Prepupal Differentiation of Drosophila Imaginal Discs: Identification of Four Genes Whose Transcripts Accumulate in Response to a Pulse of 20-Hydroxyecdysone

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ABSTRACT

We have isolated and initially characterized a novel set of four genes expressed during the prepupal differentiation of imaginal discs of *Drosophila melanogaster*. These four ecdysone-dependent genes are named EDG-42A, EDG-64CD, EDG-78E and EDG-84A-1 based on their respective chromosomal locations. Their expression is like that expected for genes encoding proteins that participate in the formation of the pupal cuticle. Transcripts complementary to these genes accumulate in imaginal discs during an 18-hr *in vitro* culture period that begins with a a 6-hr pulse of 20-hydroxyecdysone (20-HE). Transcripts for three of these genes were not detected in imaginal discs following culture in the absence or the continuous presence of 20-HE (1 μg/ml). Transcripts corresponding to EDG-64CD exhibit delayed accumulation in the continuous presence of 20-HE. Transcripts corresponding to three of the genes are only detected in the prepupal stage of development. Only EDG-64CD is complementary to transcripts present at other stages of development. One of the genes, EDG-78E, encodes a pupal cuticle protein. This is the first reported isolation of a set of steroid hormone-responsive genes that require first the presence, then removal of hormone for transcript accumulation.

The differentiation of animal cells involves the coordinate action of numerous gene products. The underlying basis for coordinating gene expression during differentiation has, however, not been well investigated. The formation during metamorphosis of the pupal cuticle by imaginal discs and the larval epidermis of *Drosophila melanogaster* offers an excellent opportunity to study gene regulation during differentiation. Pupal cuticle formation is regulated by the steroid hormone, 20-hydroxyecdysone (20-HE) (FRISTROM *et al.* 1985). Furthermore, imaginal discs form a pupal cuticle *in vitro* which facilitates the study of the underlying cellular and molecular basis of cuticle formation. The pupal cuticle itself is a complex structure composed of an outer epicuticle, and two inner lamellate regions that contain the nitrogenous polysaccharide chitin and a specific set of pupal cuticle proteins (PCPs) (SILVERT *et al.* 1984; WOLFGANG, FRISTROM and FRISTROM 1986).

During metamorphosis, imaginal discs first undergo morphogenesis (*e.g.*, form appendages) and then, during the prepupal and early pupal periods, differentiate

to form the pupal cuticle. We established the role of 20-HE in the morphogenesis and differentiation of Drosophila imaginal discs *in vitro* by determining the hormonal requirements for particular developmental steps (FRISTROM, LOGAN and MURPHY 1973; FRISTROM *et al.* 1982; DOCTOR, FRISTROM and FRISTROM 1985; FRISTROM and LIEBRICH 1986). Morphogenesis requires 20-HE and occurs in the continuous presence of the hormone. The formation of the pupal cuticle *in vivo* occurs after the hormone titer that increased at puparium formation has returned to intermolt levels (FRISTROM *et al.* 1985). Correspondingly, imaginal discs form the pupal cuticle in vitro when incubated first with and then without 20-HE. Formation of the pupal cuticle is first detected 6 hrs after 20-HE withdrawal (FRISTROM *et al.* 1982) when most of the hormone has dissociated from its receptors (YUND and FRISTROM 1975; YUND *et al.* 1978). Therefore, the initiation of morphogenesis appears to be a primary response to 20-HE, the formation of the pupal cuticle a secondary response. The differing hormonal regimens required to initiate these two processes establish the necessary temporal order for the first steps of metamorphosis: morphogenesis begins as the hormone titer rises, elaboration of the pupal cuticle begins as the hormone titer peak declines. Having identified this regulatory hierarchy by analysing disc development in vitro, we have extended our studies to investigate its underlying molecular basis.

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This paper is dedicated to the memory of Odessa Eugene.

Most proteins from untanned cuticles are quantitatively extracted in aqueous denaturing solvents (KIMBRELL *et al.* 1988). Analysis of the 7 M urea-extractable proteins of the pupal cuticle has led to the identification of eight major and approximately 12 minor proteins (DOCTOR, FRISTROM and FRISTROM 1985). These proteins fall into two classes: low molecular weight pupal cuticle proteins (L-PCPs: 8,000–25,000) and high molecular weight pupal cuticle proteins (H-PCPs: 40,000–82,000). The synthesis of these proteins is biphasic. The L-PCPs are synthesized first; they are easily detected 10 hr following puparium formation. The H-PCPs are synthesized later; they become abundant 14–16 hr after puparium formation (DOCTOR, FRISTROM and FRISTROM 1985). This biphasic synthesis results in the deposition of the L-PCPs in an outer lamellar region of the pupal cuticle, the H-PCPs in an inner lamellar region (WOLFGANG, FRISTROM and FRISTROM 1986).

Analysis of the temporal pattern of accumulation of the protein constituents of the pupal cuticle leads to specific predictions for the pattern of transcript accumulation for genes that encode these proteins. First, we expect these transcripts to accumulate in cultured imaginal discs only following a hormone pulse (FRISTROM *et al.* 1982). Second, because most Drosophila cuticle proteins exhibit stage specificity (CHIHARA, SILVERT and FRISTROM 1982), we expect transcripts that encode PCPs to be present in prepupae and early pupae, and be absent at other stages of development. Third, we expect the accumulation of transcripts encoding L-PCPs to precede the accumulation of transcripts that encode H-PCPs. Finally, because the PCPs are secreted proteins, we expect their mRNAs to be associated with membrane-bound polysomes.

In this report we describe the isolation and preliminary characterization of four genes that are complementary to transcripts that accumulate in response to a pulse of 20-HE in a manner expected for genes that encode pupal cuticle proteins.

