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EARLY EFFECTS OF ESTROGEN ON THE SYNTHESIS OF OVIDUCT NUCLEAR PROTEINS

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SUMMARY

Labelling of oviduct nuclear proteins was studied *in vitro* up to 4 h after estrogen stimulation of primed chicks. The incorporation of [^{3}H]-leucine into histones slightly decreased and that into nonhistones increased by 30% after 4 h stimulation. The specific radioactivities of nonhistones were at least 3-fold higher than those of histones. Estrogen caused an increase in the labelling of nonhistones extractable with 0.35 M NaCl and unextractable residual proteins. The specific radioactivities of nonhistones were high in the nuclear matrix fraction and in the $MgCl_2$ soluble chromatin fraction as compared to the $MgCl_2$ insoluble chromatin fraction. Estrogen stimulation appeared to increase particularly the relative radioactivity of nonhistones of 50 000, 40 000 and 34 000 molecular weight.

INTRODUCTION

Estrogen stimulation of primed chicks rapidly causes transcriptional changes in target tissues. In the oviduct excessive amounts of messenger RNA for ovalbumin are produced and translated. Nuclear nonhistone proteins are thought to be involved in tissue-specific transcription [1] and in the transcriptional response of target tissues to steroid hormones [2]. The nuclear acceptor sites for estrogen [3] and progesterone [4] may be nonhistone proteins.

A substantial amount of work has been done on the synthesis of nonhistone proteins of total chromatin in response to estrogen stimulation. Changes have been reported in the amount and the rate of synthesis of specific uterine proteins following estrogen stimulation [5–7]. A preferential labelling of a group of nonhistone proteins has been described in the oviduct 24 h after the administration of estrogen [8]. The changes have been observed in nucleolar nonhistone proteins as well as in the total chromatin proteins, and they have been shown to be independent of cell division [9]. However, very little is known about the response of chromatin proteins in subnuclear fractions to estrogen stimulation. Such information would help to understand the role of chromatin proteins in estrogen-induced gene activation in more detail.

The present study differs from the previous ones by two novel approaches. First, the study focuses on the early (up to 4 h) effects of estrogen stimulation on the synthesis of oviduct nuclear proteins in order to describe more specifically the changes possibly triggering hormone response. Second, nuclei are fractionated into newly described subnuclear components including matrix, and transcriptionally active and inactive chromatin. Each of the subfractions show characteristic functional and structural properties (see discussion), which may be relevant to the understanding of the hormone response. From previous studies it is clear that fractionation of chromatin is necessary in order to show specific changes in gene expression. In this study radioactive nuclear proteins are extracted with various salt concentrations or fractionated on hydroxylaptite columns. The fractions are further analysed on polyacrylamide gels using double-labelled proteins, and specific changes in synthetic rates can be shown.

MATERIALS AND METHODS

Experimental and labelling conditions

5-Day old chicks received a primary injection series with β-estradiol benzoate (Sigma) for 10 days. The hormone was dissolved in sesame oil (10 mg/ml) and a daily dose of 1 mg was administered in the leg muscle. After the primary injection program the injections were stopped for at least two weeks and some chicks then received a single dose of 1 mg of the hormone as a secondary injection. After various periods of time the chicks were killed and their oviducts were removed. The external collagen sheath was dissected and the oviducts were cut into small pieces with a pair of scissors. 15 ml Of leucine-free Eagle's minimal essential medium containing 30 mM Tris pH 7.4 and [^{3}H] or [^{14}C]-leucine (52 or 0.32 Ci/mmol, 4 or 1 μCi/ml, respectively) were added and the samples were shaken at 37° in close vessels for 1 h if not otherwise stated.

Preparation of chromatin

Oviduct nuclei were purified by sedimentation through 2 M sucrose as described [10]. Chromatin was prepared by repeated extraction of nuclei with

80 mM NaCl containing 20 mM EDTA, pH 7.0 [8]. The crude chromatin obtained was finally homogenized in 10 mM Tris, pH 8.0 and kept overnight at 0°. The solution was layered over 1.6 M sucrose and centrifuged in a Spinco SW-25 rotor at 63 000 *g* for 3 h. Chromatin was collected from the bottom of the tube.

