20-Hydroxyecdysone stimulates the accumulation of translatable yolk polypeptide gene transcript in adult male *Drosophila melanogaster*

(steroid hormone action/juvenile hormone action/vitellogenin/ecdysone/RNA blot)

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ABSTRACT Yolk polypeptide (YP) synthesis is hormonally stimulated during maturation of adult female *Drosophila melanogaster*. Synthesis of the three YPs is sex specific and occurs in fat body cells and follicle cells of adult females. However, males have been shown to produce YPs when treated with the steroid hormone 20-hydroxyecdysone (20-HE). By using a cell-free translation system as an assay for YP mRNA, we found that 20-HE also causes the accumulation of translatable YP message in males. In addition, hybridization of cloned copies of genes for both YP1 and YP3 to total RNA from males showed that 20-HE caused the appearance of YP gene transcripts in males. Eight hours after treatment of males with 20-HE, YP gene transcript levels had increased at least 25-fold to approximately 2.7×10^6 copies of YP1 gene transcript per adult male fly. In normal adult females, there were 42×10^6 copies per fly by 24 hr. There was neither detectable YP synthesis nor translatable YP gene transcript in either normal 1- to 3-day-old males or 24-hr-old males treated with a juvenile hormone analogue. This evidence shows that 20-HE acts to regulate the levels of translatable YP mRNA in male *Drosophila*.

Although in vertebrates expression of dimorphic characters is often regulated by sex-specific hormones (1), in insects sexual characteristics are typically determined by a cell's genotype rather than by its hormonal environment (2). Despite the lack of sex hormones, some hormonally regulated sex-specific polypeptides are found in insects, one group being the vitellogenins or yolk polypeptides (YPs). In *Drosophila melanogaster*, the three YPs (3) become the most rapidly synthesized polypeptides secreted into the hemolymph of young adult females, but these polypeptides do not appear in the males (4). The three YP genes (*Yps*) not only are sex limited in their expression but also are sex linked (5, 6). Synthesis of the YPs in females occurs both in the fat body cells (7, 8) and in the follicle cells of maturing oocytes (4, 9–11).

Synthesis of the YPs in the female has been shown to be under hormonal control. When the abdomen of a female is isolated from the anterior endocrine organs shortly after adult eclosion, the normal increase in YP synthesis which occurs during the first day of adult life is blocked (12). Treating the isolated abdomens with either the insect steroid hormone 20-hydroxyecdysone (20-HE) or a juvenile hormone analogue (JHA) restores the increase in YPs (4, 13). Although the roles of the two hormones in vitellogenesis are not interchangeable, they are both involved in the regulation of YP synthesis (14).

In vertebrates as in insects, vitellogenins are synthesized only in females. In birds and amphibians, this is due to lower estrogen titers in males than in females. Vitellogenin synthesis can be induced in the livers of roosters (15) or male *Xenopus*

(16) by estrogen treatment. Although 20-HE is not a sex steroid in insects, male *Drosophila* are induced to synthesize YPs when treated with 20-HE (10). Males treated with JHA showed no response.

The experiments described here test whether 20-HE acts on males to stimulate YP synthesis by increasing levels of YP mRNA or by increasing the translational rate of constant quantities of YP message and whether JHA can stimulate a transcriptional response of the *Yps*.

MATERIALS AND METHODS

Animals. Wild-type *D. melanogaster* (strain Oregon R) eclosing over a 15-min interval were collected and offered food ad lib for the duration of the experiment. Males were injected with $0.3 \mu\text{l}$ of 0.1 mM 20-HE in 10% ethanol/Ringer or were treated topically with ZR-515 in acetone to a final dose of $0.16 \mu\text{g}$ per fly. This duration of hormonal treatment in flies of this age is known to result in a significant synthesis of YPs (10).

Extraction of RNA. Total RNAs were isolated by extracting flies with phenol/chloroform and precipitating the RNAs from the aqueous phase with ethanol as described (8). The total RNA was finally dissolved in 10 mM Tris-HCl/2 mM EDTA, pH 7.5.

Isolation of Plasmid DNA. Plasmid subclones for each of the *Yps* were constructed by Barnett *et al.* (6). DNAs for the plasmid containing the structural gene for YP1 (pYP1) and the plasmid containing the structural gene for YP3 (pYP3) were purified as described by Gergen *et al.* (17).

Translation of RNAs. The total RNAs were translated in a cell-free rabbit reticulocyte lysate essentially as directed in a New England Nuclear translation kit. Equal quantities (A_{260} units) were added to a lysate reaction mixture containing [^{35}S]methionine. The translates were prepared for immunoprecipitation or for NaDodSO₄/polyacrylamide gel electrophoresis. The solubilized proteins were layered directly on a NaDodSO₄/polyacrylamide gradient gel (4, 18) and electrophoresed. The dried gels were autoradiographed.

