Changes in Gene Expression during Larval Development of *Calliphora vicina* Induced by Ecdysterone

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The influence of the insect hormone ecdysterone on the composition of the RNA from the fat bodies of *Calliphora* larvae was investigated by hybridization experiments. DNA was iodinated *in vitro* with ^{125}J. In saturation experiments, total RNA was hybridized to total DNA; under similar conditions, poly(A) RNA was hybridized to unique DNA sequences. RNA isolated from animals treated with ecdysterone exhibits a higher percentage of hybridization than a corresponding control RNA from the same developmental stage, a result even more prominent when poly(A) RNA was hybridized with unique DNA. It is concluded that ecdysterone induces the transcription of new unique DNA sequences.

Introduction

The ecdysteroids belong to the insect hormones which induce morphogenetic changes and control differentiation processes. According to a widely accepted hypothesis, ecdysterone acts by activation of certain genes (see recent review by Karlson *et al.* [1]). When activated these genes should give rise to synthesis of qualitatively new RNA species.

It has been shown that treatment with ecdysteroids led to a stimulation of total RNA synthesis in fat body tissue of *Calliphora vicina* [2]. Ecdysterone causes the *de novo* synthesis of poly(A) containing RNA [3]. The induction by ecdysterone of a new mRNA coding for DOPA-decarboxylase in the epidermis cells of *Calliphora* has been reported by Fragoulis and Sekeris [4]. We have now investigated by means of DNA-RNA-hybridizations, whether – in addition to the quantitative changes – the composition of fat body RNA is influenced by ecdysterone and by the developmental stage of the larvae.

Nucleic acid molecular hybridizations allow quantitative estimates of the percentage of the genome complementary to a given RNA preparation. However, the technique depends upon obtaining RNA or DNA with high specific radioactivity. This could not be achieved by labelling procedures *in vivo*. Therefore we have performed iodination *in vitro* to obtain [^{125}J]DNA with a high specific activity.

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Materials and Methods

Chemicals

Carrier free Na^{125}J (in NaOH solution) was purchased from NEN-Chemicals, $TlCl_3$ from ICN Pharmaceuticals, Poly(U)-Sepharose-4B from Pharmacia, Hydroxyapatite (Bio Gel HTP) from Bio-Rad Laboratories and Ecdysterone (β-ecdysone) from Rohto, Osaka (Japan). Pronase and Deoxyribonuclease I were obtained from Boehringer, Mannheim. S1 nuclease from *Aspergillus oryzae* was a gift from Dr. J. Niessing, Marburg. All other chemicals used were of reagent grade.

Breeding of Calliphora, preparation of fat bodies and ecdysterone treatment

Calliphora vicina R.-D. (*Calliphora erythrocephala* Meigen) was reared on beef meat at 25 °C and relative humidity of 65%. In order to synchronize development of the larvae, blowflies were allowed to deposit eggs only for 45 min daily. Under these conditions the larvae left the meat 6 days after egg deposition and pupated when 7 days old.

In our experiments we used RNA prepared from fat bodies of 5 days old larvae and white puparia. Three hours before preparation of the fat body tissue, each animal received an injection of a) 20 ng ecdysterone in 5 μl insect saline, or b) 5 μl insect saline (control animals).

Isolation and purification of RNA; preparation of poly(A)-RNA

RNA from fat bodies was prepared as previously described [2]. Poly(U)-sepharose chromatography of RNA was performed as published by Molloy *et al.* [5] with the exception that the elution buffer did not contain formamide. The poly(A) fraction was

eluted at 37 °C with 0.1 M Tris buffer (pH 7.4) containing 0.2% sodium-dodecylsulphate. The RNA solution was dialysed for 12 hours against water, then lyophilized and dissolved in hybridization buffer A (1 mM Tris, pH 7.4, 0.5 M NaCl, 0.1 mM NaEDTA) immediately before hybridization procedure.

Isolation of DNA; preparation of unique DNA

DNA was isolated from 2 days old pupae according to the method of Hastings and Kirby [6].

