STEROID HORMONE RECEPTOR FRACTION STIMULATION OF RNA SYNTHESIS: A CAUTION

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Summary

A crude chick oviduct receptor fraction stimulated rifampicin resistant RNA synthesis in a cell-free transcription system containing oviduct chromatin. Such crude receptor fractions were found to generate increased RNA synthesis by template independent processes. In order to critically evaluate the stimulatory effects of unpurified steroid receptor on transcription, a number of criteria are proposed. 1) Receptor preparations must be free of DNA and assayed with RNA polymerase in the absence of template. 2) RNA synthesis must be sensitive to actinomycin D, deficiencies in nucleotides and produce a macromolecular RNase sensitive product. 3) The reaction should be hormone-dependent and hormone specific.

INTRODUCTION

Determination of the precise mechanism by which steroid hormones alter cellular function is a fundamental question in eucaryotic regulatory biology. A recent series of papers from our laboratory (1-4) has characterized a transcriptional assay system which has provided strong suggestive evidence that the creation of new RNA polymerase initiation sites is the primary nuclear event following administration of estrogen to immature chicks.

Many of the early events of estrogen action in the chick oviduct can be mimicked by *in vivo* administration of progesterone to chicks which have received prior injections of DES[1] (14 days) followed by withdrawal (12 days) from hormone (5, and unpublished observations). Subsequent hormone responses are believed to be receptor mediated (4). Therefore, it seemed logical to investigate the effects of crude receptor fractions upon *in vitro* chromatin tran-

[1]DES, Diethylstilbestrol; TESH, 10 mM Tris-HCl, 1 mM Na_2EDTA, and 12 mM 1-thioglycerol, pH 7.4.

scription. Initial studies made use of cell extracts which contained androgen, estrogen, glucocorticoid and progesterone receptors because of the multiple hormone effects upon translation products in the chick oviduct (5-7).

During the course of these studies an artifactual stimulation of RNA synthesis by crude receptor fractions was encountered. As a result, it is possible to formulate a set of criteria which should be satisfied when characterizing real receptor mediated transcriptional events.

MATERIALS AND METHODS

Reagents: Actinomycin D was obtained from Merck. Streptolydigin was a gift from the Upjohn Co. Sources of all other reagents have been described previously (1,2,8).

Receptor Preparation: Cytoplasmic and total cell receptor preparations were prepared by homogenizing oviducts from estrogen-primed White Leghorn chicks in TESH or TESH buffer containing 0.3 M KCl at pH 7.4 (8). Receptors in the supernatant from 110,000 x g centrifugation were complexed with steroid by the addition of 10^{-7}M progesterone, estradiol, dihydrotestosterone, and cortisol. After a two-hour binding period, steroid-receptor complexes were precipitated by the addition of ammonium sulfate (8). The pellet, resuspended in TESH constituted the receptor fraction used in the transcriptional assays.

Chromatin Preparation: White Leghorn chicks were stimulated with DES for a period of 12-14 days and subsequently withdrawn from hormone for 12-18 days. The oviducts from these animals were removed and processed into chromatin as previously described (1).

Rifampicin Resistant Initiation Sites on Chromatin: Following incubation of the receptor fraction or TESH buffer control with chick oviduct chromatin, rifampicin resistant RNA synthesis was measured as previously described (1,2) using E. coli RNA polymerase purified by the method of Bautz and Dunn (9). Briefly, chromatin was incubated with RNA polymerase for 15 minutes at 37°C - a sufficient time to allow for the formation of stable initiation complexes. RNA synthesis was started and reinitiation inhibited by the simultaneous addition of rifampicin, heparin, and nucleoside triphosphates, one of which contained a [^{3}H] or [^{32}P] label. Exact conditions for transcription appear under Table I. The reaction was terminated after 15 minutes by the addition of ice-cold 5% CCl_3COOH and the RNA products measured by filtration on glass fiber filters (Reeve-Angel). RNA product size distribution was measured as previously reported (1).

RESULTS

Table I shows the results of incubating crude receptor fraction with chromatin. There was an increase in rifampicin resistant RNA synthesis which paralleled the addition of increasing amounts of the receptor fraction. This increase was statistically significant ($P < .05$) at the 100 µl dose. The increase did not occur if the receptor fraction was heated to 90° for 10 minutes prior to incubation with chromatin.

