin D, cordycepin blocks the induction by ostradiol of the specific protein fraction.

DISCUSSION AND CONCLUSION

We demonstrated that the enzymatic removal of the phosphate groups from the phosphoproteins abolishes their capacity to promote RNA synthesis. Moreover, phosphorylation of the acidic nuclear proteins constitutes part of the mechanism for positive control of transcription. The location of acidic protein phosphorylation (cytoplasm or nucleus) and whether or not this process is cyclic AMP-dependent are still unknown. The role played by protein kinases which are not dependent on cyclic AMP and which are so abundant in the nucleus also remains to be established. Neither *in vivo*, nor *in vitro* did oestradiol increase the cyclic AMP level in uterus.

Allfrey's studies on acidic nuclear proteins during the cell cycle in synchronized HeLa cells indicate that phosphorylation is maximal in the S phase and in the period encompassing late M and early G_1. Phosphorylation of the nuclear proteins is minimal in the late S and G_2 phases when RNA synthesis is also suppressed [19]. In our own experiments under the conditions used (synchronization by thymidine double block), the level of 3'5' AMP is minimal at the end of the G_2 phase and during mitosis; the maximum value is reached during the G_1 phase [20].

Most of the results described in the present work were obtained following *in vitro* assays. The interpretation of these results present certain problems which merit discussion.

The results show that a hormone (oestradiol) bound to a specific receptor stimulates the nucleolus of which no specific response can be expected. Generally, *in vivo*, this effect appears to be the earliest one observed, the bulk of m RNA synthesis taking place later. Our *in vitro* results show m RNA synthesis depends on the level of phosphorylation of the acidic nuclear proteins which are themselves tissue-specific. How are these events connected? It is tempting to recall Gorski's IP[21] or Bauleu's KIP[22], the synthesis of which could be the first specific event from which all the series of specific biological consequences could derive.

Yet, the synthesis of IP is, itself, a major subject for discussion. The 8 S cytoplasmic receptor for oestradiol is present in the rat uterus at birth and reaches its peak concentration (which is higher than that found in the mature animal) on the 10th day of life. However, it is well known that at this age, the animal fails to respond to oestradiol. Lindner and Kaye find that in the 10-day-old rat, the potential target cell is equipped to elaborate the nuclear receptor and that IP synthesis can be induced by oestradiol yet oestradiol fails to stimulate both general protein synthesis (a response that appears only between the 10th and 15th day after birth) and thymidine incorporation, which begins to respond to the hormone only between the 15th and 20th day of life [23]. The fact that IP synthesis can be induced by oestradiol in very immature animals could suggest the existence of pre-formed IP mRNA. However, cordycepin blockade of IP biosynthesis rules out this hypothesis: IP biosynthesis depends on Hn RNA-poly A and consequently on a new message. The well known fact that Actinomycin D blocks IP biosynthesis shows the necessity of nucleolar participation in its synthesis. The role of IP therefore, remains to be clearly established.

In conclusion two early effects of oestradiol can be observed in *in vitro* experiments: stimulation of nucleolar RNA synthesis and stimulation of IP synthesis, itself depending on the transcription of a new message. This latter event could correspond to the short and early peak observed in mRNA synthesis [24].

The interaction of the events that oestradiol triggers at puberty depends on other pre-requisite hormonal mechanisms related to the growth process occurring before puberty. Thus, further study of very young animals would be of great interest and work in this field is being pursued in this laboratory by biochemical means or E.M.

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DISCUSSION

Jensen:

In your experiments where you treat the extracted RNA polymerase with the estrogen-receptor complex and get a stimulation of the total polymerase but not of the nucleoplasmic polymerase, I understand you use calf thymus DNA as the template. Now if you were to use chromatin from uterine nuclei, do you find the same effect or perhaps a greater stimulation? I'm thinking of the report by Griffiths and co-workers, who found greater stimulation by androgen-receptor complex when their template was chromatin from the target tissue. I wondered if you had done similar experiments with estrogen.

Mousseron-Canet:

The best result we obtained is with crude nucleolar RNA polymerase which is tightly bound to the gene. This system is very close to the one to which you are just referring.