MATERIALS AND METHODS

General procedures: Isolation and *in vitro* incubation of imaginal discs were carried out as described by DOCTOR, FRISTROM and FRISTROM (1985) with minor modifications (FECHTEL 1986). Using the method of BENTON and DAVIS (1977), a Drosophila genomic DNA library (MANIATIS *et al.* 1978) was screened to select cloned fragments that exhibited a signal when probed with [^{32}P]cDNA (NATZEL, HAMMONDS and FRISTROM 1986) prepared from RNA isolated from hormone-pulse treated imaginal discs and no signal when counter-screened with [^{32}P]cDNA prepared from RNA isolated from imaginal discs cultured either in the absence or continuous presence of 20-HE (using equal amounts of RNA prepared from discs after 15 and 18 hr of total culture with each hormone regimen). Cloned Drosophila genomic DNA inserts were subcloned into pSP65 (Promega Biotech, Inc.) or pIBI76 (International Biotechnologies, Inc.) following restriction enzyme digestion. Single-stranded RNA probes were prepared using either SP6 or T7 RNA polymerase as recommended by Promega Biotech, Inc. Complementary RNA (cRNA) probes were prepared according to CRAIG, MCCARTHY and WADSWORTH (1979). DNA was labeled with [^{32}P]CTP by nick translation as described by NATZLE, HAMMONDS and FRISTROM (1986). RNA was isolated as described by ULLRICH *et al.* (1977) with modifications as noted in CHIRGWIN *et al.* (1979). RNA was isolated from membrane-bound or cytoplasmic ribosomes as described by NATZLE, HAMMONDS and FRISTROM (1986). RNA blots were prepared using glyoxal denaturation as described by MANIATIS, FRITSCH and SAMBROOK (1982) using Biodyne A nylon transfer membranes (now called Biotrans A, I.C.N., Inc.). Restriction maps were constructed following size fractionation of restriction enzyme digested DNA using single and multiple digestions. Ambiguities in restriction maps were resolved as described by FECHTEL (1986).

In situ **hybridization to Drosophila polytene chromosomes:** Chromosome squashes were prepared as described by BONNER and PARDU (1976). Techniques for hybridization and autoradiography were as described by CRAIG, MCCARTHY and WADSWORTH (1979) using the acetylation step suggested by HAYASHI *et al.* (1978) to reduce nonspecific background. Tritium labelled probes were synthesized as cRNAs of the entire lambda phage DNA or as single-stranded probes made from a subcloned fragment using SP6 or T7 RNA polymerase.

Immunoprecipitation of *in vitro* translation products prepared using hybrid-selected RNA: Hybrid selection of RNA was carried out as described by SNYDER, HIRSH and DAVIDSON (1981) using 10 μg of DNA from each recombinant lambda phage spotted onto nitrocellulose filters using a dot blot manifold (Schleicher & Schuell). Two dots were prepared for each genomic clone and processed as described. Two dots from each of the four clones were hybridized together in 50 μl of hybridization mix containing 10 μg of polyA$^+$ RNA isolated from hormone pulse treated imaginal discs.

The methods employed for *in vitro* translation of imaginal disc RNA were as described by NATZLE, HAMMONDS and FRISTROM (1986) except that hybrid-selected RNA was used as template. Six labelled amino acids were added to translation mixes: ^{3}H-labelled leucine, lysine, phenylalanine, proline, and tyrosine (Amersham TRK 550) along with [^{35}S] methionine (Amersham). This increased the likelihood of visualizing immunoprecipitated translation products by fluorography as analysis of the amino acid composition of the pupal cuticle proteins demonstrated that they are, overall, low in methionine and high in proline and tyrosine (FRISTROM *et al.* 1986).

Immunoprecipitation was conducted as described by DOCTOR, FRISTROM and FRISTROM (1985) except that centrifugation to remove cellular debris was omitted and dilution and wash steps were scaled down to 1 ml. Following translation, reaction mixtures were pretreated with prepared *Staphylococcus aureus* ghosts (IgG-Sorb; Enzyme Center, Boston, MA) prior to immunoprecipitation to remove *S. aureus* binding proteins. Translation products were precipitated using pupal cuticle protein antisera previously shown to precipitate both L-PCPs and H-PCPs (FRISTROM *et al.* 1982; DOCTOR, FRISTROM and FRISTROM 1985). Equal volumes of each antiserum were mixed and used for immunoprecipitation.

Polyacrylamide gel electrophoresis (stacking gel 5%; running gel 10%) was used to size fractionate translation products. Preparation of gels and fluorography were as described by NATZLE, HAMMONDS and FRISTROM (1986). Kodak SB-5 film was exposed for 14 days.

TABLE 1

Characterization of the ecdysone dependent gene set

| Gene | Phage clone | Plasmid subclone | RNA length (kb) | Stage specific transcript accumulation | Transcript accumulation in cultured imaginal discs | | | Transcripts enriched in membrane fraction | Immune precipitable translation product |
					No hormone	Continuous hormone	Pulse hormone		
EDG-84A-1	51	51RX1.5	0.9	Yes	−	−	+	Yes	No
EDG-78E	57	57R1.5	0.6	Yes	−	−	+	Yes	Yes
EDG-42A	615	615R3.2	3.0	Yes	−	−	+	Yes	No
EDG-64CD	63	63RS6.6	5.4, 5.0, 3.9, 3.9	No	−	+[a]	+	Yes	No

[a] Although transcripts accumulate in culture with continuous hormone, this accumulation occurs with a 6-hr time lag relative to pulse-hormone cultured imaginal discs. This gene, therefore, does not qualify as a member of the IMP gene set described by NATZLE, HAMMONDS and FRISTROM (1986). Members of the IMP gene set accumulate in continuous hormone culture within 10 hr following hormone addition.

RESULTS

Isolation of genomic clones: Six independent clones in lambdaphage Charon 4 were isolated that contain sequences complementary to RNAs that accumulate during imaginal disc differentiation in response to a pulse of 20-HE. Phage clones 51, 56, 57, 63, 611 and 615 were selected from a genomic library of *D. melanogaster* based on their differential hybridization to ^{32}P-labeled cDNA prepared from mass-isolated imaginal discs that were cultured under different hormonal regimens. In this report we present an initial characterization of four of these lambda clones: 51, 57, 63 and 615 (summary in Table 1).

In situ **hybridization to polytene chromosomes:** To determine whether the genomic clones isolated in this study are from genetically characterized regions, in situ hybridization to polytene chromosomes was employed to identify the origin of these clones in the Drosophila genome (Figure 1). Phage clone 51 hybridizes to a well-studied region: 84A on the left arm of chromosome 3. This genomic insert overlaps with a set of inserts independently isolated in the laboratory of Thomas Kaufman as part of their characterization of the Antennaepedia-Complex. The other clones were found to localize to regions of the Drosophila genome that have not been extensively studied: clone 57 to 78E, clone 63 to 64CD, and clone 615 to 42A.

At the hybridization stringency employed (0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 60°), only a single site of hybridization was observed for each clone on Drosophila polytene chromosomes (Figure 1) indicating that these clones are not complementary to members of dispersed, highly conserved gene family. Analysis of Drosophila genomic DNA blots using standard hybridization stringency (50% formamide, 5× SSC, hybridized at 37° and washed in 0.1× SSC at 55°) supports this hypothesis because the only genomic restriction fragments that hybridize to subcloned probes are identical in size to restriction fragments expected from the restriction map from the original phage (data not shown).

