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EFFECT OF PROGESTERONE ON HnRNA AND AVIDIN mRNA OF THE CHICK OVIDUCT

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SUMMARY

Nuclear pre-mRNA (HnRNA) and polysomal avidin mRNA were extracted from diethylstilbestrol and progesterone-treated immature chick oviducts. Purity of avidin antibody precipitated polysomes as well as isolated nuclei were confirmed by electron microscopy (EM). The number of perichromatin granules was counted from the oviductal epithelial cell nuclei. Form and size of RNA extracts were studied with EM. Avidin coding activity of RNA's was determined in a rabbit reticulocyte lysate system.

The nuclei and polysomes of the control animals contained the same amount of avidin coding activity per 5 g of the oviduct tissue. During the progesterone induction of the avidin synthesis the activity of nuclear pre-mRNA coding for avidin decreases while the cytoplasmic avidin mRNA increases. The total avidin coding activity increases only slightly during 24 h after progesterone. There is an initial decrease in the amount of perichromatin granules followed by an increase exceeding the level of the controls 12 and 24 h after progesterone. The EM analysis of RNA extracts revealed that the average size of pre-mRNA molecules was 12000–13000 nucleotides, whereas the size of the mRNA molecules was considerably smaller, the major portion of the mRNA fraction was about 3000 nucleotides in length. The present results suggest that the cleavage and transfer of the nuclear pre-mRNA might be the critical steps of the non-transcriptional control of avidin synthesis by progesterone.

INTRODUCTION

Through the years, various theories have been proposed to explain the basis for the induction of the specific protein synthesis by steroid hormones [1–8]. During the recent years the field has been dominated by the hypothesis of the transcriptional control [7, 9, 10]. We have approached the mechanism of steroid action by studying the induction of avidin in chick oviduct by progesterone [10, 11]. Avidin is a secretory protein of the reptilian, amphibian and avian oviduct.

In mammalian cells a portion of HnRNA, the heterogenously sized nuclear RNA, probably represents precursor to mRNA (pre-mRNA) [12]. A majority of the pre-mRNA and cytoplasmic mRNA contain a 3′-terminal poly (adenylic acid) segment which is about 200 nucleotides in length. In addition, there is accumulating evidence that the primary transcription product is clearly larger than the final mRNA [13–15].

The nuclear progesterone receptor complex is bound to the acceptor sites of the chromatin material in the chick oviduct epithelial cells [16]. Consequently, the nuclear RNA synthesis is activated [9], suggesting that progesterone may regulate the transcription. Moreover, the presence of cytoplasmic mRNA for a specific steroid induced protein and its absence in the unstimulated tissue has been regarded as evidence for the regulation of transcription by the steroid [7, 10, 17–19]. However, the nuclear RNA (pre-mRNA) species reflect apparently more closely the transcriptional events than does the cytoplasmic polysomal mRNA. In the present study the avidin coding activity of the nuclear pre-mRNA and polysomal mRNA from progesterone-treated immature chick oviducts is studied.

MATERIAL AND METHODS

Animals

One-day-old immature Leghorn chicks (strains Ti 53 and Ti 453, Turun Muna Hatchery, Turku, Finland) were injected daily with 0.5 mg of diethylstilbestrol in order to stimulate the oviducts to grow. On day 10 they were injected with 5 mg of progesterone. Because the yield of mRNA was very low (20–40 μg/of polysomal RNA and 3–4 μg of pre-mRNA per 10 g of the oviduct tissue), oviducts from 30 chicks were pooled for the RNA extractions. The oviducts were frozen immediately by placing the tissues on dry ice.

Extraction of pre-mRNA

The tissues were homogenized in Waring blendor (13,500 rev/min) for 30 s at +4°C with 2.5 ml of a buffer/g of tissue (0.25 M sucrose, 4 mM $MgCl_2$, 10 mM KCl, 1 mM methanethiol in 20 mM Tris–HCl pH 7.5). Only a few nuclei were broken as observed under the light microscope. The purity of the isolated nuclei was checked under an electron microscope. After the washing no ribosomal contamination of the nuclei was observed. Of the various RNAse inhibitors heparin was added to the homogenate (500 mg/ml) to block ribonuclease activity as was suggested by

