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On the Mechanism of Hormone Action

XVIII. Alterations of the Nature of RNA Synthesized in Isolated Fat Body Cell Nuclei as a Result of Ecdysone and Juvenile Hormone Action

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The RNA synthesized in isolated fat body cell nuclei of blowfly larvae under the influence of ecdysone and juvenile hormone was studied by sucrose density centrifugation and RNA-DNA-hybridization.

RNA produced in isolated nuclei of larvae in the early third instar has a polydisperse sedimentation pattern, showing a maximum at about 14 S, while RNA synthesized in nuclei of larvae in a later developmental stage has, in average, lower sedimentation coefficients. No significant differences in sedimentation properties could be seen between RNA of control and hormone-treated nuclei.

An investigation of the hybridization of RNA synthesized in hormone-treated nuclei showed that the RNA produced under the influence of ecdysone or juvenile hormone is qualitatively different from that of the controls. This points to a specific effect of the hormones on the genome. The antagonistic effect of ecdysone and juvenile hormone on RNA synthesis is discussed on the basis of hybridization studies.

In a previous communication we have reported the stimulating effects of ecdysone and juvenile hormone on RNA synthesis in isolated fat body nuclei of the blowfly Calliphora erythrocephala. These results were consistent with similar effects observed with ecdysone in epidermis cell nuclei of the blowfly and point to a direct action of both hormones on the cell nucleus.

The effects of ecdysone and juvenile hormone on RNA biosynthesis in isolated nuclei can give information about the mechanisms of hormone action, the interrelationship between both hormones and the

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degree of responsiveness of nuclei from different tissues to the same hormone. An important prerequisite for these investigations is the characterization of the RNA synthesized under the influence of the hormones. In this work we have analysed this RNA by two methods: sucrose density centrifugation and RNA-DNA-hybridization.

Materials and Methods

Animals

*Calliphora erythrocephala* Meig larvae were reared as previously described. Only larvae during the feeding period of the third instar were used for these experiments.

Isolation of nuclei and RNA synthesis in vitro

The isolation technique described in our previous communication was used. This consisted essentially of: a) homogenization of fat body cells in a buffer containing 0.25 M sucrose, 0.025 M potassium chloride, 0.02 M magnesium chloride, 0.012 M ascorbic acid and 150 IU/ml penicillin G in 0.05 M tris-HCl buffer, pH 7.55, b) centrifugation over 10% Ficoll and c) filtration through Millipore filters type NC. For the analytical characterization of RNA synthesized in vitro large quantities of nuclei were required. For example, three Millipore filters type NCWP with a diameter of 25 mm were used for the preparation of nuclei from 250 larvae (with an average weight of 40—55 mg).

The filters with adhering nuclei were washed and maintained before incubation in an hypotonic medium, containing 6 μmoles mercaptoethanol and 60 IU penicillin G in 190 μl 0.065 M tris-HCl buffer, pH 7.9. The concentration of nuclear material was 30—60 μg protein/nucleus incubation batch. Incubation was carried out for 10 min with hormones and then for 15 to 20 min with RNA precursors in a water bath at 25 °C. The end concentrations in 300 μl incubation medium were 0.5 μmoles each of ATP, GTP and CTP (Boehringer), 3.0 μmoles creatine phosphate, 10 μg creatine phosphokinase, 3.0 μmoles mercaptoethanol, 6 μmoles magnesium sulfate, 60 IU penicillin G and 20 μmoles tris-HCl, pH 7.9. The concentration of the hormone in this medium was 1 μg/ml ecdysone or 0.1 μg/ml juvenile hormone. Ecdysone was prepared by chemical synthesis and was a gift of Schering AG, Berlin. The juvenile hormone preparation was kindly provided by Prof. H. Roeller and consisted of a mixture of 24% juvenile hormone, 22% of the trans, cis, trans form and 54% of the all-trans form of juvenile hormone. The determinations of protein, DNA and RNA were made as previously described.

*RNA extraction*

We used the method of Schütz, Gallwitz, and Sekeris for the isolation of RNA. After incubation, the nuclei were suspended in a buffer containing 0.14 M NaCl, 140 μg/ml polyvinyl sulfate, 4 mg/ml bentonite and 0.05 M sodium acetate, pH 5.0. An equal volume of water-saturated phenol was added and the mixture vigorously shaken at 65 °C for 10 minutes. The emulsion obtained was centrifuged for 5 min at 3500 x g and the aqueous phase extracted until no interphase could be seen. The final aqueous phase was shaken with ether to remove traces of phenol and precipitated with two volumes cold ethanol at −20 °C.