Transcript accumulation in cultured imaginal discs: The pattern of transcript accumulation in cultured imaginal discs was examined. The results (Figure 2) demonstrate that each cloned insert is complementary to transcripts that accumulate during the differentiation of the imaginal discs. Except for clone 615, complementary transcripts begin to accumulate within 6 hr following the removal of 20-HE from the culture medium. With one important exception (see below), these transcripts are absent or at low levels in the imaginal discs cultured in the continuous presence or absence of 20-HE. The presence of low levels of transcripts in discs after short *in vitro* incubations may result from *in vivo* exposure of the imaginal discs to 20-HE. Using discs mass-isolated from approximately 500 g of larvae, it is difficult to avoid an *in vivo* exposure to 20-HE. A pulse of 20-HE is mimicked when discs that have been exposed to 20-HE *in vivo* are incubated *in vitro* without hormone. Consequently, transient early accumulation of transcripts in imaginal discs incubated without 20-HE is not considered significant when increased levels of transcripts are not detected after prolonged disc culture in the absence of 20-HE.

Phage clone 51 is complementary to a 0.9-kb transcript that accumulates in imaginal discs only after a hormone pulse. Transcripts are readily detected 6 hr after the pulse (12 hr total culture time, Figure 2, panel a, lane 12P). This 0.9-kb transcript persists 9 hr after maximal transcript accumulation (Figure 2, panel a, lane 24P).

Phage clone 57 cross-hybridizes with a 0.6-kb transcript that accumulates only following a hormone pulse (Figure 2, panel b, lanes labeled P). The transcripts are first detected at low levels 6 hr after hormone withdrawal (12 hr total culture time, Figure 2, panel b, lane 12P) and, like those complementary to clone 51, exhibit peak accumulation at the 15-hr time point. In contrast to those of phage clones 51 and 63, transcripts complementary to phage clone 57 were not detected in discs cultured for 24 hr.
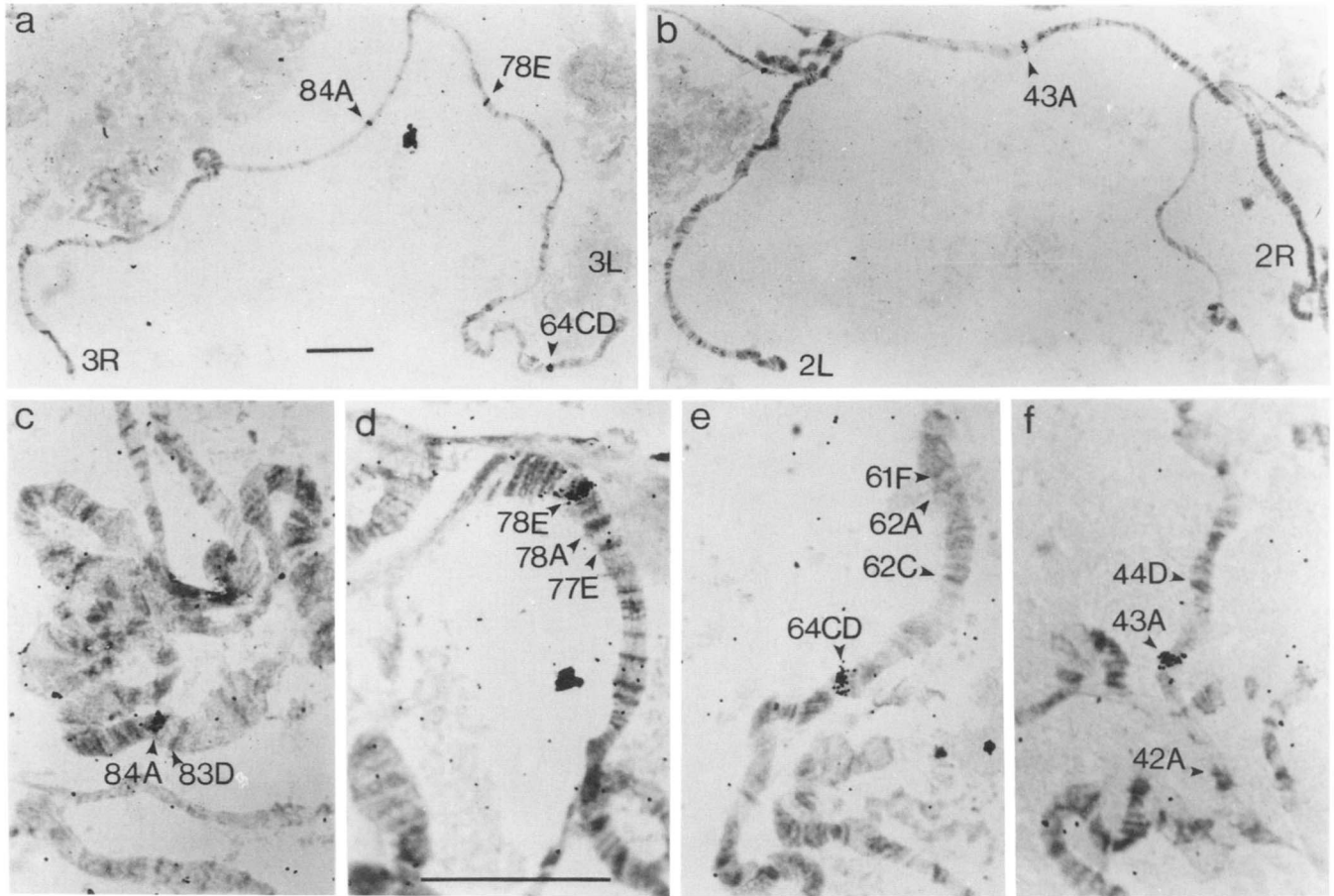
FIGURE 1.—Chromosomal localization of genomic clones. DNA sequences complementary to clones 51, 57, 615 and 63 were localized cytologically by hybridizing ^{3}H-labeled probes to salivary gland chromosome squashes from a *giant* stock of Drosophila. Panel a shows the hybridization of clones 51 (84A), 57 (78E), and 63 (64C,D) to the third chromosome. Panel b shows the hybridization of clone 615 to the second chromosome. Panels c–f show the chromosomes used to determine the cytological location for each clone. Panel c depicts a chromosome squash hybridized with clone 51; panel d, clone 57; panel e, clone 63; panel f, clone 615. The magnification bar represents 0.5 mm.