Schimke *et al.*[20]. However, we found that heparin did not improve the yields of mRNA. In contrast, the activity of mRNA decreased by 40%. Pre-mRNA was extracted from the nuclear fraction. The nuclear pellet (IEC, +4°, 10,000 rev/min, 15 min) was suspended in the homogenization buffer and filtered through cheese cloth. The suspension was layered on the equal vol. of 0.32 M sucrose in the homogenization buffer. The nuclear fraction (7000 *g*, 10 min) was washed and extracted by the method of Georgiev and Samarina[21]. Polydextrane sulphate (0.1 mg/ml) was added as RNase inhibitor. The combined extracts were centrifuged on a sucrose gradient (15–30%) in the extraction buffer 24,000 rev/min for 6 h (MSE superspeed, swing-out rotor No 25). The combined pre-mRNA fractions [22] were kept under a continuous flow dialysis for 4 h in buffer (3 mM $MgCl_2$, 0.32 M sucrose in 100 mM Tris–HCl pH 7.6). Sodium dodecyl sulphate (0.5%) was added and pre-mRNA fractions were stirred with an equal vol. of water-saturated phenol at 4°C for 30 min. The water phase was collected and an equal vol. of a buffer (0.5% SDS in 100 mM Tris–HCl pH 9.0) was added twice to the phenol layer [23]. 0.1 vol. of 1 M NaCl was added to the combined water phases. RNA was precipitated and washed three times with ethanol at −20°C. An excess of SDS was precipitated with 0.5 M KCl. pre-mRNA was dissolved in water (6 μg/ml).

Extraction of polysomal mRNA

The polysomal mRNA was extracted from the supernatant fraction of the same pooled sample as pre-mRNA. The polysomes were isolated by the method of Noll [24]. Avidin antibody serum prepared in a rabbit [25] was added into the polysomal pellet. Electron microscopy of the polysomal pellet showed that it was free of other cytoplasmic components. The specificity of avidin antibody serum was tested by adding excess amounts of ovalbumin but ovalbumin did not replace avidin. Chick avidin antibody precipitated only 5–12% of the purified frog avidin when the result was compared to the biotin-[C^{14}]-bentonite determination of the frog avidin [25]. An equal vol. of water-saturated phenol was added in the antibody precipitate suspension and the RNA was then further processed as pre-mRNA with an exception that mRNA was trapped on a nitrocellulose filter (Millipore, HA 0.45) [23]. Finally mRNA was dissolved in distilled water (30 μg/ml).

Electron microscopy of avidin polysomes, HnRNA and avidin mRNA

Precipitated avidin polysomes were mounted directly on grids and stained with uranyl acetate.

HnRNA and mRNA preparations (in 0.1 M Tris, 0.01 M EDTA, 10% v/v mercaptoethanol) of 50 μl and 5 μl of cytochrome-c solution (1 mg/ml in 0.2 M ammonium acetate) were mixed and ramped on a hypophase of 0.1 M Tris—0.01 M EDTA or prepared by microdrop adsorption method using glutaraldehyde drops to facilitate protein film formation. Grids were subsequently immersed in alcohol, dried and rotary shadowed with Pt using the distance of 10 cm. and the angle of 7–8°.

Cell-free system

All the RNA extractions were repeated three times and the messenger activity of pre-mRNA and polysomal mRNA was tested three times in a cell-free reticulocyte lysate derived from three different rabbits [26]. 1 μCi of C-14 labelled protein hydrolysate (S.A. 57 mCi/m atom carbon, the Radiochemical Centre, Amersham, England) and 400 μl of the RNA solution (10–20 μg of pol-mRNA or 2–3 μg of pre-mRNA) were incubated with the cell-free system at 37°C for 90 min. Avidin was assayed by an antibody precipitation method [25]. Avidin antibody 20 μl/0.5 ml of the lysate) was added. The precipitate was trapped and washed on the millipore filter (HAWP 02500). The filter was counted in 10 ml of aquafluor by Wallac Decem liquid scintillation counter. All the results of the repeated experiments were similar with the exception that the extent of the incorporation of [^{14}C]-amino acids into avidin was directly proportional to the activity of the lysate and to the amount of mRNA added. The endogenous protein synthesis (hemoglobin synthesis) of the lysate varied from 23200 c.p.m. to 60800 c.p.m./ml of the lysate. The RNA samples frozen in distilled water at −20°C showed a slight decrease (approx. 30%) in the activity when stored for 2 months.

Avidin assay. Avidin was assayed by the antibody precipitation method as described earlier [22].