*DNA isolation*

DNA was isolated from crude nuclear preparations of *Calliphora* pupae by the method of Marmur with different modifications consisting essentially in: a) a treatment with 1.7% sodium dodecyl sulfate for 30 min at 4 °C, b) incubation with ribonuclease for 1 hour and c) incubation with pronase for 12 hours. DNA from salmon sperm was purchased from Calbiochem.

 Sucrose density gradient analysis

Sedimentation analysis were made with linear sucrose gradients (5 — 20%, 5 — 30% or 15 — 30%) containing 0.14 M sodium chloride and 0.05 M sodium acetate, pH 5.0. Centrifugation was carried out under different conditions (see figure legends). The gradients were collected with an ISCO fraction collector and the fractions precipitated on paper discs (Schleicher & Schuell paper 2043b) with ice cold 5% perchloric acid, washed twice with cold 5% perchloric acid, methanol and ether, dried and counted in a Nuclear Chicago scintillation counter. The sedimentation coefficients were estimated according to Martin and Ames with cytoplasmic RNA from rat liver or yeast RNA as standard.

 Hybridization

Hybridization was carried out according to Drews and Brawerman and Nygaard and Hall. DNA was denaturated by boiling for 20 min in 0.04 M KCl and 0.03 M tris-HCl buffer, pH 7.8 and rapidly cooled at −20 °C. Freshly denatured DNA and 14C or tritium-labelled RNA were incubated in 1 ml of 0.6 M NaCl and 0.06 M sodium citrate, pH 7.0, in tightly

3 G. Schütz, D. Gallwitz, and C. E. Sekeris, European J. Biochem. 4, 149 [1968].
6 W. Furrmein, S. J. Berry, and M. Swindlehurst, Biochim. biophysica Acta [Amsterdam] 149, 190 [1967].
closed scintillation vials at 67 °C for 18 hours. After annealing, the samples were cooled and treated with 5 μg ribonuclease dissolved in 1 ml distilled water for 20 minutes. The samples were diluted with 15 ml of 0.9 M NaCl and 0.09 M sodium citrate, pH 7.0, collected by suction filtration on nitrocellulose filters (Sartorius SM 11306, 27 mm, 0.45 μl) washed with about 80 ml of the same buffer, dried and counted by liquid scintillation.

**Results**

**Sedimentation characteristics of in vitro synthesized RNA**

The sedimentation pattern of RNA synthesized in isolated nuclei from young *Calliphora* larvae is shown in Fig. 1. The nuclear RNA fraction extracted by the hot phenol method consisted essentially of a large peak of low molecular weight material (2 to 5 S), a 18 S peak and minor fractions at 10 S, 14 S and 21 - 23 S.

![Fig. 1](image1.png)

Fig. 1. Sucrose density gradient sedimentation profiles of RNA synthesized in isolated fat body cell nuclei from young larvae with an average weight of 28 mg. The nuclei were isolated as described in Methods. After incubation the RNA was extracted by the hot phenol method and samples were centrifuged for 3.3 hours at 60,000 rev/min in the SW 65 rotor of the Spinco Model L-65 preparative ultracentrifuge at 0 °C on linear sucrose gradients of 5 to 30%. --- A254 nm; \( \bullet \) --- \( ^{14} \)C-counts/min.

The newly synthesized RNA had a maximum at about 14 S and its polydisperse sedimentation pattern was quite different from the absorption peaks at 254 nm. RNA synthesized by nuclei from larvae older than 4 days and with a weight higher than 40 - 50 mg showed lower sedimentation values (Fig. 2).

![Fig. 2](image2.png)

Fig. 2. Changes of sedimentation coefficients of RNA synthesized in isolated nuclei from larvae in different stages of the feeding period.

The differences between sedimentation patterns of controls and hormone-treated nuclei were investigated (Fig. 3). No significant changes could be detected after incubation with ecdysone or juvenile hormone in any of the stages of the feeding period.

![Fig. 3](image3.png)

Fig. 3. Sucrose density gradient sedimentation profiles of RNA synthesized in isolated fat body nuclei after hormone treatment. The incubation and extraction were made as indicated in Fig. 1. Centrifugation was for 3.5 hours at 60,000 rev/min. Linear sucrose gradients of 5 to 30%. --- A254 nm; \( \bullet \) --- \( ^{14} \)C-counts/min.