Immunoprecipitation of YPs. The cell-free translation lysates were diluted 1:3 with 0.1 M NaCl/2 mM EDTA/10 mM Tris-HCl, pH 7.4 (NET buffer) and 0.05% Triton X-100. To each sample, 0.1 vol of anti-YP antiserum (designated anti-YP) was added, and the mixture was incubated for 12–15 hr at 0°C. Anti-YP was prepared for all three YPs by injecting rabbits with homogenates of soluble egg proteins. After precipitation, 0.1 vol of heat-killed *Staphylococcus aureus* (10%, wt/vol) was added and the mixture was incubated for 2 hr at 0°C. The precipitates

Abbreviations: YP, yolk polypeptide; 20-HE, 20-hydroxyecdysone; JHA, juvenile hormone analogue; Yp, structural gene for YP.

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were pelleted and washed three times with NET buffer. The proteins were solubilized, layered on a NaDodSO₄ gel, and electrophoresed. An autoradiogram was made of the dried gels.

Hybridization Assay for Yp Transcripts. The procedure for RNA transfer was essentially as described by Thomas (19).

The baked filters were prehybridized in buffer (20) at 42°C for 20 hr. The ³²P-labeled probes were made by nick-translation of the plasmids containing the YP genes. The nick-translation procedures were essentially as given for the New England Nuclear nick-translation kit. The labeled probes were passed through a 1.5-ml Bio-Gel P-60 column in a 10 mM Tris·HCl/2 mM EDTA, pH 8.2. The void volume was collected and added to the hybridization buffer (20).

The hybridization was done at 42°C for 20 hr. The blots were washed once in 2× and 1× SSPE (1× SSPE is 0.15 M NaCl/0.01 M sodium phosphate/1 mM EDTA) plus 0.05% NaDodSO₄ at room temperature. The blots were then washed with two changes of 0.1× SSPE plus 0.05% NaDodSO₄ for 30 min at 65°C. With these hybridization conditions, cross-hybridization between the pYP1 probe or the pYP3 probe and the other two YP gene transcripts was reduced to an undetectable level (6). The damp blots were wrapped in Saran Wrap (Dow) and autoradiographs were made with preflashed Kodak X-Omat AR x-ray film and a Dupont Cronex Lightning Plus intensifying screen at -70°C.

Quantitation of YPs and YP Transcripts. Autoradiograms of the gels containing radiolabeled YPs were scanned with a Joyce-Loebl microdensitometer, and the areas of the YP peaks were measured with a polar planimeter (4). Comparison of the peak areas for various concentrations of label demonstrated linearity for this method. For *in vivo* labeled polypeptides, the YPs were

normalized by comparison with the sum of six peak areas for constitutive polypeptides. The immunoprecipitated translates were normalized on the basis of A₂₆₀ units of total RNA added to the cell-free lysate mixture.

To quantitate the number of transcripts present, a RNA blot was constructed that contained known quantities of cloned YP DNA and RNAs from normal females. The stringency of the hybridization conditions was established to allow for equivalent association between RNA·DNA duplexes and DNA·DNA duplexes (21). The quantitated RNA standards were then included on the blots. The copies per sample were computed by scanning the autoradiograms with a Joyce-Loebl microdensitometer and then comparing the measured peak areas with the known standard. The data presented represents the values for RNA preparations from two different experimental groups.

Because the number of fat body cells in adults has never been determined (22), the number of Yp transcript copies per fat body cell is based on the mean (± SEM) number of fat body cells found in larval males (2,160 ± 54) (22). Although the larval fat body cells undergo histolysis by the second day after eclosion (22), we assume here that the number of fat body cells in adults reaches the same number found in the larvae.

RESULTS

Accumulation of YP Transcripts During Normal Adult Female Maturation. Before we investigated the artificially induced response of the males, we examined the parameters of YP synthesis in females during normal maturation. The rapid increase in the rate of YP synthesis in adult females demonstrated previously (4) was confirmed for our experimental ani-