Salt extraction of chromatin was carried out by homogenization with 10 strokes of a Teflon–glass homogenizer in 0.35 M NaCl containing 10 mM Tris pH 8.0. After 30 min on ice, the homogenate was centrifuged at 100 000 *g* for 30 min. The supernatant was collected and the pellet was homogenized once more as above. The resulting pellet was homogenized in 0.6 M NaCl containing 10 mM Tris, pH 8.0 and centrifuged as above, Finally the remaining pellet was homogenized in 2.0 M NaCl containing 10 mM Tris pH 8.0 and centrifuged to separate a soluble exctract and the residual proteins. For the determination of radioactivity, aliquots of the extracts were precipitated with cold 20% trichloroacetic acid, collected on glass fiber filters and washed two times with scintillation fluid. For gel electrophoresis the salt extracts were dialysed against 0.1% sodium dodecyl sulfate, freeze-dried and analysed on urea-sodium dodecyl sulfate-acrylamide gels as described elsewhere [8–11].

Fractionation of chromatin

Oviduct nuclei were prepared as above, lysed and digested with DNase II [10]. Three fractions were collected: nuclear matrix (100000 *g* pellet), $MgCl_2$ insoluble chromatin (precipitable with 3 mM $MgCl_2$), and $MgCl_2$ soluble chromatin (collected at 100 000 *g* for 16 h). The fractions have been characterized elsewhere [10, 12].

For hydroxylapatite fractionation of nuclear proteins the samples were dissolved in 5 vol. of 6 M urea, 2.4 M NaCl and 1.2 mM sodium phosphate, pH 6.8 (total vol. 1 to 2 ml) and sonicated in a Branson sonifier for 6 × 10 s. Proteins were fractionated on hydroxylapatite columns according to MacGillivray *et al.* [13] as described elsewhere [8, 11]. However as a modification to our previous method, only one nonhistone fraction was collected. The present nonhistone fraction contains the two previous nonhistone fractions. In the present version of hydroxylapatite chromatography two fractions were eluted: I (mainly histones) with the equilibration buffer, 5 M urea, 2 M NaCl and 1 mM phosphate buffer, pH 6.8; II (nonhistone) with 5 M urea, 0.5 M NaCl, 0.2 M phosphate buffer, pH 6.8 containing 0.2% sodium dodecyl sulfate. Gel electrophoresis of the fractions was carried out as described above.

Protein was determined according to Lowry *et al.* [14].

RESULTS

Labelling of oviduct chromatin proteins with [^{3}H]-leucine was studied in an *in vitro* incubation.

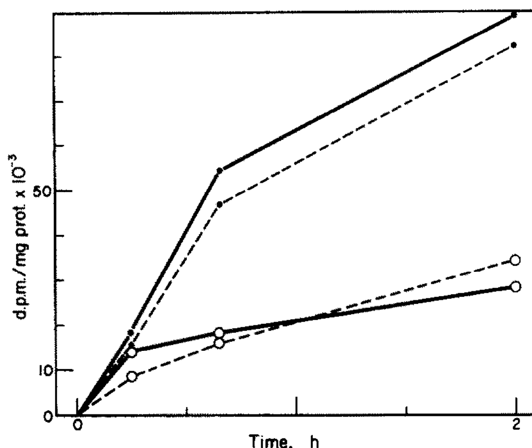

Fig. 1. Time-course of [^{3}H]-leucine incorporation into chromatin proteins of chick oviduct incubated at 37°. Oviducts were prepared from primed control chicks (---) or stimulated chicks 4 h after secondary stimulation (——). The proteins were fractionated on hydroxylapatite columns into fraction I (histones, ○) and fraction II (nonhistones. ●).