Phage clone 63 is complementary to two, 3.0- and 3.9-kb, transcripts in imaginal discs. These transcripts are readily detected 6 hr after hormone withdrawal (12 hr total culture time, Figure 2, panel c, lane 12P). With culture in continuous hormone transcripts are detected after 18 hr total culture time (Figure 2, lane: 18C). Maximum levels of transcript accumulation are similar after both a hormone pulse and in the continuous presence of hormone; however, there is a six hour lag in the initiation of transcript accumulation in the continuous presence of 20-HE. The signal observed below the 3.0-kb length class is seen only when total RNA is used; when poly(A$^+$) RNA is employed (Figure 3c) no signal below 3.0 kb is detected. Therefore, we do not believe these signals indicate the presence of additional transcript length classes.

Phage clone 615 cross-hybridizes with a 3.0-kb transcript that accumulates only after a hormone pulse. Transcripts are first detected 9 hr following hormone withdrawal (15 hr total culture time, Figure 2, panel d, lane 15P). Maximum accumulation is 12 hr after hormone withdrawal (18 hr of culture). Thus, tran-

scripts that correspond to clone 615 accumulate more slowly after a hormone-pulse than those complementary to transcripts 51, 57 and 63. Again, some signal is detectable below the 3.0-kb transcript length class. Since this is not observed when poly(A$^+$) RNA is used, we conclude that the 3.0-kb transcript length class represents the product of this locus.

These RNA blot experiments demonstrate that phage clones 51, 57, 63 and 615 are complementary to transcripts whose accumulation is dependent on 20-HE. Transcripts corresponding to these genes fail to accumulate in the absence of hormone. Phage clones 51, 57 and 615 are complementary to transcripts that fail to accumulate in imaginal discs cultured in the continuous presence of 20-HE (1.0 μg/ml). Thus, these three genes exhibit the pattern of transcript accumulation in cultured imaginal discs expected for genes that encode pupal cuticle proteins.

Transcript accumulation during Drosophila development: The pupal cuticle is the major differentiation product formed by imaginal discs during the prepupal and early pupal stages of development. The
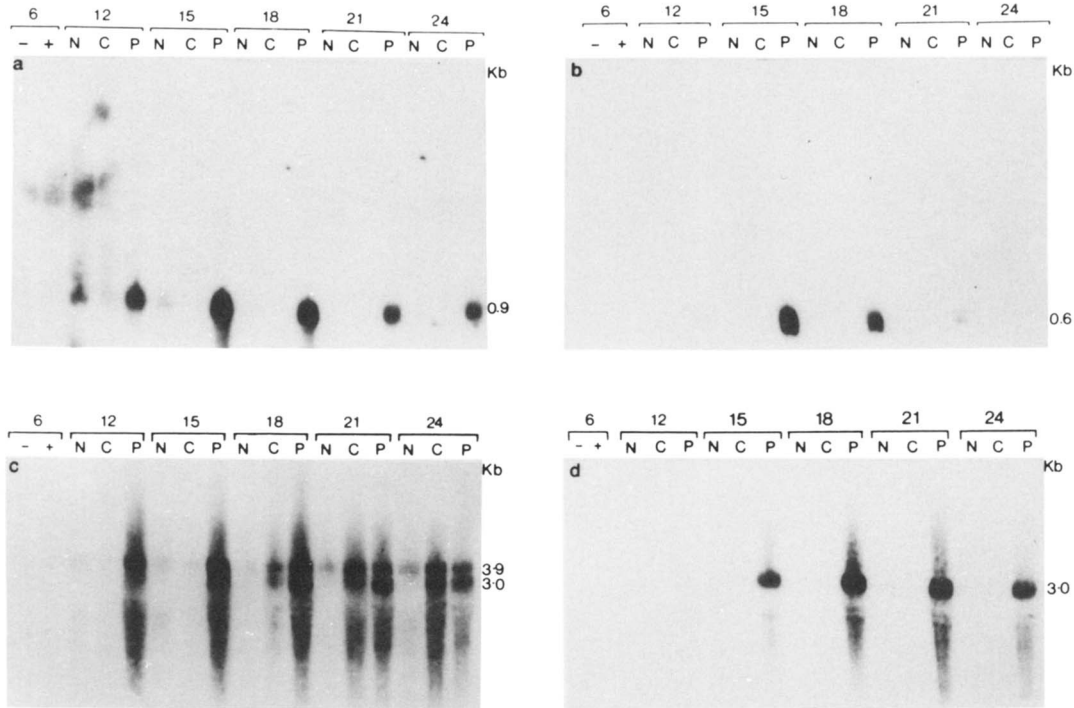
FIGURE 2.—RNA blots of imaginal disc transcripts complementary to genomic clones. Imaginal discs from a single isolation were cultured with continuous hormone (C), 1 μg/ml 20-HE; without hormone (N); or with a hormone pulse (P, 1 μg/ml 20-HE for the first 6 hr followed by continued culture without hormone). Samples were withdrawn from culture at 6, 12, 15, 18, 21 and 24 hr and frozen. RNA was isolated from each sample and denatured with glyoxal prior to electrophoresis in 1% agarose gels. The DNA was transferred from the gel to Biodyne A transfer membranes. RNA blots were hybridized with nick-translated DNA from phage clones 51 (panel a), 63 (panel c), and 615 (panel d) or a single-stranded RNA probe prepared using pIBI57R1.5, 57 (panel b). Kb = kilobases.

major proteins isolated from the pupal cuticle are stage specific (CHIHARA, SILVERT and FRISTROM 1982). To determine further whether the products of the ecdysone-dependent genes cloned in this study are likely to encode pupal cuticle proteins, the stage specificity of transcripts complementary to these clones was examined (Figure 3).

Phage clone 51 corresponds to a 0.9-kb transcript length class that does not appear to exhibit stage specific accumulation (Figure 3, panel a). Further analysis has established, however, that clone 51 encodes several different transcripts of the same length, one of which is specific to the prepupal stage of development (Figure 4). This gene cluster was also identified by PULTZ et al. (1988) because of its presence in the Antennapedia-Complex between the *labial* and *proboscipedia* genes. They did not detect the presence of EDG84A-1 in their characterization of this cluster because of its failure to cross-hybridize with other genes in this cluster. PULTZ et al. (1988), however, identified additional genes on this and adjacent genomic segments that we did not detect.