FIG. 1. *In vivo* YP synthesis in normal adult males and females and in hormonally stimulated males. Hormonally stimulated flies were either injected with 0.3 μ l of 0.1 mM 20-HE or were topically treated with 0.16 μ g of ZR-515 in acetone 8 hr prior to collection of blood. Hormonally treated flies and untreated flies were injected with 2 μ Ci or [³⁵S]methionine in 0.2 μ l of Ringers solution 1 hr before collection of blood. The hemolymph polypeptides were resolved on a 0.1% NaDodSO₄/9–12% polyacrylamide slab gel. The figure shows an autoradiogram of the dried gel. Lanes: 1, 24-hr males; 2, 48-hr males; 3, 72-hr males; 4, 20-HE-treated 24-hr males; 5, 20-HE-treated 48-hr males; 6, 20-HE-treated 72-hr males; 7, JHA-treated 24-hr males; 8, JHA-plus-20-HE-treated 24-hr males; 9, 1-hr females; 10, 24-hr females. The labeled bands that appeared in the area of the YPs in the JHA-treated males (lane 7) also appeared in the untreated 24-hr males (lane 1). These polypeptides did not migrate precisely with the YPs and were not immunoprecipitable. They probably were synthesized by the remaining larval fat body cells because they were not present in 2- and 3-day-old males (lanes 2 and 3), when the larval fat body cells disappear (23, 24). NT, no treatment.

Table 1. Quantities of YPs and YP mRNAs in normal males and females and hormonally treated males

Sex	Age, hr	Treatment	Incorporation of [³⁵ S]methionine*			Hybridizable transcript		
			YP1		YP3	pYP1		pYP3
			<i>In vivo</i>	<i>In vitro</i> [†]	<i>In vivo</i>	Copies, no. × 10 ⁻⁶ /fly	% of 24-hr female	% of 24-hr female
Female	0	None	3	<2	<2	1.2	3	<0.2
Female	24	None	100	100	100	42.2	100	100
Male	24	None	<2	<2	<2	<0.1	<0.2	<0.2
Male	24	20-HE, 8 hr	34	13	33	2.7	7	39
Male	24	JHA, 8 hr	<2	<2	<2	<0.1	<0.2	<0.2

Each gel was autoradiographed for two time periods to ensure linearity of the exposure.

* Expressed as % of that found in 24-hr-old female.

[†] Cell-free translation.

males by injecting [³⁵S]methionine into 10 siblings of the flies to be extracted for RNA. After 1 hr the flies were bled, and the newly synthesized radiolabeled polypeptides were visualized by autoradiography after separation of the hemolymph proteins by NaDodSO₄ gel electrophoresis. The three YPs appeared in the hemolymph of 24-hr-old females (Fig. 1; Table 1). Hemolymph from 1-hr females contained only small amounts of newly synthesized YPs. In these animals, the rate of YP synthesis increased 37-fold between 1 hr and 24 hr.

To determine if the increase in YP synthesis during normal female maturation is based on an increase in translatable YP message, total RNA was extracted from females less than 15 min after eclosion (0 hr) and from 24-hr adult females. Equal quantities of total RNA from these flies were translated in a cell-free lysate containing [³⁵S]methionine. The translation mixtures were incubated with anti-YP and the resultant precipitates were resolved by NaDodSO₄ gel electrophoresis. Although immunoprecipitable precursors to YPs were found in translates of RNA from 24-hr females, none were detectable in translates of 0-hr females (Fig. 2). Thus, there is an increase in the amount of translatable YP mRNA between eclosion and 24 hr which coincides with the increased rate of *in vivo* labeling of the YPs during this period, confirming our earlier report (14).

To measure the levels of Yp transcripts, equal amounts of RNA from 0-hr females and 24-hr females were denatured and resolved by agarose gel electrophoresis. The RNAs were blotted to nitrocellulose and then probed with ³²P-labeled pYP1. The autoradiogram of the filter showed the presence of Yp1 transcript in RNA from 24-hr females (Fig. 3A). Yp1 transcript was detected in the RNA from freshly eclosed females only after the autoradiogram was exposed for a considerably longer period. When pYP3 was used as probe, the hybridization signal showed a similar pattern: large quantities of Yp3 transcript in 24-hr females and only a few copies in 0-hr females (Fig. 3B). These results confirm those of Barnett (25) who showed an increase in hybridizable YP transcript for all three YPs. Quantitative analysis of the transcripts present in the 24-hr female showed that the mature adult females contained approximately 42 × 10⁶ copies of Yp1 transcript per fly (Table 1). In the 0-hr females there were approximately 1.2 × 10⁶ copies per fly. This represents a 35-fold increase in the amount of Yp1 transcript in the total fly occurring in the first 24 hr after eclosion.