The specific radioactivities of histones and nonhistones increased up to 2 h (Fig. 1). The rate of incorporation into hydroxylapatite fraction I (histones) was practically similar and into hydroxylapatite fraction II (nonhistones) slightly higher in oviducts of estrogen-stimulated (4 h secondary stimulation) chicks as compared to oviducts of control chicks. This does not indicate however that the rate of synthesis of nuclear proteins is increased after hormone stimulation, as the concentration of radioactive precursor leucine may also be higher in stimulated cells. This question is not considered relevant for this study, as the emphasis is on the *relative* rates of incorporation rather than on absolute rates of synthesis. This limitation also concerns data to be presented later.

Estrogen stimulation of chicks influenced initially incorporation of [^{3}H]-leucine into hydroxylapatite

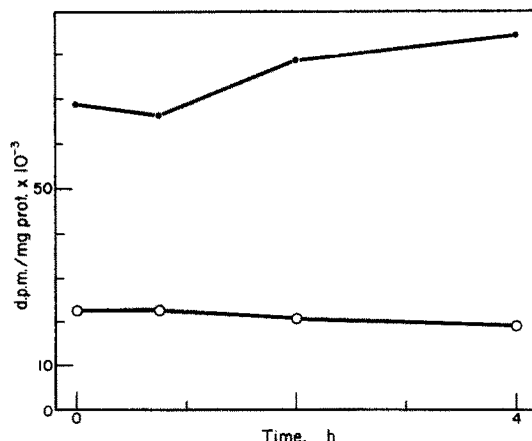

Fig. 2. Effect of estrogen stimulation (1 mg/chick) on the incorporation of [^{3}H]-leucine into oviduct chromatin proteins. Primed chicks received a secondary stimulation of estrogen and the oviducts were prepared after various periods of estrogen action. Hydroxylapatite fraction I (histones, ○) and hydroxylapatite fraction II (nonhistones, ●).

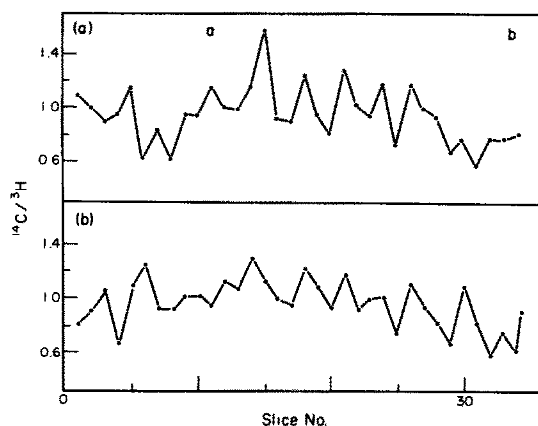

Fig. 3. Polyacrylamide gels of nonhistone proteins (hydroxylapatite fraction II) from stimulated and control oviducts. Stimulated oviducts were incubated in the presence of [^{14}C]-leucine and control oviducts in the presence of [^{3}H]-leucine. Secondary stimulation for 2 h (a) and 4 h (b). Molecular weight standards are marked as (a) bovine serum albumin, molecular weight 68 000, and (b) lysozyme, molecular weight 17 000.

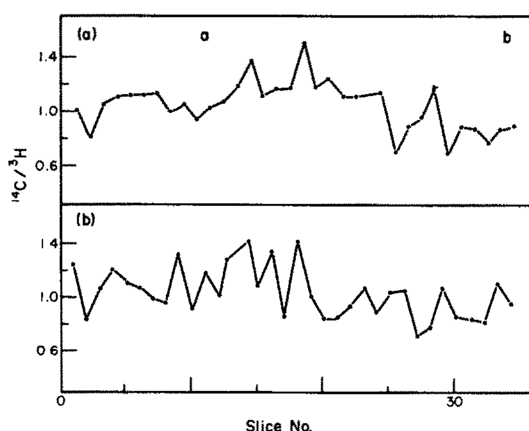

Fig. 4. Polyacrylamide gels of nonhistone proteins extractable with 0.35 M NaCl from stimulated (^{14}C-label) and control (^{3}H-label) oviducts. Secondary stimulation for 2 h (a) and 4 h (b).

fraction II (nonhistones). The increased labelling was observed as early as 2 h after a secondary stimulation (Fig. 2). The rate of incorporation into hydroxylapatite fraction I (histones) slightly decreased up to 4 h of secondary stimulation.