Phage clone 57 cross-hybridizes to two transcripts in addition to the one responsible for its isolation in the differential screen. The 0.6-kb transcript, whose accumulation is ecdysone dependent in imaginal discs, was detected only during the prepupal developmental

stage (Figure 3b). Although in low abundance in prepupal RNA, this 0.6-kb transcript is enriched in hormone-pulse treated imaginal discs (Figure 3, panel b, lane: Disc P). Phage clone 57 cross-hybridizes with a 1.5-kb transcript that is present in imaginal discs. (This transcript is not seen in Figure 2b because a subcloned probe was employed.) This 1.5-kb transcript is most abundant in imaginal discs cultured without hormone; it was not detected in imaginal discs cultured with continuous hormone (Figure 3, panel b, lane: disc C). This transcript was detected in hormone pulse treated imaginal discs but at a lower level than that observed for discs cultured without hormone. This suggests that this gene corresponds to transcripts whose prevalence is affected by the presence of 20-HE, but not in the manner expected for a gene encoding a pupal cuticle protein. Phage clone 57 also cross-hybridizes with a 0.8-kb transcript that accumulates predominantly during embryogenesis. The genes complementary to the 0.9- and 1.5-kb transcripts were not studied further.

Phage clone 63 is complementary to 5 transcript length classes. A 1.0-kb transcript was detected only during embryogenesis (15–18-hr animals, Figure 3, panel c, lane g). This transcript was not found in imaginal discs and was not further characterized. The pattern of transcript accumulation for the 5.4-, 5.2-,
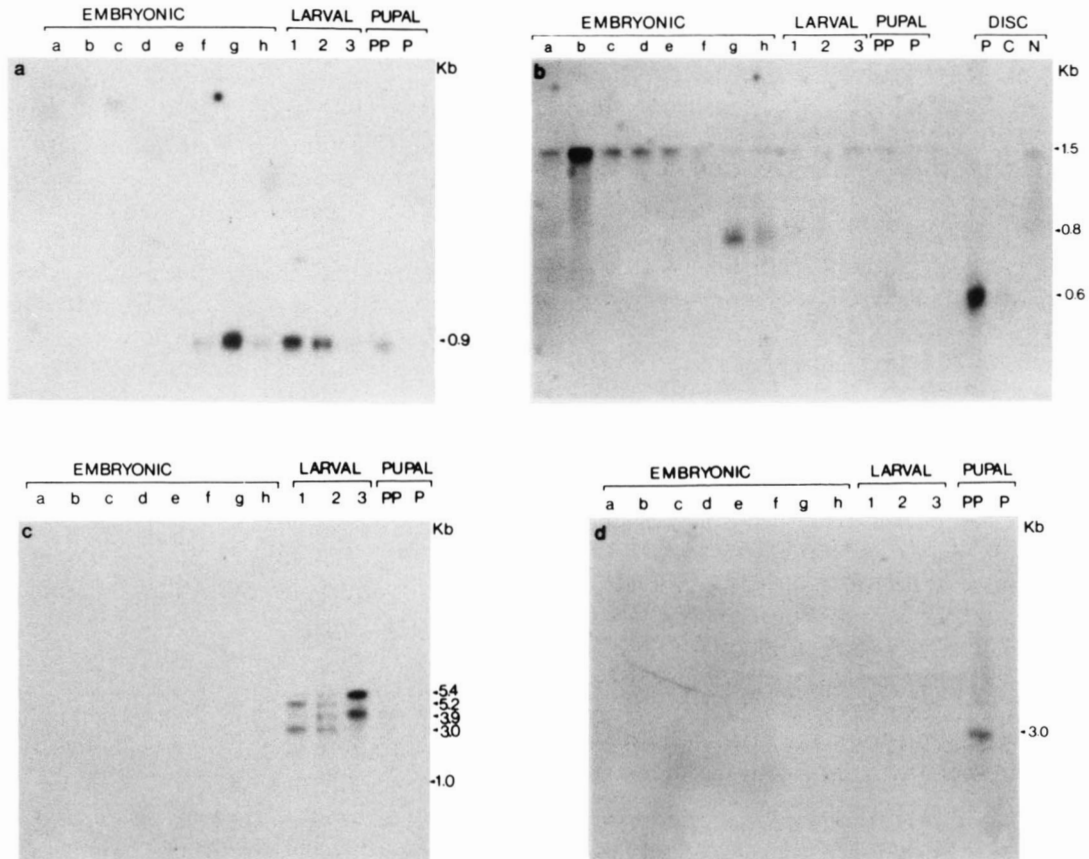
FIGURE 3.—Transcript accumulation during Drosophila development. Poly(A⁺) RNA (5 μg/lane) was treated with glyoxal and size fractionated by electrophoresis in 1% agarose gels. The size-fractionated RNA was transferred to Biodyne A nylon membranes and hybridized with nick-translated DNA from phage clones 51 (panel a: EDG-84A-1), 57 (panel b: EDG-78E), 63 (panel c: EDG-64CD), and 615 (panel d: EDG-42A). RNA was isolated from embryos (a) 0-2, (b) 2-3, (c) 3-6, (d) 6-9, (e) 9-12, (f) 12-15, (g) 15-18, and (h) 18-21 hrs old. RNA was also isolated from first instar (1), second instar (2), and third instar (3) larval stages and from prepupae (PP) and pupae (P).

3.9-, and 3.0-kb transcripts shows complex developmental regulation. The 3.9- and 3.0-kb transcripts predominate in imaginal discs following hormone stimulation.

Phage clone 615 cross-hybridizes with a 3.0-kb transcript whose accumulation is detected only during the prepupal developmental stage.

In summary, Figure 3 shows prepupal stage specificity for two ecdysone-dependent transcripts specified by clones 57 and 615. Furthermore, a subclone (pIBI51RX1.5) derived from clone 51 corresponds to a transcript that is detected only during the prepupal developmental stage (Figure 4). Phage clone 63 is complementary to transcripts whose expression is not limited to prepupae. The developmental accumulation of transcripts corresponding to clone 63 is complex suggesting either differentially processed transcripts derived from a single DNA interval or the presence of a multigene family whose members are differentially expressed. The pattern of ecdysone-dependent transcript accumulation in imaginal discs (Figure 2) combined with the restricted developmental accumulation during the prepupal develop-

mental stage (Figures 3 and 4) is consistent with our hypothesis that three of these genes (EDG-84A, EDG-78A and EDG-42A) encode pupal cuticle proteins.