The stimulation of RNA polymerase activity and the sedimentation analysis of the RNA synthesized after incubation with the hormone could be interpreted as a rather general and unspecific increase in RNA synthesis under the influence of both hormones. In order to detect changes in the population of RNA molecules after hormone treatment a more sensitive method of analysis was required. One of the most sensitive procedures available at the moment...
for the investigation of this problem is the RNA-DNA-hybridization technique. We therefore tried to analyse the in vitro-synthesized RNA by this method.

**RNA-DNA-hybridization**

Some characteristics of the hybridization technique used in this work were examined in preliminary experiments. The results can be summarized as follows: a) No radioactivity could be detected on nitrocellulose filters after incubation in standard conditions without DNA. b) The maximal denaturation of DNA, as determined by the increase of UV-Absorption, was reached 15 min after boiling. c) The RNA synthesized in vitro hybridized specifically with *Challiphora* DNA; only a very low activity could be observed after annealing with salmon sperm DNA. d) The maximal hybridization was reached with DNA/RNA ratios of about 5:1. For the experiments with DNA-saturation we routinely used 100 μg DNA and 20 μg RNA. e) The RNA-saturation curves varied somewhat from experiment to experiment. In some cases the maximal incorporation was reached with RNA : DNA ratios of 6 : 1, while more RNA was required in other experiments. For hybridization-competition studies we have used a ratio of labelled RNA : DNA of 6 : 1.

For the analysis of the RNA synthesized in hormone-treated nuclei, we have used the method of DNA saturation, which has been already employed by different authors to determine differences in RNA after hormone treatment\(^\text{10,11}\), after UV radiation\(^\text{12}\) and also to characterize hybridized RNA\(^\text{13}\). This method does not require excessively high quantities of RNA and eliminates the problem of RNA-RNA interactions in hybridization-competition studies. The hybridizability of RNA synthesized in control nuclei and hormone-treated nuclei from the same isolation batch was measured; the result was expressed as percent of the controls. In this manner it was possible to compare the hormonal effects of different experiments independent of the variation in the level of hybridization. The results of these experiments are presented in Table 1. RNA synthesized by ecdysone- or juvenile hormone-treated nuclei hybridized significantly more than the RNA of control nuclei. This effect could not be imitated by a juvenile hormone isomer devoid of juvenile hormone activity in vivo.

In order to test the results obtained with the technique of DNA saturation, hybridization-competition studies were performed. Only one concentration (Labelled RNA : unlabelled RNA = 1:1) was tested, because it is difficult to isolate enough RNA for this kind of experiments. RNA from ecdysone-treated nuclei was able to compete more efficiently than the controls by about 12 - 15% of the initial hybridization. The same experimental design with juvenile hormone gave similar results. These results can be considered as an additional support of the data obtained with the more accurate method of DNA saturation.

In other series of experiments we studied the interaction between ecdysone and juvenile hormone. In a previous communication we have reported an interaction between both hormones at the level of the nucleus\(^\text{1}\). This result tempted us to compare the nature of the RNA synthesized in isolated nuclei after treatment with ecdysone and juvenile hormone by the method of competitive hybridization. Some of these experiments are summarized in Table 2. The degree of inhibition of hybridization by a given amount of unlabelled RNA varies considerably from one experiment to the other, as has already been described\(^\text{8}\) in other systems with high concentrations of competing RNA. Independent of the level of inhibition, juvenile hormone-treated nuclei synthesized a RNA which could compete better with the

<table>
<thead>
<tr>
<th>Hormone treatment (see Methods)</th>
<th>Hybridization capacity as [%] of controls</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>1 μg/ml Ecdysone</td>
<td>143</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.1 μg/ml Juvenile hormone</td>
<td>137</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>0.1 μg/ml cis,cis,trans isomer of juvenile hormone</td>
<td>88</td>
<td>—</td>
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Table 1. The effects of hormones on hybridization of in vitro synthesized RNA: The RNA synthesized in isolated nuclei after hormone treatment was extracted by the hot phenol method and its hybridization capacity tested as indicated in Methods. DNA/RNA = 5:1. The hybridization capacity was calculated and expressed as % over controls. The P values were calculated from 10 experiments.

RNA induced by ecdysone than the controls and vice versa. It seems that one part of the ecdysone-evoked RNA synthesis can be also induced by juvenile hormone and this fact could be explained as the activation of some genes by both hormones; the possible explanation of these results will be discussed below.