The possible differential effects of hormone stimulation on the labelling of polypeptide groups was studied with double-labelled nonhistone proteins on polyacrylamide gels. The control oviducts were labelled with [^{3}H]-leucine and the stimulated oviducts with [^{14}C]-leucine. The ratio of [^{14}C] and [^{3}H] radioactivity was recorded in gels containing chromatin proteins of hydroxylapatite fraction II, both from stimulated and from control oviducts (Fig. 3). Among nonhistones three groups of polypeptides, at calculated molecular weights of 50 000, 40 000 and 34 000, preproducibly displayed a relative increase in specific radioactivity after 2 and 4 h of secondary stimulation with estrogen. Stimulation also appeared to decrease the rate of labelling of some polypeptide groups.

A differential salt extraction of chromatin proteins was performed to characterize binding properties and specific radioactivities of chromatin protein fractions (Table 1). The chromatin prepared had a protein/DNA ratio of 2.6. Almost half of the proteins were nonhistones extractable with 0.35 M NaCl. The histones were recovered in two fractions: H 1 was extracted with 0.6 M salt and the other histones with 2 M salt. Together these fractions amounted to 39% of the total chromatin protein. The unextractable residual protein constituted 14% of the total protein. As expected, the labelling of the histone fractions was low in comparison to that of the nonhistone fractions, of which the residual proteins displayed the highest specific radioactivity. Estrogen stimulation caused an insignificant 10–20% increase in the specific radioactivity of the nonhistone fractions.

In order to detect changes in labelling rates of polypeptide groups a double-label experiment was carried out as described above. The salt extractable proteins were separated on polyacrylamide gels and [^{3}H] and [^{14}C] radioactivities were monitored along gels (Fig. 4). Estrogen-stimulation appeared to increase particularly the relative labelling of polypeptides at molecular weights of 50 000 and 40 000 among the nonhistones extractable with 0.35 M NaCl. In this case the groups of polypeptides may be identical to those pointed out in Fig. 3 as the two fractions contain the bulk of nonhistones.

The distribution of radioactivity was further studied in the three subfractions of oviduct nuclei (Table 2). Distinct differences were observed in the specific

Table 1. Incorporation of [^{3}H]-leucine into oviduct chromatin proteins fractionated by differential salt extraction

	Primed control		Estrogen stimulation 4 h
Fraction	% of total protein*	Radioactivity d.p.m./mg protein $\times 10^{-3}$	Radioactivity d.p.m./mg protein $\times 10^{-3}$
0.35 M NaCl extractable proteins	46	63 ± 12	75 ± 8.0
0.6 M NaCl extractable proteins	18	8.6 ± 0.1	6.3 ± 0.9
2.0 M NaCl extractable proteins	21	5.7 ± 0.1	6.0 ± 1.3
Residual proteins	14	110 ± 62	121 ± 12

* The amounts refer to fractionations from oviducts of primed control chicks. Means ± S.E.M. of 4 experiments.

Table 2. Incorporation of [^{3}H]-leucine into histone and nonhistone proteins of oviduct chromatin fractions

Fraction	%* of total protein	A Primed control d.p.m./mg prot. $\times 10^{-3}$	B Estrogen stimulation (2 h) d.p.m./mg prot. $\times 10^{-3}$	B/A $\times$ 100	Estrogen stimulation 4 h d.p.m./mg prot. $\times 10^{-3}$	C/A $\times$ 100
Crude nuclear matrix	43					
HAP† I (histones)	–26	30 ± 6.7	33 ± 4.1	1.10	36 ± 5.4	1.20
HAP II (nonhistones)	–74	61 ± 18	67 ± 3.9	1.09	65 ± 9.8	1.07
$MgCl_2$ insoluble chromatin	43					
HAP I (histones)	–72	10 ± 1.6	7.4 ± 1.8	0.74	6.6 ± 1.1	0.66
HAP II (nonhistones)	–28	34 ± 5.8	31 ± 3.4	0.92	40 ± 6.1	1.18
$MgCl_2$ soluble chromatin	14					
HAP I (histones)	–26	16 ± 5.0	18 ± 3.1	1.12	16 ± 1.7	1.02
HAP II (nonhistones)	–74	68 ± 15	58 ± 4.7	0.85	67 ± 8.8	0.98