Electron microscopy of nuclear RNA particles

Perichromatin particles (35–45 nm dia.) were counted from the epithelial cell nuclei as described earlier [27].

RESULTS

Injection of progesterone clearly increases the messenger activity for avidin in the polysomal mRNA fraction (Fig. 1). Avidin mRNA activity is found in the immature, untreated control chick oviducts. Also the yield of polysomal mRNA increases clearly up to 24 h after the injection of progesterone. Nuclear pre-mRNA can be translated in a heterologous cell-free system. The yield of the nuclear pre-mRNA is fairly constant up to 24 h after progesterone. The messenger activity of nuclear RNA shows changes opposite to that of the polysomal one (Fig. 1).

After the salt extraction of the purified nuclei nuclear pre-mRNA fraction varied extensively in size being however composed of considerably larger molecules than cytoplasmic avidin mRNA molecules (Figs. 2–3). The average size of the HnRNA can be estimated to be about 12000–13000 nucleotides whereas in the avidin mRNA fraction the average molecular size was found to be about 3000 nucleotides.

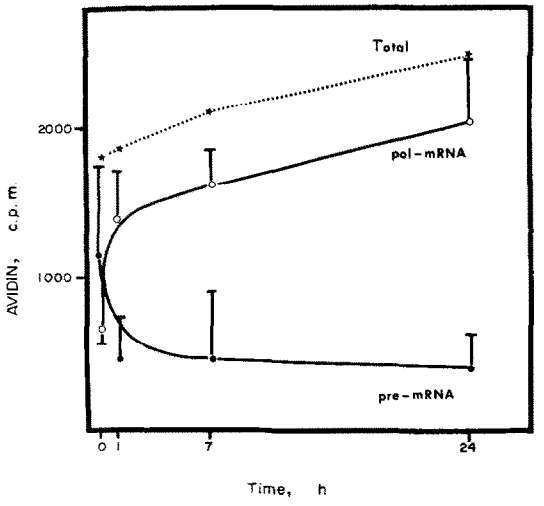

Fig. 1. Translation of avidin nuclear pre-mRNA and polysomal mRNA (pol-mRNA) obtained from chick oviducts treated with progesterone for the time indicated. Cell-free system for translation is derived from rabbit reticulocytes. "Total" represents the total avidin coding activity of 5 g of the oviduct (±S.D.).

Morphometric analysis of the electron micrographs of the epithelial cell nuclei showed that there was an initial decrease in the amount of perichromatin granules followed by an increase exceeding the level of the controls 12 and 24 h after progesterone (Fig. 4).

DISCUSSION

Our present results are in agreement with the earlier findings [10, 17] that the messenger activity for avidin in the polysomal mRNA fraction clearly increases after the administration of progesterone. However, contradictory to the earlier reports is the present finding that avidin mRNA is located in the immature, untreated control chick oviducts. The lower sensitivity of the earlier avidin assay method can account for this discrepancy in results. The S.A. of polysomal mRNA coding for avidin per μg of mRNA does not change since the yield of polysomal mRNA increases clearly up to 24 h after the injection of progesterone. While the polysomal messenger activity for avidin increases during the induction by progesterone the messenger activity of nuclear RNA shows changes opposite to that of the polysomal one. This is most probably due to the transfer of nuclear RNA into the cytoplasm and polysomes. On the basis of the present results we assume that there is no or only a slight activation of transcription of avidin pre-mRNA from its genetic template by progesterone, whereas the major control of avidin synthesis occurs at the level of the transfer of pre-mRNA into cytoplasm and/or at the translational level.

An accumulating evidence shows that there is a giant nuclear RNA (HnRNA) containing mRNA sequences [14, 15]. In the present study, the electron microscopic analysis of the RNA extracts showed that nuclear pre-mRNA molecules were significantly larger than the cytoplasmic mRNA molecules. However the nuclear RNA cleaved easier to smaller sequences than the polysomal one. Apparently the sucrose gradient fraction was homogenous and its size was about 10,000 nucleotides. Only a small portion of the pre-mRNA molecules contain avidin mRNA sequences. The present results do not demonstrate whether nuclear pre-mRNA is a polymer of avidin mRNA's or whether it contains long non-informative part. Apparently, pre-mRNA has to be cleaved in order to form smaller cytoplasmic mRNA molecules. However, the EM comparison of HnRNA and avidin mRNA length gives indirect evidence of their true relation. The size of the cytoplasmic mRNA molecules is considerably larger than what is expected. The molecular weight of avidin is 68.000 and it consists of four subunits and 5.2% hexose and 3.4% glucosamine. Thus the molecular weight of a subunit peptide is about 15.500. The minimum size of mRNA coding for this peptide is about 500 nucleotides. The size of the avidin mRNA molecule was 5–6 times larger, which indicates that one messenger may code for the entire avidin molecule and still the major portion of the mRNA might play a non-informative role.

