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crooked legs encodes a family of zinc finger proteins required for leg morphogenesis and ecdysone-regulated gene expression during *Drosophila* metamorphosis

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SUMMARY

***Drosophila* imaginal discs undergo extensive pattern formation during larval development, resulting in each cell acquiring a specific adult fate. The final manifestation of this pattern into adult structures is dependent on pulses of the steroid hormone ecdysone during metamorphosis, which trigger disc eversion, elongation and differentiation. We have defined genetic criteria that allow us to screen for ecdysone-inducible regulatory genes that are required for this transformation from patterned disc to adult structure. We describe here the first genetic locus isolated using these criteria: *crooked legs* (*crol*). *crol* mutants die during pupal development with defects in adult head eversion and leg morphogenesis. The *crol* gene is induced by ecdysone during the onset of metamorphosis and encodes at least three protein isoforms that contain 12-18 C_2H_2 zinc fingers. Consistent with this sequence motif, *crol* mutations have stage-specific effects on ecdysone-regulated gene expression. The *EcR* ecdysone receptor, and the *BR-C*, *E74* and *E75* early regulatory genes, are submaximally induced in *crol* mutants in response to the prepupal ecdysone pulse. These changes in gene activity are consistent with the *crol* lethal phenotypes and provide a basis for understanding the molecular mechanisms of *crol* action. The genetic criteria described here provide a new direction for identifying regulators of adult tissue development during insect metamorphosis.**

Key words: *Drosophila*, Metamorphosis, Imaginal disc development, Transcriptional regulation, Zinc finger, Ecdysone

INTRODUCTION

The spectacular biological transformations associated with insect and frog metamorphosis provide an ideal context for understanding the hormonal regulation of key developmental pathways, including tissue morphogenesis, remodeling and programmed cell death (Gilbert et al., 1996). We are studying the metamorphosis of the fruit fly, *Drosophila melanogaster*, as a model system for understanding how steroid hormones direct stage- and tissue-specific biological responses during development. Pulses of the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone) signal the destruction of larval tissues and their replacement by adult tissues and structures during *Drosophila* metamorphosis (Riddiford, 1993). The external structures of the adult head and thorax develop from imaginal discs that proliferate and undergo pattern formation during larval development (Cohen, 1993). Pulses of ecdysone then signal the imaginal discs to evert and differentiate into their appropriate adult form (Fristrom and Fristrom, 1993; von Kalm et al., 1995). Although a great deal is known about how imaginal discs are spatially patterned, much less is understood about how these patterns are elaborated into specific adult structures in response to ecdysone during metamorphosis. In this paper, we define genetic criteria that allow us to identify genes that regulate this hormone-driven transformation, from patterned disc to differentiated adult structure.

Two high titer pulses of ecdysone direct the animal through the early stages of metamorphosis (Riddiford, 1993). A high titer ecdysone pulse at the end of larval development triggers puparium formation initiating the prepupal stage of development. This phase is terminated by another ecdysone pulse, approximately 10 hours after puparium formation, that triggers adult head eversion and signals the onset of pupal development (Sliter and Gilbert, 1992). Initial insights into the mechanisms by which ecdysone directs these developmental responses arose from studies of the puffing patterns of the larval salivary gland polytene chromosomes (Becker, 1959; Ashburner et al., 1974). Ecdysone directly induces a small set of early puffs in the polytene chromosomes. Some of these puff genes encode transcription factors that both cross-regulate their own activity and induce large sets of secondary-response late puff genes (Russell and Ashburner, 1996; Thummel, 1996). The late genes, in turn, appear to direct the appropriate stage- and tissue-specific developmental responses to ecdysone during metamorphosis.

Two early puff genes have been characterized in detail: the *Broad Complex* (*BR-C*) (DiBello et al., 1991) and *E74* (Burtis

et al., 1990). The *BR-C* and *E74* genes are complex and contain multiple nested ecdysone-inducible promoters that direct the synthesis of families of related protein isoforms. The *BR-C* encodes multiple protein isoforms that can be distinguished by their use of one of four possible pairs of C_2H_2 zinc fingers, while the E74A and E74B proteins share an identical ETS DNA-binding domain. The *BR-C* and *E74* are widely expressed and are required for the proper development of both larval and imaginal tissues during metamorphosis (Kiss et al., 1988; Thummel et al., 1990; Boyd et al., 1991; Emery et al., 1994; Fletcher et al., 1995).