Expression of YP Genes in Adult Males. Normal adult males 24, 48, and 72 hr old were bled after being injected with [³⁵S]methionine. When the newly labeled polypeptides from the hemolymph were visualized by autoradiography after separation by NaDodSO₄ gel electrophoresis, there was no indication that the YPs were being synthesized (Fig. 1). However, treatment of 16-hr males with 20-HE led to the initiation of YP synthesis and the appearance of labeled YPs in the hemolymph

of the males after 8 hr, confirming previous results (10). YP synthesis in the 24-hr males that had been treated with 20-HE for 8 hr reached about 1/3 of the level of YP synthesis in normal 24-hr females. Treatment of 2- or 3-day-old males also stimulated YP synthesis to about 1/10 of normal females, indicating that the responsiveness of the males may be age related (Fig. 1). In contrast to treatment with 20-HE, JHA caused no ap-

FIG. 2. Cell-free translation of total RNAs from normal males and females and hormonally treated males. Total RNAs (0.05 A₂₆₀ unit/25-μl incubation mixture) of flies were translated in a nuclease-treated rabbit reticulocyte lysate containing 60 μCi of [³⁵S]methionine. The translation products were incubated with anti-YP, and the antigen-antibody complexes were adsorbed to *S. aureus*. The adsorbed polypeptides were resolved on an 0.1% NaDodSO₄/9–12% polyacrylamide slab gel. The figure shows an autoradiogram of the dried gel. Lane 1 shows total translation products of total RNA from 24-hr females. Lanes 2–6 show immunoprecipitable translation products of total RNA as follows: 2, 24-hr females; 3, 0-hr females; 4, 24-hr males; 5, JHA-treated 24-hr males; 6, 20-HE-treated 24-hr males. T, total translation mixture; IP, immunoprecipitated translation mix; NT, no treatment; PYP, precursors of YPs.

FIG. 3. Hybridization of RNAs from normal adult males and females and hormonally stimulated males. Total RNA (0.38 A_{260} unit per lane) was denatured by reaction with glyoxal and resolved on a 1.5% agarose gel. After the RNAs were transferred to nitrocellulose, the paper was hybridized with nick-translated DNA of clone pYP1 or pYP3 and exposed for 6 hr with a Cronex Lightning Plus screen at -70°C . (A) Hybridization signal to clone pYP1. (B) Hybridization signal to clone pYP3. Lanes: 1, normal 24-hr females; 2, normal 0-hr females; 3, normal 24-hr males; 4, 24-hr males treated with 20-HE; 5, 24-hr males treated with JHA; 6, normal 24-hr females. NT, no treatment.

parent stimulation of YP synthesis in 24-hr males.

To determine whether 20-HE caused an increase in the levels of translatable YP message in males, total RNA was extracted from 24-hr males treated with either 20-HE or JHA or from untreated controls. Ten of each group of these flies were used in the *in vivo* [^{35}S]methionine incorporation experiments reported above. Equal quantities of total RNA from untreated males or males treated with 20-HE or JHA were translated in a cell-free lysate and immunoprecipitated. The autoradiogram of the NaDodSO₄ gel showed the presence of the 47,000-dalton immunoprecipitable YP precursors only in the translation products of RNA extracted from 20-HE treated 24-hr males (Fig. 2). The 46,000-dalton precursor for YP3 was not detected, and we assume that this was due to insufficient copies of YP3 mRNA in the RNA extract to produce enough of the polypeptide to create a detectable signal on the autoradiogram. Even though the primary translation products for YP1 and YP2 run coincidentally at 47,000 daltons under these conditions, we can still make comparative quantitative estimates for YP1 because 20-HE stimulates the synthesis of YP1 and YP2 equally in both females and males (4, 10). Translation products from 20-HE-treated males contained 1/10 of the immunoprecipitable YP precursors found in translates from 24-hr females (Table 1). The translates from JHA-treated males and untreated 24-hr males did not contain any detectable immunoprecipitable YP material (Fig. 2). This demonstrates that 20-HE stimulates the male to accumulate translatable YP mRNA.

To test for the presence of Yp transcripts, equal amounts of total RNA from 20-HE-treated, JHA-treated, and untreated 24-hr males were denatured and resolved by agarose gel electrophoresis. The RNAs were then blotted to nitrocellulose, and the filter was hybridized with ^{32}P -labeled pYP1 DNA. The autoradiogram showed that RNA extracted from 20-HE-treated males contained sequences complementary to pYP1 (Fig. 3A). This represented 2.7×10^6 copies of Yp1 transcript per fly (Table 1). Neither the JHA-treated nor the untreated male

RNAs contained detectable Yp1 transcripts (fewer than 10^5 copies per fly). From these data, we conclude that treatment with 20-HE causes an increase of at least 25-fold in Yp1 transcripts per fly over untreated or JHA-treated males.