The ethanol precipitated DNA was resolved in 0.18 M equimolar phosphate buffer ($Na_2HPO_4 - NaH_2PO_4$), pH 6.8 and further purified by hydroxyapatite chromatography [7]. The purified DNA was dissolved in SSC-buffer (0.15 M sodium chloride, 0.015 M sodium citrate), sonicated twenty times for five seconds by a Branson Sonifier, step 5, then heated to 100 °C for 10 min and quickly cooled to 55 °C. The DNA was allowed to renature to a cot-value of 60 and was then separated into unique and repetitive sequences by hydroxyapatite column chromatography [8]. The unique DNA fraction was eluted with 0.18 M phosphate buffer (pH 6.8) and precipitated with ethanol.

In vitro iodination of DNA

DNA from the unique DNA fraction was iodinated according to the method of Orosz and Wetmur [9].

The reaction mixture contained in a volume of 2 ml: 10^{-5} M KJ, 3 mCi of carrier free [^{125}J]iodine (NaJ), 0.1 M/0.04 M sodium acetate-acetic acid, pH 5.0, 5×10^{-4} M $TlCl_3$ and 20 μg of DNA, which was denatured at 100 °C for 5 min. The solution was incubated at 60 °C for 30 min and then rapidly cooled to 4 °C. The iodinated DNA was separated from the reaction mixture on a Sephadex-G-25 column, (1×27 cm) equilibrated and eluted with water.

The labelled DNA in the void volume was dialysed at 60 °C for 5 h against buffer B (0.4 M NaCl, 15 mM NaH_2PO_4, and 2 mM NaEDTA-NaOH; pH 6.0) to remove unstable side products of the iodination reaction. The resulting [^{125}J]DNA was dialysed at 4 °C for 12 h against water, then lyophilized and dissolved in hybridization buffer A.

The specific activity of the unique DNA was 5.55×10^6 cpm/μg DNA, and of total DNA 3.9×10^6 cpm/μg.

DNA-RNA-hybridization

DNA-RNA hybridization was done in solution. The reaction mixture contained in a volume of 250 μl buffer A the appropriate amount of RNA and

a) 0.064 μg [^{125}J]DNA (2.5×10^5 cpm) when hybridized with total fat body RNA;
b) 0.45 μg [^{125}J]DNA (2.5×10^6 cpm) (unique DNA) when hybridized with poly(A) RNA.

The hybridization mixtures were heated in a conical tube of silicone for 10 min at 100 °C and then transferred to a 69 °C bath.

At various times, aliquots of 10 μl were removed and diluted in 2.1 ml of buffer C (0.03 sodium acetate, pH 4.5, 0.3 M NaCl, 0.003 M $ZnCl_2$). To 1 ml of this solution, 25 μg denatured calf thymus DNA and 150 μg bovine serum albumin were added as carriers, and the reaction mixture immediately precipitated with 1 ml of 10% trichloracetic acid. A second aliquot of 1 ml was incubated at 45 °C for 1 hour in the presence of single-strand specific nuclease from *Aspergillus oryzae* (Vogt [10]). The amount of radioactive DNA resistant to digestion was determined by precipitation as above. The precipitates were collected on glass fiber filters (GF/C; Whatman) washed five times with 1 M HCl containing 0.1 M tetrasodium-pyrophosphate, twice with ethanol and dried. The radioactivity was determined with a toluene based scintillation fluid in a Berthold counter BF 5003. The amount of the DNA-RNA hybrids was expressed as the radioactivity in the S1 nuclease digest divided by the radioactivity in the non-digested aliquot.

Renaturation of DNA

The DNA was dissolved in SSC buffer and sheared by sonifying. The sheared DNA was denatured at a concentration of 630 μg/ml in a cuvet with 1 mm light path. The rate of renaturation was pursued by measuring the hypochromicity at 260 nm in a recording spectrophotometer Beckman Acta CII.

The iodinated DNA (0.26 μg/ml) was renatured with an excess amount of unlabelled DNA (1.7 μg/ml) which effectively drives the reaction. Aliquots were removed and the fraction of renatured DNA was expressed as described in the former chapter.