Interestingly, aspects of the *BR-C* and *E74* mutant phenotypes bear resemblance to one another. For example, some *E74B* mutant pupae display cryptocephalic phenotypes and short, twisted legs (Fletcher et al., 1995). This latter phenotype, referred to as the malformed leg phenotype, is a characteristic of some heteroallelic combinations of *BR-C* mutant alleles and is associated with incomplete disc eversion during prepupal development (Kiss et al., 1988). *BR-C* and *E74* mutants also show similar effects on the transcription of some ecdysone-regulated target genes, providing a molecular framework for interpreting the similarity in their developmental phenotypes (Guay and Guild, 1991; Karim et al., 1993; Fletcher and Thummel, 1995).

These related mutant phenotypes suggest that a number of ecdysone-inducible regulatory genes may circuit into these complex developmental pathways. The identification of these genes based on their puffing pattern in the larval salivary gland polytene chromosomes, however, has strict limitations. First, not all genes that are expressed in the salivary glands form puffs in the polytene chromosomes. Second, at least some genes that affect adult tissue development are not expressed in larval tissues (Appel et al., 1993). Third, mutant phenotypes can only be discerned after the difficult and time-consuming effort of reverse genetics. Indeed, the absence of genetic screens for mutants that affect metamorphosis has been a significant hindrance to progress in this area of research.

In an effort to circumvent these difficulties, we have established a set of genetic criteria that allow us to identify new ecdysone-inducible genes that play a role in adult tissue development during metamorphosis. We describe here the first results of this effort, the isolation of a new pupal lethal mutant called *crooked legs* (*crol*). *crol* mutants die during pupal development with two phenotypes reminiscent of mutations in the *BR-C* and *E74*: an inability to properly evert their head and defects in leg morphogenesis. Furthermore, *crol* mutations specifically affect the transcription of a number of ecdysone-induced genes during the prepupal-pupal transition, including the *EcR* ecdysone receptor gene (Koelle et al., 1991), as well as the *BR-C*, *E74* and *E75* early puff genes. The *crol* locus encodes at least three related protein isoforms that contain 12-18 clustered C_2H_2 zinc fingers, suggesting that *crol* manifests its effects on metamorphosis by directly regulating gene expression. Finally, *crol* transcription is induced by ecdysone during late larval and prepupal development, and appears to be expressed in a number of ecdysone target tissues including imaginal discs, salivary glands and the central nervous system. The *crol* gene thus appears to be a new regulator of the prepupal and early pupal genetic responses to ecdysone. The identification of *crol* provides a means of extending our studies of metamorphosis beyond the confines of the puffing response in the larval salivary glands, directly into the morphogenesis of adult tissues.

MATERIALS AND METHODS

Fly stocks and phenotypic characterization

The *crol* alleles are available through the Berkeley *Drosophila* Genome Project as *l(2)04418*, *l(2)06470* and *l(2)k08217* (Spradling et al., 1995). The $crol^{4418}$ allele was kindly provided by S. Shaver and A. Hilliker as part of a collection of prepupal and pupal lethal mutants (S. A. Shaver, M. B. Sokolowski, and A. J. Hilliker, personal communication). The $crol^{6470}$ and $crol^{k08217}$ alleles were provided by T. Laverty and G. M. Rubin. *l(2)04418* and *l(2)06470* are derived from the mutagenesis described in Karpen and Spradling (1992) while the *l(2)k08217* allele is from Torok et al. (1993). The *y; cn*^{1}*; ry*^{506} parental stock was provided by A. Spradling. *Df(2L)esc*^{10} was obtained from the Bloomington stock center. Other stocks are described in Lindsley and Zimm (1992). Flies were raised on a standard cornmeal/molasses/yeast medium at 25°C.

For lethal phase analysis, 300 eggs were collected from *y w;* $crol^{4418}$ *cn/CyO* y^+ flies after an 8 hour egg laying period, and transferred to a Petri dish with hard agar and fresh yeast paste. Hatched first instar larvae were collected after 30 hours and *crol* homozygous mutant larvae were distinguished from their *crol/CyO* y^+ siblings by the yellow phenotype of their mouth hooks and denticle belts. *CyO* y^+ homozygotes die during embryogenesis. The number of *crol* homozygous larvae was 33% of the total, indicating no embryonic lethality. To examine possible larval lethality, 80 $crol^{4418}$ homozygous and $crol^{4418}$*/CyO* y^+ heterozygous first instar larvae were transferred to different vials and allowed to develop for about 3 days, after which both mutant and control third instar larvae were counted. To analyze prepupal or pupal lethality, 60 third instar larvae of both genotypes were collected and allowed to develop at 25°C. The vials were checked every day for 7 days in order to score for a delay in pupariation as well as progression through metamorphosis and adult eclosion.