* The amounts refer to fractionation from oviducts of primed control chicks. † HAP = hydroxylapatite fraction. Means ± S.E.M. of 4 to 6 experiments.

radioactivities of the fractions. The nonhistone proteins of nuclear matrix and of the $MgCl_2$ soluble chromatin were labelled two times more actively than those of the $MgCl_2$ insoluble chromatin fraction. This is particularly interesting as the ovalbumin gene sequences are enriched in these two fractions and as the $MgCl_2$ soluble chromatin is enriched in estrogen receptors after hormones stimulation [12]. Hydroxylapatite fraction I (histones) was most actively labelled in the crude nuclear matrix fraction. As this fraction contains a number of highly labelled, poorly soluble nonhistone proteins, which may contaminate the histone fraction, it is not clear whether histones of this fraction are actually labelled more actively than those of the two other fractions. Estrogen stimulation caused only moderate changes in specific radioactivities of the protein fractions. The largest change was the decrease in specific radioactivity of histones in the $MgCl_2$ insoluble chromatin fraction (the difference between the control and 4 h stimulation is significant at $P < 0.2$, two-tailed Student's *t*-test).

DISCUSSION

Chick oviduct is a useful tissue to study the mechanisms of estrogen action, because hormone administration is accompanied with a massive production of mRNA for ovalbumin. Such an extensive and specialized response is unique among the responses of tissues to steroid hormones. In pretreated chicks the response can be elicited within a few hours, reducing the effects of differential cell proliferation in studies on early hormone action. For this reason attention was paid in this study to the early period of steroid hormone action. Labelling of nuclear proteins was investigated in subnuclear fractions characterized recently by structural and functional properties. The fractionation of nuclear components is of particular interest, since estrogen is likely to regulate the expression of a limited number of genes [15]. There are no more than a few thousands of high affinity acceptor sites for steroid hormones in a cell nucleus [16,17]. We have presented evidence that both estrogen receptors and ovalbumin gene sequences are concentrated in the $MgCl_2$ soluble chromatin [10,12], and that the extent of concentration appears to depend on hormone stimulation [18].

The nuclear subfractions used in this study have been characterized elsewhere [12]. Morphological studies of the crude nuclear matrix fraction have recently been extended [19]. Typical matrix network has been observed in electron micrographs in agreement with other workers [20, 21]. Additionally, an active incorporation of [^{3}H]-thymidine into the matrix fraction [22] has been confirmed.

It is of interest that the incorporation of [^{3}H]-leucine into histones and nonhistones of the matrix fraction and of the $MgCl_2$ soluble (transcriptionally active) chromatin fractions is more active than the incorporation into the $MgCl_2$ insoluble (transcriptionally inactive) chromatin. Ovalbumin gene sequences show a similar distribution in the subnuclear fractions [12]. However, estrogen stimulation (up to 4 h) caused insignificant changes in the labelling of the total proteins in the chromatin fractions. This may suggest that the changes taking place during altered gene expression affect a few proteins and go undetected, or require no newly synthesized proteins. No reliable double-labelled gel electrophoretic analysis could be done with these fractions due to the amount of radioactivity available.

Detailed studies on the effect of estrogen stimulation on the synthesis of individual polypeptides could be carried out with protein fractions from the total chromatin fractionated on hydroxylapatite columns or with differential salt extraction. Analysis of double-labelled proteins on polyacrylamide gels revealed a relative active synthesis of polypeptides at molecular weights of 50 000, 40 000 and 34 000. Estrogen stimulation for 4 h increased the labelling of the total nonhistones by about 20%, and the labelling of the three polypeptides considerably more than that. It will be seen in the future work how the actively labelled polypeptides are localized in the subfractions of nuclei and whether they are involved in the mechanisms of hormone action controlling gene expression.

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