RNA fractionation into membrane-bound or cytoplasmic polysomes: transcripts that encode pupal cuticle proteins are expected to fractionate into membrane-bound rather than free polysomes because these proteins are secreted (for review, see SABATINI *et al.* 1982). Identification of transcripts enriched in the cytoplasmic fraction would exclude cloned genes from further consideration as pupal cuticle protein genes. The fractionation of free and membrane-bound polysomes and subsequent RNA isolation using imaginal discs has been previously described (NATZLE, HAMMONDS and FRISTROM 1986). All the transcripts that exhibit ecdysone-dependent accumulation encoded by these cloned inserts are significantly enriched in membrane-bound polysomal RNA fractions isolated from the hormone pulse treated imaginal discs (Figure 5). This suggests that the products of the EDG gene set are either secreted or are membrane proteins.

Structure of genomic clones: Restriction sites were mapped for each cloned Drosophila insert and regions

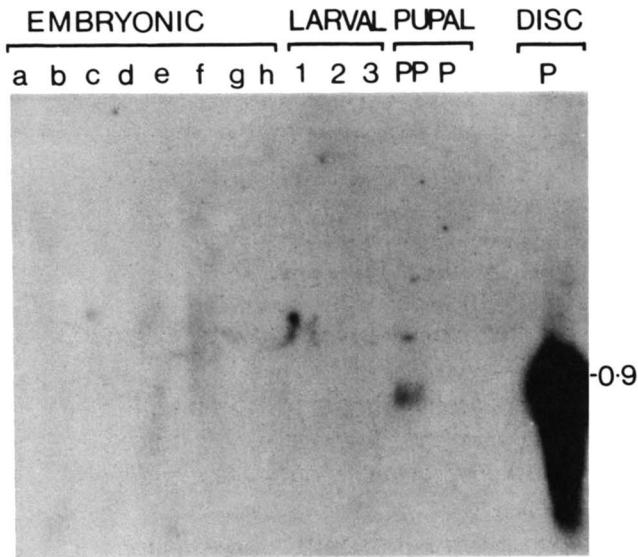FIGURE 4.—Pupal-specific transcript accumulation during Drosophila development. A RNA blot was prepared as described in Figure 3 and hybridized with single-stranded, ^{32}P-labeled RNA transcribed from subclone pIBI51RX1.5. The 0.9-kb transcript (EDG-84A-1) that is greatly enriched in RNA from hormone pulse treated imaginal discs (Disc, lane P) is detected only during the prepupal stage of development (Pupal, lane PP). Symbols are as in Figure 3.
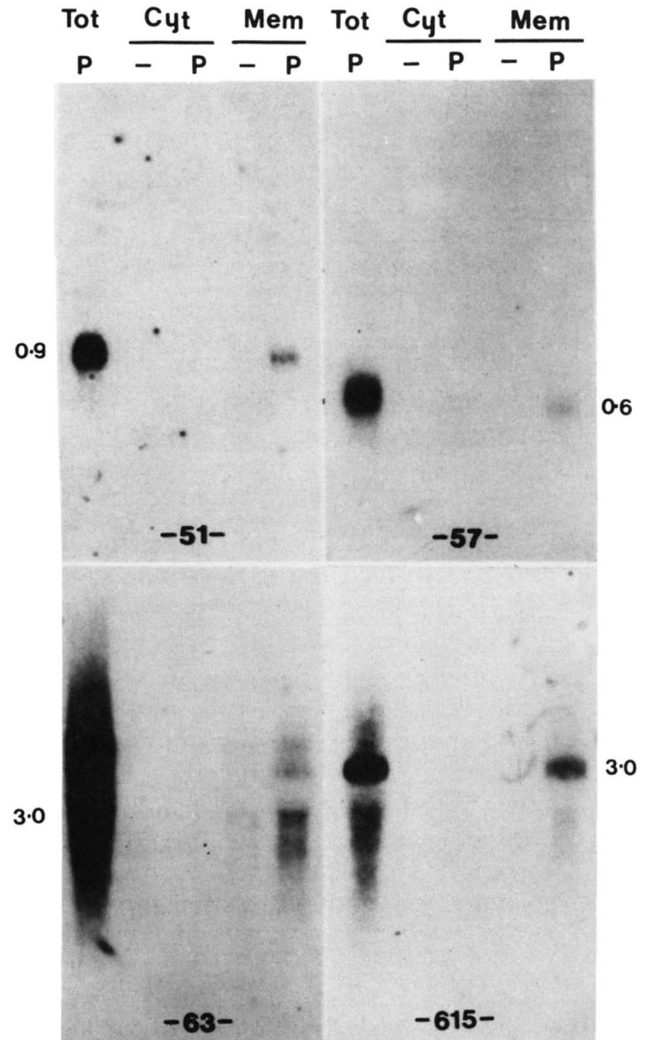

FIGURE 5.—Enrichment of transcripts in RNA isolated from membrane-bound polysomes. RNA was isolated from imaginal discs cultured with a six hour hormone pulse (P) or without hormone (−). Unfractionated total RNA was isolated from pulse-treated imaginal discs and 20 μg of this RNA was loaded in the first lane (Tot=total, P=pulse) of a 1% agarose gel. RNA from a different isolation of imaginal discs was fractionated into cytoplasmic (Cyt) or membrane-bound (Mem) polysomal fractions and 5 μg of this fractionated RNA was loaded into the remaining lanes. The RNA was subjected to electrophoresis at 80 V, transferred to Biodyne A paper, and hybridized with nick-translated DNA from phage clones 51, 57, 63 and 615.

complementary to transcripts whose accumulation is ecdysone dependent in cultured imaginal disc were identified (data not shown). Direction of transcription was determined using single-stranded RNA probes generated using Sp6 or T7 RNA polymerase and the RNA polymerase initiation sites located in the pSP65 and pIBI76 vectors. These data are compiled in Figure 6.

Phage clone 51 has two DNA intervals complementary to labelled cDNA prepared from hormone-pulse treated discs (data not shown). The strongest signal is associated with the 1.5-kb XbaI-EcoRI fragment from clone 51. A weak signal was also found corresponding to the left-most transcript on the map of clone 51 (Figure 6). We chose to focus on the 1.5-kb XbaI-EcoRI fragment because transcripts that cross-hybridize with this DNA interval exhibit the prepupal stage specificity expected for a pupal cuticle protein gene. This gene is named EDG-84A-1.