Results

Renaturation kinetics of Calliphora DNA and integrity of iodinated DNA

In preliminary experiments we found that nucleic acid preparations labelled to greater than 10^7 cpm/μg lost the capacity for hybridization. Therefore, DNA labelled up to 5×10^6 cpm/μg was used in our experiments.

The stability of [^{125}J]DNA was tested on polyacrylamide gels. The iodinated DNA showed the same electrophoretic mobility before and after the hybridization procedure (Fig. 1).

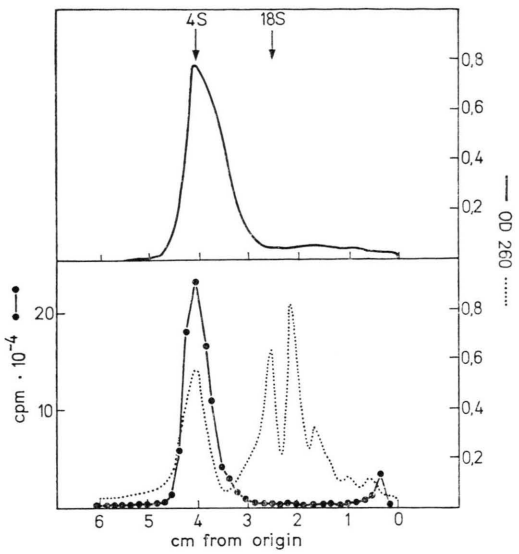

Fig. 1. Polyacrylamide gel electrophoresis of DNA before and after *in vitro* labelling with ^{125}J. —— Unique sequence DNA from 1 day old pupae; ●—● unique sequence DNA after iodination (both DNA solutions sonicated); ···· total RNA from white puparia as marker.

The integrity of [^{125}J]DNA after the labelling procedures was tested by comparing the renaturation behaviour of iodinated und unlabelled DNA. From Fig. 2 it can be seen that both DNA solutions reassociated with the same velocity until cot 50. At higher cot values, the reaction rate of iodinated DNA was lower. 76% of the [^{125}J]DNA anneals with a cot of 1000 whereas 96% of the unlabelled DNA reannealed after this time.

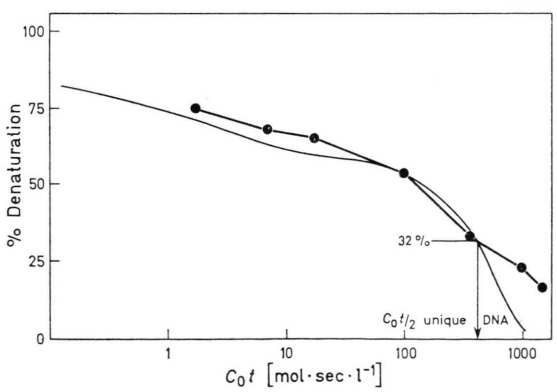

Fig. 2. Renaturation of sheared and denatured DNA from 9 days old *Calliphora*. —— Unlabelled DNA; ●—● [^{125}J]-DNA. Renaturation was measured by following the hypochromicity at 260 nm.

By hybridizing RNA with DNA in solution, two reaction products are formed simultaneously: a) DNA-RNA-hybrids and b) DNA-DNA double-strands. To correct the values for the DNA which reanneals as DNA-DNA duplex, [^{125}J]DNA was incubated alone for the same length of time and the amount of nuclease S1 resistant radioactivity was substracted from the total label.

We did not choose longer hybridization times than 7 days, corresponding to a Rot value of 5000, because aliquots taken later gave differing values probably resulting from degradation.

To minimize the competing DNA-DNA reaction we annealed

a) [^{125}J]RNA to filter bound DNA;
b) RNA to filter bound [^{125}J]DNA.

Since a variable amount of DNA was lost from the filters, we did the hybridization reaction in solution using posthybridization filtration to separate DNA-RNA hybrids (*cf.* Scherberg and Refetoff [11]).

Hybridization of total RNA with total DNA

In these series of experiments we hybridized a vast excess of total fat body RNA with [^{125}J]DNA (RNA : DNA = 10^4 : 1) and analyzed the resulting hybrids by measuring the amount of labelled material resistant to single strand specific nuclease. We compared RNA derived from 5 days old ecdysterone treated larvae with RNA from untreated control animals.