When the filter was stripped of the pYP1 DNA and rehybridized with ^{32}P -labeled pYP3 DNA, the autoradiogram showed that the RNA extracted from 20-HE treated males contained sequences complementary to pYP3 as well (Fig. 3B). No hybridization signal for pYP3 was detected in the RNAs from either JHA-treated males or untreated males. When compared to the 24-hr females, the male accumulated approximately 1/3 of the Yp3 transcripts. Thus, 20-HE is acting on the accumulation of Yp3 transcripts as well as Yp1 transcripts.

DISCUSSION

In order to determine the level at which the steroid hormone 20-HE acts to stimulate the rate of YP synthesis in male *Drosophila*, we measured three parameters that reflect the process of Yp expression: the rate of [^{35}S]methionine incorporation into YPs synthesized *in vivo*, the amount of RNA-directed YP synthesis in a cell-free translation system, and the quantities of Yp transcript hybridizable to cloned Yp DNAs. We found that all three of these parameters increased during normal maturation of adult females, but Yp expression was not detectable by any of the criteria in normal males. However, in males stimulated with 20-HE there was an increase in the rate of YP synthesis, the quantities of translatable YP mRNA, and the amount of Yp transcript. From these data we conclude that the steroid hormone 20-HE controls Yp expression by regulating the amount of Yp mRNA in *Drosophila* fat body cells.

For a comparison with the males, normal Yp expression in females was examined. We found that the rate of YP synthesis increased nearly 37 times in the first 24 hr of adult life in females and that the amount of Yp transcript coordinately increased with the YP synthesis rate. An increase in Yp transcript during

adult female maturation was also reported by Barnett (25) but no quantitative data were presented.

Both measures of YP RNA—cell-free translation and hybridization to cloned Yp genes—showed that stimulated males contained about 1/10 of the amount of YP message contained in 24-hr-old females, but the flies allowed to incorporate [³⁵S]-methionine *in vivo* were translating and secreting YPs at about 30% of the female rate. The significance of the discrepancy between the YP mRNA detected and the YPs produced is not yet understood. The level of Yp gene expression was always less in stimulated males than in normal females, perhaps due to the fact that males were exposed to hormones for 8 hr whereas females had been exposed for about 24 hr.

Ecdysones have been shown to affect the accumulation of gene transcripts or translatable mRNAs of other proteins in *Drosophila*. The mRNA levels for the polypeptide P1 (26) and dopa decarboxylase (27) in the ecdysone-deficient mutant *ecd¹* and some proteins in *Drosophila* Kc-H cell lines (28) were found to increase after treatment with ecdysone or 20-HE. Although none of these proteins showed a sensitivity to juvenile hormone stimulation, vitellogenin mRNA levels in *Locusta migratoria* are indeed regulated by juvenile hormone (29).

That the adult male does not usually produce Yp transcript must indicate there are basic differences in Yp control between males and females. This difference could be due to either sexually dimorphic hormone levels or to a difference in the abilities of male and female fat body cells to respond to the same hormonal milieu. Evidence from a number of studies suggests that males and females have similar levels of juvenile hormone (4, 9, 10, 22, 23, 30–32) and 20-HE (ref. 33; A. M. Handler, personal communication). If there are no large differences in hormone titers between male and female, then the difference in response must be intrinsic to the cells themselves. The lower sensitivity of males to 20-HE (10 μ M 20-HE for males; 0.1 μ M 20-HE for females) (4, 24) may be due to the paucity of specific cellular factors such as hormone receptors (34) or to the structure of chromatin in the region of the regulated gene (35, 36).

Quantitative estimates of the amount of Yp1 transcript accumulating in a male 8 hr after treatment with 20-HE shows that there are approximately 2.7×10^6 copies per fly or about 1,200 copies per fat body cell. Even though the accuracy of this estimate depends on obtaining a more exact census of adult fat body cells, this value is within the range of quantitative measurements for other systems after steroid hormone stimulation. During the secondary response of male *Xenopus* liver to estradiol, vitellogenin mRNA was found to be 1,100 molecules per cell at 12 hr (37, 38). Even though 20-HE is present in *Drosophila* males, we do not know whether the stimulation of Yp expression in males by 20-HE can be considered to be a secondary response.

The rapid accumulation of Yp transcripts in males treated with 20-HE leads us to conclude that regulation of the Yp occurs at a pretranslational level. The results are consistent with the hypothesis that the hormone is acting to induce transcription of the Yps, although other possibilities have not been ruled out. Because 20-HE causes a change in the amount of YP mRNA in males, it raises the question of whether the hormone has a similar effect in females. Because follicle cells respond only to JHA by synthesizing YPs, one also wonders whether there are tissue-specific actions of the two hormones on the accumulation of YP mRNAs.

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