Phage clone 57 encodes at least the 3′ end of the EDG-78E gene. EDG-78E transcripts accumulate exclusively during the prepupal developmental stage; this accumulation is ecdysone dependent in imaginal discs. Although this 0.6-kb transcript could easily lie within the 1.5-kb EcoRI restriction fragment to which it has been mapped, it is also possible that the EDG-78E gene extends beyond the region contained in lambda clone 57. Regions corresponding to the other two transcripts present in imaginal discs (Figure 3) were not localized.

Phage clone 63 may specify a differentially proc-

essed transcript as all four transcripts cross-hybridize with only the 6.6-kb SalI-EcoRI fragment of this genomic clone. The DNA interval complementary to the 1.0 kb, low abundance transcript found in embryos was not identified.

Phage clone 615 may specify the entire 3.0-kb transcript for the EDG-42A gene (Figure 6). However, it is also possible that the pIBI615R.8 subclone corresponds to intronic regions of the primary transcript. If this is the case, the 3′ end of the EDG-42A gene is not contained in clone 615. cDNA prepared from hormone pulse treated imaginal disc RNA does not hybridize to pIBI615R.8 and no signal was detected
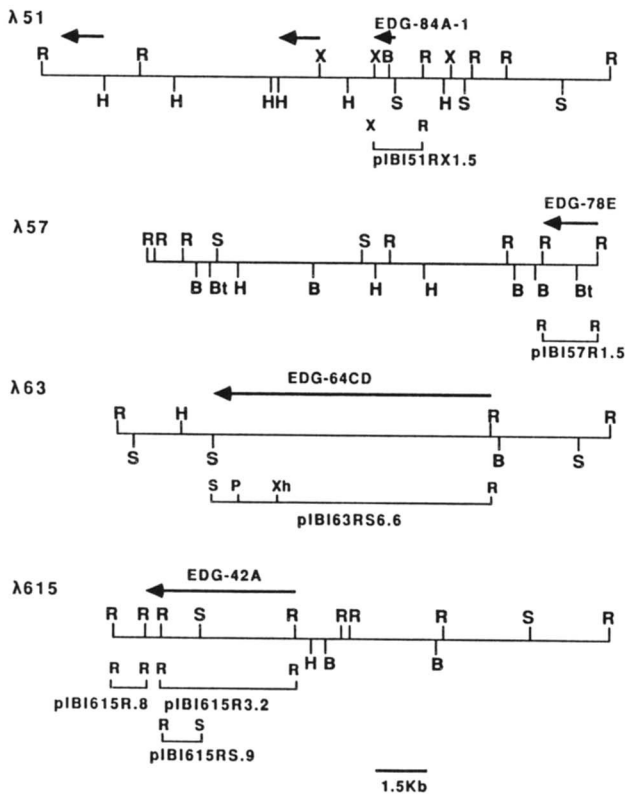
FIGURE 6.—Structure of genomic clones. Restriction sites were mapped for each phage clone and regions corresponding to transcripts found in pulse-treated imaginal discs were mapped with respect to location and direction of transcription. pIBI-76 (International Biotechnologies, Inc.) subclones used in this report are indicated beneath the restriction map. Vectors are pSP65 (data not shown; Promega Biotech, Inc.) or pIBI-76 (International Biotechnologies, Inc.). Subclones are named as follows: the first two numbers refer to the lambda clone from which the subclone was derived. Letter designations refer to the enzyme used for subcloning and the final number is the size of the fragment. E=*Eco*RI, H=*Hin*dIII, S=*Sal*I, X=*Xba*I, B=*Bam*HI, Bt=*Bst*EII, P=*Pst*I, Xh=*Xho*I. The measurement bar (right, bottom) represents 1 kb.

when ^{32}P-labeled pIBI615R.8 was used to probe RNA blots using either nick-translated or single-stranded RNA probes (data not shown).

***In vitro* translation of hybrid-selected RNA:** RNA isolated from hormone pulse treated imaginal discs was hybrid-selected using clone 57 DNA and then was used as template for *in vitro* translation. The translation products were subjected to immunoprecipitation using mixed polyclonal antisera prepared against purified pupal cuticle proteins (SILVERT *et al.* 1984; DOCTOR, FRISTROM and FRISTROM 1985). Figure 7 (lane: I.ppt 4) demonstrates that the product of the EDG-78E gene is immunoprecipable and hence likely to be a low molecular weight pupal cuticle protein (L-PCP). We cannot determine which pupal cuticle protein the EDG-78E gene encodes because the presence of signal sequences should alter the apparent molecular weights of *in vitro* translation products compared to those of the native proteins (Figure
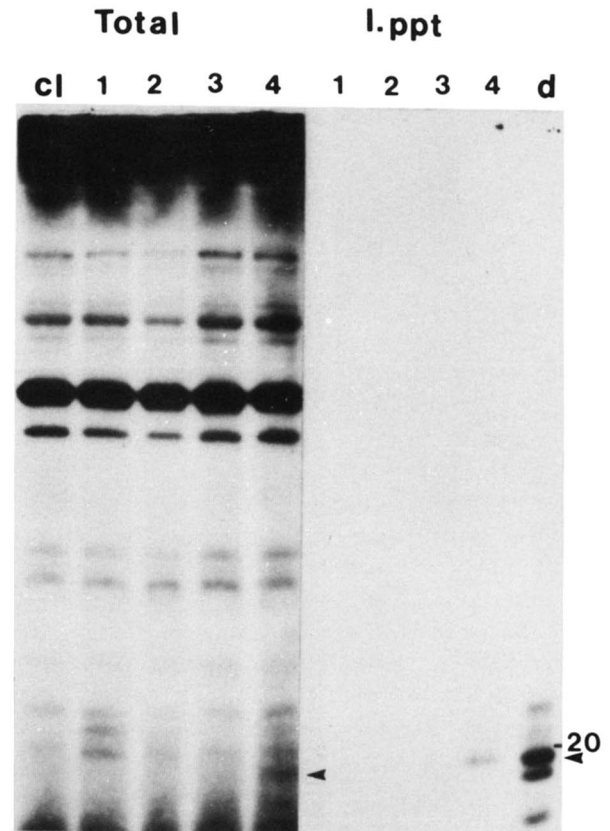

FIGURE 7.—*In vitro* translation and immunoprecipitation of hybrid-selected RNA. Lambda clones 51, 57, 63 and 615 were used to hybrid-select RNA isolated from imaginal discs cultured with a hormone pulse. The RNA was eluted and translated *in vitro*. Total RNA from imaginal discs was also translated. Translation products were immunoprecipitated using complex antisera prepared against purified pupal cuticle proteins. Immunoprecipitated translation products were subjected to electrophoresis using denaturing polyacrylamide gels. A 5% acrylamide stacking gel was used above a 10% gel. The left panel presents a fluorograph of the total translation reaction (Total); the right panel is a fluorograph depicting the remaining proteins following immunoprecipitation (I.ppt). Immunoprecipitated products (arrow, lanes I.ppt 4 and d) are visible in the unprecipitated samples (arrow, lanes total 3 and 4). Cl=cleared lysate control (no exogenous RNA added to translation reaction mixture), 1=clone 63, 2=clone 615, 3=clone 51, 4=clone 57, d=total disc RNA, 20=20,000 size marker.