Our recent results [22] indicate that the activation of nuclear non-specific ribonuclease may account for the cleavage of giant pre-mRNA to small active mRNA and the onset of avidin synthesis, since the

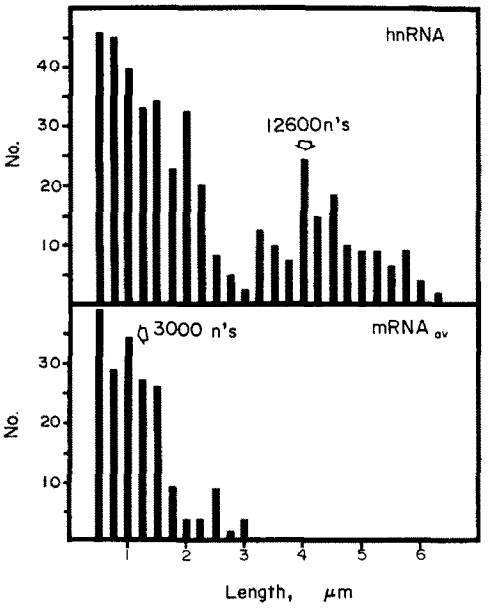

Fig. 2. Histograms of the molecular size of HnRNA and avidin mRNA ($mRNA_{av}$) from the chick oviduct. The limits of size classes is ±0.125 μm. The total number of measured HnRNA molecules is 423 and that of $mRNA_{av}$ 176. The molecular fragments below 0.5 μm in length are not included.

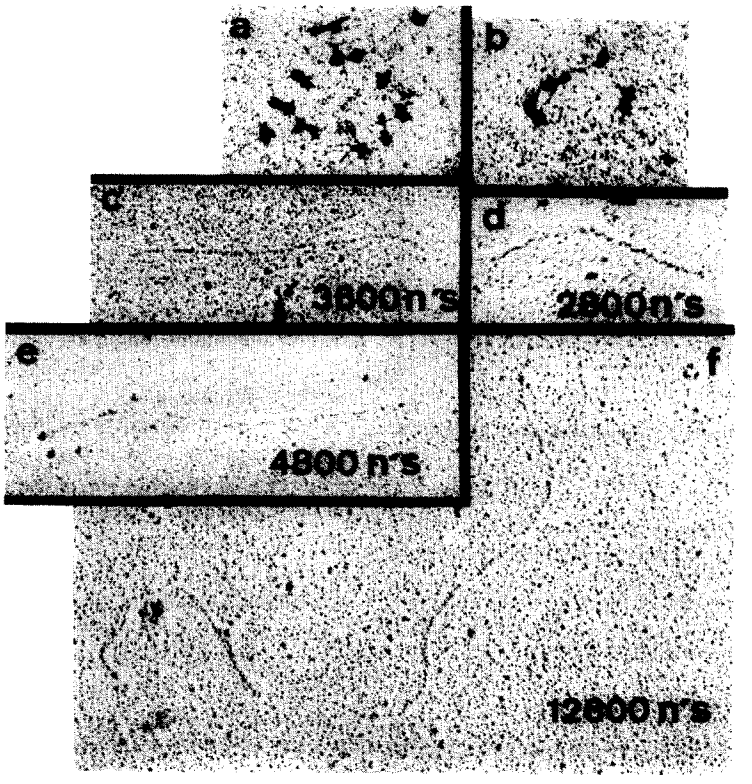

Fig. 3. Electron micrographs of avidin polysomes (a–b, magnification 100.000 dia.), avidin mRNA (c–d) and HnRNA (e–f) isolated from progesterone stimulated chick oviduct.

activation is concomitant with the induction of avidin. The small fraction of avidin mRNA which has relatively long half-life (24 h or more) [9] has better chance to be translated in the polysomes, when the activated cytoplasmic ribonuclease removes shortlife mRNAs. The mechanism by which progesterone exerts its effect on ribonuclease is not known. It may be a direct allosteric effect on the enzyme [28] or a release of ribonucleases from the lysosomes [29].