Fig. 3 shows that a considerable degree of transcription could be observed in fat body tissue of

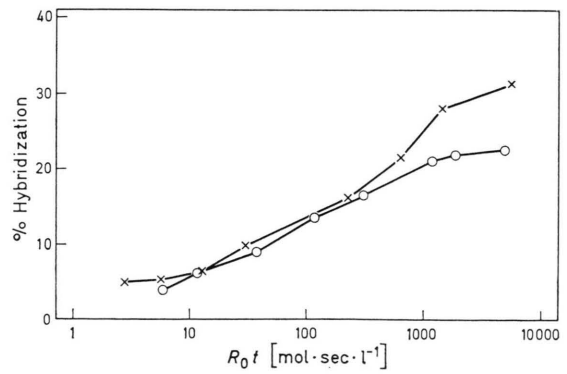

Fig. 3. Hybridization of [^{125}J]DNA with a vast excess of total RNA derived from ○—○ 5 days old larvae (controls); ×—× 5 days old larvae treated each with 20 ng ecdysterone 3 h before preparation of the fat bodies. 0.06 μg [^{125}J]DNA (specific activity 3.9×10^6 cpm/μg) was hybridized in solution with 725 μg RNA in a volume of 250 μl.

five days old larvae. Total RNA from fat bodies hybridized with 21.5% of the *Calliphora* DNA. But the amount of DNA transcribed into RNA becomes 31% when the RNA derived from ecdysterone treated animals. Assuming that transcription is largely asymmetric, this represents 62% of the sequences in total DNA, whereas only 43% of the DNA is transcribed in control animals which had not received an injection with ecdysterone.

Hybridization of unique DNA with poly(A) RNA

To separate unique DNA sequences from repeated DNA sequences we renatured sheared *Calliphora* DNA as described in Methods. Fig. 2 indicates that the renaturation curve of unlabelled DNA shows a plateau between cot 20 and cot 70, that means that in this area the nonrepeated sequences begin to renature. The different parts of the renaturation curve were normalized and compared with the theoretical curve (Laird and McCarthy [12]). The best fit of the curves was reached when we took 64% of the DNA as last fraction comprising the unique sequences and 36% as repeated sequences. The cot/2 for the unique sequence fraction was 430 mol $\times$ sec $\times$ 1^{-1}.

The slow renaturing fraction of *Calliphora* DNA with a cot/2 of 275 (mol $\times$ sec $\times$ 1^{-1}) may be used to provide estimates of the diversity of sequences transcribed from unique sequences, which are believed to code for mRNA. By hybridizing unique DNA with poly(A) RNA we get an estimate of at least a part of the genetic information which is transcribed into mRNA in dependance of the developmental stage and under the influence of ecdysterone. Therefore we have separated repetitive DNA sequences from single copy sequences by means of hydroxyapatite column chromatography after reannealing a denatured DNA sample to a cot of 60. Poly(A) RNA was isolated by affinity chromatography of total RNA on poly(U) sepharose as mentioned in Materials and Methods.

The analysis of the resulting poly(A) containing RNA on polyacrylamide gels shows that the size of the isolated molecular species ranges in the messenger region between 4S and 22S [2].

The hybridization time was 168 hours, sufficient to reach saturation under the conditions of reaction. Since saturation values represent only a small percentage of the input DNA, we used 0.45 μg [^{125}J]-DNA (2.5×10^6 cpm) in 250 μl reaction volume, that is ten times the concentration used in the hybridization with total RNA.

Self annealing of unique [^{125}J]DNA was measured in a parallel experiment to be about 1.2%. This value was substracted from the values obtained for poly(A) RNA hybridization with unique DNA.