TABLE I

Stimulatory Effects of Chick Oviduct Cytoplasmic Receptor Fraction on In Vitro RNA Synthesis[a]

Addition	[^{3}H]UMP Incorporated (Mean ± SEM)	Per Cent Control
Buffer (Control)	4612 ± 473	100
Receptor Fraction		
5 µl	3920 ± 478	85
10 µl	5110 ± 323	111
50 µl	5829 ± 99	126
100 µl	7085 ± 599	154
Heated Receptor Fraction		
100 µl	5023 ± 456	109

[a]TESH buffer, various amounts of a total cellular receptor fraction or heated receptor fraction (90°, 10 minutes) were incubated with 10 µg of chromatin for 1 hour at 23°. RNA polymerase preinitiation complexes were then established by a subsequent incubation (37°, 15 minutes) with 20 µg of E. coli RNA polymerase and a mix containing 20 µmole Tris-HCl pH 7.9, 0.25 µmole $MnCl_2$, 12.5 µmole $(NH_4)_2SO_4$ pH 7.9, and 0.50 µmole β-mercaptoethanol. RNA synthesis was carried out at 37° (15 minutes) by the addition of nucleoside triphosphates (37.5 nmole of ATP, GTP, CTP, and [^{3}H]UTP [115 cpm/pmole]) together with the drug rifampicin (10 µg) and 200 µg of heparin in a final volume of 250 µl. CCl_3COOH precipitable counts were measured as described in Methods.

When a fixed amount of the receptor fraction was incubated with variable amounts of E. coli RNA polymerase in the absence of chromatin template, as in Figure 1, there was also an increase in rifampicin resistant RNA synthesis. This was the amount of RNA synthesis one expected from ∿1 µg of DNA or 10-20 µg of DNA as chromatin. However, the receptor fraction contained no DNA or chromatin detectable by the diphenylamine assay of Burton (10). The same "stimulation" of RNA synthesis was seen to occur if the receptor fraction was passed through a small (1 ml) DEAE-cellulose column in TESH containing 0.3 M KCl prior to ammonium sulfate precipitation. Under these conditions, steroid hormone receptors passed through the column, but 99.9% of an applied [^{3}H] labeled DNA sample (1.43 µg; 36,300 cpm) was retained. Chromatin samples were also

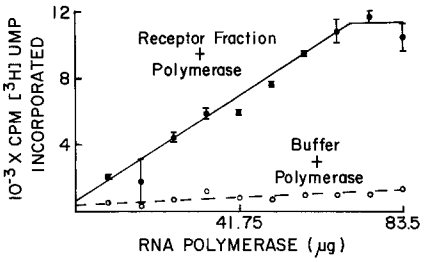

Figure 1 Template Independence of Receptor Fraction Promoted Increase in RNA Synthesis

Total receptor fraction (60 μl) or TESH buffer (60 μl) was incubated with various amounts of RNA polymerase at 37° in the absence of chromatin template. RNA synthesis was then carried out and assayed as in Table I.

TABLE II

Nucleotide Specificity of Template Independent Receptor Fraction Promoted RNA Synthesis

	[^{3}H]UMP Incorporated[a] (Mean ± SEM)		
Labeled Nucleotide	Receptor Fraction[b]	Polymerase[c]	Receptor Fraction + Polymerase
[^{32}P]CTP	245 ± 42	729 ± 214	4085 ± 508
[^{32}P]ATP	343 ± 36	1113 ± 331	3973 ± 147
[^{32}P]GTP	728 ± 399	427 ± 89	8904 ± 507

[a]RNA synthesis carried out as in Figure 1 except that a constant amount of enzyme (20 μg) was used and [^{3}H]UTP was replaced by either [^{32}P]CTP, [^{32}P]GTP, or [^{32}P]ATP at the same specific activity.

[b]RNA polymerase omitted from the reaction.

[c]Receptor fraction omitted from the reaction.

retained by the column. Our calculations indicate that if the RNA synthesis shown in Figure 1 was due to DNA contamination, the receptor fraction had to contain DNA at a concentration of 40 mg/ml. Since the diphenylamine reaction was negative, this explanation was an impossibility.

Changing the labeled nucleotide from [^{3}H]UTP to α-labeled [^{32}P]CTP,

[^{32}P]ATP, or [^{32}P]GTP brought about similar results. Table II shows that very little RNA synthesis occurs in either the enzyme or the receptor fractions alone. Combination of the two fractions resulted in dramatic increases of TCA precipitable counts. Thus the stimulatory effect was not nucleotide specific. Furthermore, sucrose gradient analysis (not shown) of the product demonstrated significant material sedimenting at greater than 18S. Omission of the three unlabeled nucleotides from the RNA synthesis reaction only slightly decreased the number of acid precipitable counts. This suggested that the product might consist of some sort of polymer.