Lethal phases were also determined in animals that carried $crol^{4418}$ in combination with *Df(2L)esc*^{10} as well as controls that carried *y; cn/Df(2L)esc*^{10}. 100 eggs were collected from *y w*; *Df(2L)esc*^{10}*/CyO* y^+ animals mated with *y w;* $crol^{4418}$ *cn/CyO* y^+ animals, and 32% of the hatched first instar larvae were *yellow* mutants, indicating complete survival of the $crol^{4418}$*/Df(2L)esc*^{10} embryos. Similarly, 100 embryos collected from *y w; Df(2L)esc*^{10}*/CyO* y^+ animals mated with *y; cn;* ry^{506} animals yielded 48% *yellow* mutant first instar larvae, indicating that there are no early haploinsufficient phenotypes associated with the *crol* locus. Lethal phase analysis of later stages was performed using either 80 first instar larvae, or 70 third instar larvae of the same genotypes: *y w;* $crol^{4418}$*cn/Df(2L)esc*^{10} and *y; cn/Df(2L)esc*^{10} as a control.

For phenotypic characterization, $crol^{4418}$*cn* homozygous or $crol^{4418}$*cn/Df(2L)esc*^{10} hemizygous wandering third instar larvae were collected and allowed to develop at 25°C. Animals were followed for 7 days and scored for morphological markers representative of successive pupal stages (Bainbridge and Bownes, 1981). To document the phenotypes, each pupa was embedded in a small drop of water (10 μl) to dissolve the glue and gently detached from the wall using a wet paintbrush. About 15-20 pupae were transferred to a 1.5 ml Eppendorf tube containing 1 ml of boiling water, and left for 2 minutes at 95°C. The pupal case was then gently dissected in water for analysis of pharate adults. All animals were viewed by dark-field microscopy and photographed with Kodak Ektar 25 color print film. Legs were dissected in PBS, cleared by incubation overnight in 10% KOH at room temperature, dehydrated in ethanol and mounted in Euparal.

lacZ staining

Staged third instar larvae and prepupae were dissected in PBS and fixed in 2.5% glutaraldehyde, 50 mM sodium cacodylate, pH 7.0, in

PBS for 10-15 minutes. Organs were then rinsed three times with PBS and incubated in a staining solution containing 0.4% X-Gal for 16-20 hours at 25°C (Ashburner, 1989). After two rinses in PBS, the stained organs were mounted in 50% glycerol in PBS and viewed and photographed using a Zeiss Axiophot microscope.

Northern blot hybridization and organ culture

Third instar larvae were maintained on food containing 0.1% bromophenol blue and staged essentially as described (Andres and Thummel, 1994). Staged prepupae were synchronized at the white prepupal stage (0 hour prepupae) and allowed to age at 25°C for the appropriate time. For the northern blot hybridization shown in Fig. 6B, *crol* alleles were balanced over a *CyO* y^+ chromosome in a *y w* background. Homozygous animals were identified by the yellow phenotype of their mouth hooks and denticle belts. For the northern blots shown in Figs 5 and 8A, *yellow* animals were collected from the following crosses: for *crol*$^-$ mutants, *y w; Df(2l)esc*^{10}*/CyO* y^+ virgin females were crossed to *yw; crol*^{4418}*cn/CyO* y^+ males, and for control animals, *y w; Df(2l)esc*^{10}*/CyO* y^+ virgin females were crossed to *y; cn*^{1}*; ry*^{506} males. In all cases, 10 μg of total RNA was used in each lane. Hybridization with *rp49* was used as an internal control for loading, transfer and hybridization conditions.

RNA was extracted, fractionated by formaldehyde agarose gel electrophoresis and blotted as described (D'Avino et al., 1995). Filters were hybridized, washed and stripped as described (Karim and Thummel, 1991). DNA probes are described in Andres et al. (1993), except the *Brg-P9* cDNA probe, described in Emery (1995), and the *Sb* probe, which was synthesized from the *Eco*RI-*Sal*I fragment at the 5′ end of the cDNA described by Appel et al. (1993). This fragment does not contain the coding region for the Sb protease domain. Probes were labeled by random priming (Prime-It kit, Stratagene) of gel-purified fragments.