Some recent findings can be explained on the basis of our present results. The target cell nuclei contain several thousand specific steroid receptors which are supposed to mediate the hormonal effects. However, for a steroid-specific protein there is apparently only one gene per haploid genome [7]. The number of steroid–receptor complexes is comprehensible, if they act directly on the several nuclear pre-mRNA or ribonuclease molecules. Recently, Liao *et al.*[30] have shown a binding of steroid–receptor complexes by ribonucleoprotein. The significance of this binding might be a direct action of steroid–receptor on ribonucleoprotein. Scherrer[13] has proposed that tissue-specific recognizing proteins determine the selection of the cleavage of giant nuclear RNA. Progesterone–receptor complex may serve as a recognizing protein for the avidin mRNA sequences and protect against the activated ribonuclease. Various transcription blockers, as actinomycin D [31] and streptolydigin [32], do not block the avidin induction by progesterone, but they rather induce the avidin synthesis. The induction of avidin synthesis by actinomycin D [31] can be also partially based on the ribonuclease system, since it also activates non-specific ribonuclease [32]. The non-specific induction of avidin synthesis by a mechanical trauma [31] can be explained, if there is a pre-existing nuclear messenger for avidin, since the effect of ligature on avidin synthesis is most prominent in the untreated immature chick oviduct [33]. The decrease of nuclear pre-mRNA is reflected as an initial decrease of the perichromatin granules in the chick oviduct epithelium cell nuclei after progesterone injection [27] which has been confirmed by the present results. Perichromatin granules

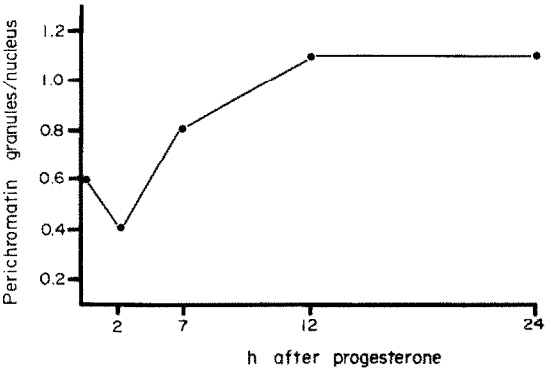

Fig. 4. The change of the number of perichromatin granules per chick oviduct epithelial cell nucleus after progesterone injection.

are supposed to contain mRNA sequences in the nuclei [34].

From the data presented here, we conclude that we have identified avidin mRNA activities in the nuclear and cytoplasmic fraction of the chick oviduct. These can be translated in a heterologous cell-free system. Progesterone stimulates cytoplasmic, but decreases nuclear messenger activity for avidin. Simultaneously an increased ribonuclease activity has been found. Thus, the transfer and cleavage of the nuclear pre-mRNA is activated by progesterone. This is a potential mechanism of the non-transcriptional control of avidin synthesis in the chick oviduct. It is tempting to speculate that the transcription of the avidin mRNA is induced by estrogen whereas the major effect of progesterone is the promotion of the translation of the avidin mRNA.

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DISCUSSION

Hamilton. I was intrigued by your EM polyribosomes where you suggest that 8–10 ribosomes per messenger. Are they molecules and I would like to know, did you take those samples that you analyzed from sucrose gradients?

Tuohimaa. These polysomes are the bottom fraction of the sucrose gradient. After the sucrose gradient polysomes are precipitated by an avidin antibody.

Hamilton. What was the pH of your homogenization medium that you used initially?

Tuohimaa. It was 7.5.

O'Malley. Your mRNA appears to be much larger than one would predict for a 15,000 molecular weight protein which should be only about 400 nucleotides. Since a number of messages are reported to have about 200–400 extra sequences, one would expect that this mRNA would be no larger than 600–800 at most you would think you would want nucleotides, yet you report 3,000. Do you have any explanation for this?

Tuohimaa. We cannot yet completely exclude the possibility of an aggregation. An aggregation is especially poss-

ible during the sucrose gradient centrifugation. On the other hand, the RNA aggregates would not be resistant to the stretching technique for electron microscopy. Thus, the molecules observed under an electron microscope are not aggregates. The size of the messenger for avidin could theoretically be also as large as 2000 nucleotides if it codes for the entire avidin molecule, but still there are several extra nucleotides in our extracted messenger.