Discussion

The RNA synthesized in isolated fat body cell nuclei of the blowfly Calliphora erythrocephala showed a polydisperse sedimentation pattern in sucrose-density gradient centrifugation, with a maximum at about 14 S. This maximum was shifted to lower sedimentation values with RNA synthesized in nuclei of old larvae. An increase in ribonuclease activity of the fat body during development could be one of the explanations of this phenomenon. The RNA species synthesized in isolated nuclei have a rather low molecular weight as compared with rapidly labelled RNA synthesized in vivo, a fact which has been already observed with in vitro subcellular systems from plants, insects and mammals. The activation of ribonucleases or a partial damage of the transcriptional system during the isolation of nuclei could explain the difference between RNA synthesized in vivo and in vitro.

Hybridization studies under conditions of DNA saturation indicate a quantitative change in the nature of RNA synthesized in isolated nuclei as a result of ecdysone or juvenile hormone action. This effect suggests a specific action of both hormones on transcription. An hormone action via nuclear membrane or nucleotide pool should be excluded, because the nuclei were first preincubated in an hypotonic medium and subsequently in the presence of excess nucleotides. On the other side, the nuclei were damaged or fractionated during the isolation. A direct interaction of both hormones with the chromatin, as has been observed with cortisol, is most probable.

The RNA synthesized under the influence of the hormones had a significantly higher hybridization capacity than that of the controls. This fact could be interpreted as an increase of either the functional messenger RNA (or D1-RNA) or an increase of the RNA which is rapidly degraded in the cell nucleus (D2-RNA). D2-RNA shows a much higher hybridizability than D1-RNA. According to the theory of Georgiev on the regulation of transcription in eukaryotic cells, D2-RNA is the RNA transcribed from regions of DNA with highly repetitive sequences. These regions of the genome are supposed to be involved in the regulation of transcription. The considerable increase of RNA-hybridization after hormone treatment (ecdysone or juvenile hormone) could be due to an increased synthesis of D2-RNA, but further characterization is necessary to confirm this assumption.

The results obtained under conditions of DNA saturation were confirmed by the method of competitive hybridization. The fact that the same effect is seen using different experimental designs is very important, because results based only on competitive

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**Table 2. Hybridization-competition studies with ecdysone- and juvenile hormone-evoked RNA species in fat body nuclei**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unlabelled RNA</th>
<th>Percentage of initial hybridization</th>
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<tbody>
<tr>
<td>Ecdysone-treated nuclei</td>
<td>51 mg control nuclei</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>61 mg juvenile hormone-treated nuclei</td>
<td>66</td>
</tr>
<tr>
<td>Juvenile hormone-treated nuclei</td>
<td>56 mg control nuclei</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>56 mg juvenile hormone-treated nuclei</td>
<td>42</td>
</tr>
</tbody>
</table>

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hybridization are not always considered to be definitive evidence for qualitative changes in RNA. This technique seems to give quite variable results, especially if the RNA:DNA ratios are not high enough\textsuperscript{23}; there are different opinions about the necessity of preincubation of the DNA with the competing RNA\textsuperscript{23}, or the need of simultaneous incubation of competing and labelled RNA\textsuperscript{24}. On the other hand, large quantities of RNA are required. This is the main limitation in our system, because only relatively few animals can be prepared at the same time. The rapid degradation of the in vitro synthesized RNA is an additional complication. Under these conditions only restricted number of samples can be tested at the same time.

In a previous communication some integrations between ecdysone and juvenile hormone at the level of the nucleus were described\textsuperscript{1}. By incubating nuclei with both hormones together, RNA synthesis was partially inhibited. Patel and Madhavan\textsuperscript{25} have recently reported a similar effect on RNA synthesis in imaginal wing disks of the Ricini-silkworm after injecting a mixture of the two hormones. The working hypothesis was put forward, that some genes could be regulated by both hormones at the same time\textsuperscript{14} (without excluding the possible existence of specific ecdysone- or juvenile hormone-dependent genes). According to this model, one hormone alone would lead to activation of the ecdysone- and juvenile hormone-dependent genes, while both hormones together would inactivate them. In this manner, the fluctuating concentrations of both ecdysone and juvenile hormone in the larvae could lead either to stimulation of RNA synthesis in some stages of development or to inhibition of this production in other stages. The competition studies described in this paper, while subject to the known limitations of this hybridization technique, are consistent with the existence of ecdysone- and juvenile hormone-dependent genes. Work is now in progress to establish whether these genes produce informational RNA or whether they represent repetitive sequences involved in regulatory processes.

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\textsuperscript{24} E. G. Piker, A. V. Lichtenstein, and V. S. Shapot, Biochim. biophysica Acta [Amsterdam] 174, 758 [1968].
\textsuperscript{25} N. Patel and K. Madhavan, J. Insect Physiol. 15, 2141 [1969].