It can be seen from Table III that although the product synthesized was RNase sensitive and resistant to DNase, it was also resistant to actinomycin D and streptolydigin. In contrast, under similar conditions true RNA synthesis from a DNA template was markedly inhibited by both streptolydigin and actinomycin D. These are particularly important experiments because both actinomycin D and streptolydigin act by blocking RNA chain elongation; the former by binding to DNA template (11) and streptolydigin by binding to the RNA polymerase (12).

One enzyme whose activity might account for such results is polynucleotide phosphorylase. This enzyme acts by polymerizing nucleoside diphosphates into RNA with the elimination of P_i (13). It's activity can be inhibited by the addition of 0.4 M phosphate, pH 7.5 (14). As shown in Table III, addition of this reagent quantitatively inhibited the receptor fraction mediated synthesis of RNA. Thus, it appeared that the stimulation caused by the receptor fraction might be due entirely to polynucleotide phosphorylase activity.

DISCUSSION

Polynucleotide phosphorylase catalyzes the reaction (13):

$$n_1\text{ADP} + n_2\text{GDP} + n_3\text{CDP} + n_4\text{UDP} \xrightleftharpoons{Mg^{++}} \text{RNA} \begin{bmatrix}\text{AMP}\\ \text{GMP}\\ \text{CMP}\\ \text{UMP}\end{bmatrix} + (n_1+n_2+n_3+n_4)P_i$$

McConnell and Bonner (15) have reported that polynucleotide phosphorylase may

TABLE III

Characterization of RNA Synthesis Promoted by Chick Oviduct Receptor Fraction[a] in the Absence of Template[a]

Addition	[^{3}H]UMP Incorporated (Mean ± SEM)	Per Cent Control
None (Control)	4625 ± 196	100
DNase (10 µg)[b]	4668 ± 17	101
RNase (400 µg)[c]	351 ± 2	8
Actinomycin D (10 µg)[b]	3603 ± 807	75
Streptolydigin[d]	4325 ± 258	96
Phosphate, pH 7.5 (0.4 mM)[e]	< 100	< 2

[a]Receptor fraction (60 µl) plus polymerase (22 µg). RNA synthesis carried out as in Figure 1.

[b]Under similar conditions 95% of RNA synthesis off of 1 µg DNA template was inhibited.

[c]RNase added in huge excess because of the presence of heparin.

[d]Under similar conditions 50% of RNA synthesis off of 1 µg DNA template was inhibited.

[e]Under similar conditions RNA synthesis off of chromatin template was unaltered.

contaminate purified E. coli RNA polymerase. Both RNA primer independent and dependent forms of this enzyme have been described (14). Since our purified E. coli RNA polymerase ordinarily incorporated very low amounts of radioactive nucleotides into acid precipitable counts, it could only have been contaminated by the primer dependent from of polynucleotide phosphorylase. The addition of cellular RNA with the receptor fraction could have provided the necessary primer to stimulate polynucleotide formation catalyzed by the polynucleotide phosphorylase. The receptor fraction must also have contained nucleoside triphosphatase activity which converted large amounts of the radiochemically pure labeled nucleoside triphosphates into nucleoside diphosphate substrates. Alternatively, in addition to phosphatase activity, the receptor fraction might have con-

tained trypsin-like protease activity which converted polynucleotide phosphorylase from the primer dependent to the primer independent form (14).

Spurious enzyme activity should be carefully guarded against in studies such as these. Use of E. coli RNA polymerase may introduce polynucleotide phosphorylase or polyphosphate kinase contaminants (15). Measurement of endogenous polymerase activities may be complicated if the added receptor fraction contains a factor like that of Wilkie and Smellie (16). Proteases may selectively digest F_1 histones converting chromatin transcription to a DNA like pattern of more open template (unpublished observations). Nucleases may nick DNA template and cause an apparent increase in transcription (1). RNase activity may bring about underestimates of the amount of product synthesized. Heparin addition can correct for this potential problem (1). In the absence of spurious enzymes, care should be taken that receptor fractions do not contain trace amounts of template. Finally, because of the lipid nature of steroids one should guard against detergent stimulated increases in endogenous polymerase activity similar to that reported to result from treating chromatin with 0.5% sarkosyl (17).

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