Fig. 4 shows that the poly(A) containing RNA from fat body tissue of 5 days old larvae hybridized with 1.4% of the unique sequence DNA of the *Calliphora* genome. The poly(A) RNA from animals,

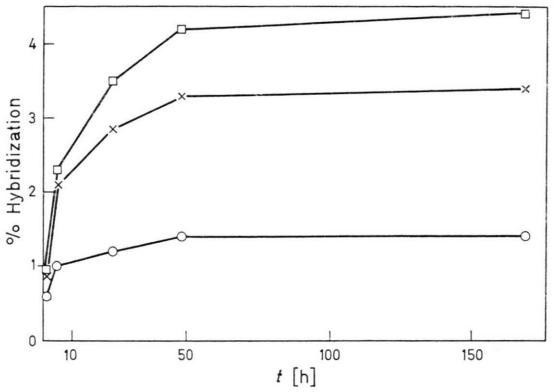

Fig. 4. Hybridization of poly(A) containing RNA with unique sequences of [^{125}J]DNA. 0.45 μg (2.5×10^6 cpm) single copy [^{125}J]DNA was hybridized with 142.5 μg of poly(A) RNA derived from fat bodies of ○—○ 5 days old larvae; ×—× 5 days old larvae treated each with 20 μg ecdysterone 3 h before preparation of the tissue; □—□ white puparia.

which had received an injection of 20 ng ecdysterone three hours before preparation of the fat bodies, hybridized to an about three fold higher amount (3.4%) with unique sequences of DNA. These data indicate that ecdysterone induces the transcription of mRNA from unique sequence DNA. In earlier experiments we could demonstrate by competitive hybridization experiments that ecdysterone stimulates the synthesis of a qualitatively new class of poly(A) containing RNA [3]. In another experiment, also shown in Fig. 4, we used poly(A) containing RNA from white puparia to hybridize with unique sequence DNA. At this developmental stage the endogeneous moulting hormone titer is at a maximum so that we could examine if the response of the fat body would be altered by high levels of endogeneous hormone. It can be seen that the poly(A) containing RNA from white puparia shows a much higher percentage of hybridization (4.4%) than the poly(A) RNA from untreated 5 days old larvae, where the endogeneous hormone titer is at a minimum.

Discussion

Eukaryotic genomes contain unique and repeated base sequences which have been detected by an analysis of the renaturation kinetics of DNA [13]. We used this method to separate the repeated DNA sequences from the unique DNA sequences which are believed to code for mRNA.

The isolation of poly(A) containing RNA by poly(U)-sepharose chromatography enables us to enrich mRNA from fat body tissue of *Calliphora*. By means of molecular hybridization we can compare the percentage of the genome that is transcribed during different developmental stages of the larvae and under the influence of the moulting hormone ecdysterone.

Our data from hybridization experiments of poly(A) RNA with unique sequence DNA show:

(i) Fat body tissue of 5 days old larvae contain an amount of poly(A) containing RNA that hybridizes with 1.4% of the unique sequences of the *Calliphora* genome.

(ii) The mRNA in white prepupae hybridizes to 4.4% of the unique sequence DNA. These mRNA copies might correlate with the physiological potency to form imaginal tissues. Cytological investigations further show that the fat body of white puparia contains more heterogeneous cells than the fat body in earlier development stages (R. Marx, personal communication). More heterogeneous cells contain more transcriptional capacity inducing a higher percentage of transcription per tissue.

(iii) After ecdysterone treatment, the poly(A) RNA hybridizes with 3.4% of the unique DNA. We interprete this increase as the result of expression of hitherto unexpressed genes; this confirms the hypothesis that ecdysteroids act by activation of certain genes. Another, less probable explanation would be that ecdysteroids alter the metabolism of mRNA by influencing the half life of poly(A) sequences, which are believed to stabilize the mRNA molecule [14].

The saturation values can be used as a measure for the differences between the RNA preparations compared in our experiments.

However, in hybridization reactions some uncertainities remain:

a) Because of the high complexity of the eucaryotic DNA and the resulting low reaction rate of hybridization it is possible that some potentially hybridizable RNA species are degraded before formation of stable duplexes with DNA.

b) It is difficult to reach a true saturation because the RNA is too heterogeneous and contains many frequency classes. It is possible that those sequences with low frequency will not react completely.

For these reasons, our hybridization data should not be interpreted as an absolute value of the percentage of total DNA transcribed.

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