7). RNA isolated from discs exposed to a hormone pulse was also hybrid selected using lambda clones 51, 615 and 63 and translated but no immunoprecipitable material was identified.

DISCUSSION

We report the isolation of a novel set of ecdysone-responsive genes that require a hormone pulse for transcript accumulation. Our preliminary characterization, summarized in Table 1, supports the hypothesis that these genes participate in the differentiation of the imaginal discs during metamorphosis. The pupal cuticle is the major, if not the only, differentiation product of the imaginal discs during the prepupal

period of development. This extracellular structure is elaborated by epithelial cells from their apical surface. The pupal cuticle is composed of an outer epicuticle (FRISTROM and LIEBRICH 1986) and an inner procuticle. The procuticle is composed of chitin and proteins and has two electron microscopically distinct lamellar regions. The outer lamellar region is secreted before the pupal molt (12 hr after puparium formation) and contains only L-PCPs. The inner lamellar region is secreted after the pupal molt and contains only H-PCPs (DOCTOR, FRISTROM and FRISTROM 1985; WOLFGANG, FRISTROM and FRISTROM 1986). DOCTOR, FRISTROM and FRISTROM (1985) demonstrated that L-PCPs are synthesized before the pupal molt, H-PCPs after the pupal molt.

The set of genes described in this report exhibit the temporal pattern of expression expected for differentiation products of the imaginal discs, in particular, that observed for pupal cuticle proteins. EDG-42A, EDG-78E and EDG-84A-1 are complementary to transcripts that accumulate in imaginal discs in vitro only after a pulse of 20-HE. These transcripts are associated with membrane-bound polysomes and accumulate only during the prepupal period of development. In contrast, transcripts complementary to EDG-64CD are not limited to prepupae. The EDG-64CD gene may, however, be expressed each time a cuticle is formed. It is possible that EDG-64CD encodes a general function that is required for cuticle synthesis. An example of a protein required for the synthesis of each cuticle would be chitin synthetase, since chitin is a component of all cuticles.

Although transcripts complementary to all four of these genes accumulate in imaginal discs under pulse-hormone conditions, EDG-64CD transcripts also accumulate under conditions of continuous hormone treatment. Because continuous exposure to high levels of 20-HE (1 μg/ml) does not occur in vivo, this result is an artifact of culture. The result, however, may indicate differences in the regulation of EDG-64CD and the other genes.

The translation product of RNA hybrid-selected using EDG-78E was immunoprecipitable with antisera prepared against pupal cuticle proteins. We conclude that EDG-78E, found on clone 57, encodes an L-PCP. Translation products prepared using hybrid selected RNA from the other three genes did not produce immunoprecipitable products. However, failure to produce an immunoprecipitable translation product does not preclude these genes from encoding pupal cuticle proteins. The presence of the signal sequence or the lack of post-translational modifications (H-PCPs are glycosylated) could result in the failure of the available antisera to recognize the in vitro translation product. In fact, the small size (0.9-kb) of the EDG-84A-1 transcript (from clone 51) and the similarities of its pattern of transcript accumulation to EDG-78E suggest that this gene also encodes a L-PCP. EDG-42A, found on clone 615, exhibits the pattern of transcript accumulation and transcript length (3.0 kb) expected for a high molecular weight pupal cuticle protein (H-PCP).

EDG-64CD, found on clone 63, shows a more complex pattern of transcript accumulation. This pattern is consistent with either the presence of a multigene family or the presence of a unique gene that is differentially processed. Two lines of evidence lead us to believe that EDG-64CD is unique in the genome and encodes a differentially processed set of transcripts. First, hybridization of clone 63 probes to Drosophila polytene chromosomes results in a single band of hybridization to region 64CD on the left arm of chromosome three. Therefore, if multiple copies of this gene exist, they must reside in a tandem array. Second, hybridization of the subclone pIBI63RS6.6 (Figure 6, Lambda Clone 63) to Drosophila genomic DNA using Southern blot analysis results in a single band of hybridization to the same size fragment expected from the restriction map of the phage clone. If a tandem array was responsible for the generation of four distinct transcript length classes, a probe exhibiting cross-homology would be expected to hybridize to different restriction fragments.

Morphogenesis of imaginal discs to form appendages occurs in the continuous presence of metamorphic concentrations of 20-HE (1 μg/ml). Whereas the elaboration of the pupal cuticle occurs only after withdrawal of 20-HE from the culture medium. Correspondingly, we have isolated two sets of ecdysone-responsive genes. One set, presumably involved in imaginal disc morphogenesis, contains four genes whose transcripts accumulate within one hour of exposure to 20-HE and remain at high levels in the continual presence of the hormone (NATZLE et al. 1986; NATZLE, FRISTROM and FRISTROM 1988; OSTERBUR et al. 1988). Here we describe a second set of genes, presumably involved in the formation-of the pupal cuticle, whose transcripts only accumulate after a pulse of 20-HE or, for EDG-64CD, after prolonged exposure (18 hr) to the hormone. It is of interest to understand the regulatory basis for the differential responses of these two sets of genes. Both are dependent on 20-HE, but the accumulation of transcripts from the second set is either inhibited or delayed by 20-HE. The half-life of binding of 20-HE to its nuclear receptors is about 30 minutes (YUND and FRISTROM 1975; YUND, KING and FRISTROM 1978). Consequently, the accumulation of the transcripts 6 hr after a hormone pulse does not occur until the hormone has dissociated from its receptor. ASHBURNER et al. (1974) and RICHARDS (1976) presented a formal model to explain delayed gene induction by 20-HE.

According to the model, 20-HE inhibits expression of these genes, but also induces the synthesis of transcriptional regulators required for their expression. Once 20-HE is removed, the genes are then expressed. Our goal in future studies is to dissect how this gene set is regulated. We are particularly interested in whether the same regulators, with differing affinities for each gene, are used to regulate all members of this gene set, or whether different genes respond to different regulators.

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