Gene structure

The genomic regions flanking the P-element insertions in *crol*^{4418} and *crol*^{6470} were isolated by plasmid rescue as described by Hamilton and Zinn (1994). The region flanking the insertion in *crol*^{k08217} was isolated by inverse PCR as described (Yeo et al., 1995). The rescued fragment from the *crol*^{4418} allele was used to map this insertion on the walk of Frei et al. (1985) by Southern blot hybridization and restriction mapping. A 2.3 kb *Hin*dIII fragment that gave the best signal in northern blot hybridizations ('probe' in Fig. 6A) was used to screen seven cDNA libraries. These included five oligo(dT)-primed and random-primed cDNA libraries constructed from RNA isolated from staged prepupae (provided by P. Hurban), one library prepared from RNA isolated from third instar larval organs cultured in the presence of 20-hydroxyecdysone and cycloheximide (Hurban and Thummel, 1993) and one library prepared from 8-12 hour embryos (Brown and Kafatos, 1988). The longest cDNAs obtained were sequenced on both strands using oligonucleotide primers and the Prism Dye Primer kit on an ABI 373A automated sequencer. A combination of DNA sequencing, Southern blot hybridization and PCR was used for intron/exon mapping. PCR amplifications were performed in an Idaho Technology air thermocycler essentially as described in Fisk and Thummel (1995). The 2.3 kb *Eco*RI-*Hin*dIII genomic fragment containing the third exon was completely sequenced on one strand. RT-PCR was performed using a Perkin-Elmer 480 DNA thermal cycler with the GenAmp RNA PCR kit (Perkin-Elmer) according to the manufacturer's instructions. To identify the *crolα* isoform, an oligonucleotide located in the sixth exon of *crolγ* (E22: 5′ TGTGGTTTCTCACCCGAATG 3′) was used to reverse transcribe (30 minutes at 42°C) RNA isolated from *Canton S* white prepupae. This cDNA was then amplified by PCR using a second primer (7A14: 5′ TACGCGCAAGGAACACTATG 3′) located in third exon downstream from the *crolγ* splice site. An amplified fragment was obtained of the appropriate size for the *crolα* isoform and its identity was confirmed by Southern blot analysis and a second round of PCR amplification using nested primers.

RESULTS

Identification and initial characterization of *crol* mutants

For our initial screen, we restricted our efforts to collections of prepupal/pupal lethal mutants, the times during development when *BR-C* and *E74* mutations lead to lethality. We further restricted our analyses to collections of single P-element-induced mutations in order to facilitate subsequent gene isolation and to avoid being misled by phenotypes due to multiple mutations. We eliminated mutations that cause a delay in puparium formation in order to avoid genes that might be involved in ecdysone metabolism. Subsequently, we selected those mutants that specifically affect the morphogenesis and/or differentiation of the imaginal discs during prepupal or pupal development, but neither their size nor morphology in late third instar larvae. Finally, we prepared northern blots from RNA isolated from mutant animals and screened for defects in ecdysone-regulated gene transcription during the onset of metamorphosis. In this way, we directed our efforts toward the isolation of genes that affect imaginal disc differentiation through the mis-regulation of target genes in the ecdysone regulatory hierarchies.

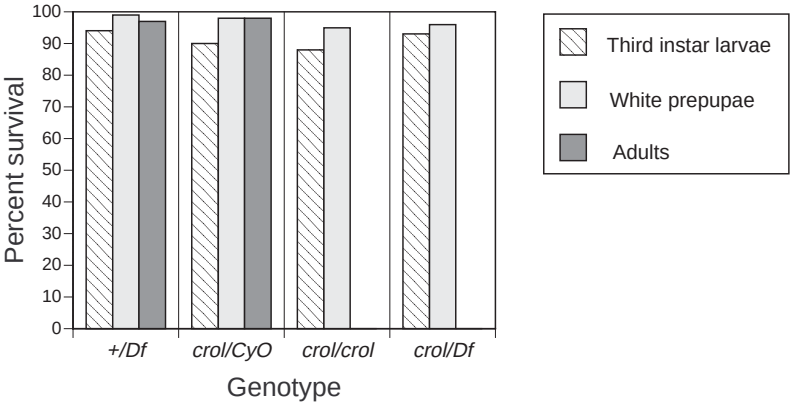

Fig. 1. Lethal phase analysis of *crol*^{4418} mutants. Lethal phases were determined in animals of four genotypes: (1) *y; cn/Df(2L)esc*^{10}(+/*Df*), (2) *y w; crol*^{4418} *cn/CyO* y^+ (*crol*/*CyO*), (3) *y w; crol*^{4418} *cn* (*crol*/*crol*) and (4) *y w; crol*^{4418} *cn/Df(2L)esc*^{10}(*crol*/*Df*), as described in Materials and methods. 80 first instar larvae were selected from each genotype and later counted as feeding third instar larvae. 60 third instar larvae were selected from genotypes 2 and 3 above, and 70 second instar larvae were selected from genotypes 1 and 4 above, allowed to develop to later stages, and white prepupae and adult flies were counted. The percent of recovered animals is plotted for each stage scored of each genotype. The number of surviving third instar larvae cannot be directly compared to the number of white prepupae and adults since these animals were derived from separate collections.

Using these criteria, we identified three P-element-induced pupal lethal mutations that define a single lethal complementation group: *l(2)04418*, *l(2)06470* and *l(2)k08217*. These mutations all behave as recessive loss-of-function alleles. Based on the mutant phenotype, we have named the corresponding locus *crol*, for *crooked legs*. As expected from the criteria used in our screen, *crol* mutants show no delay in puparium formation and their imaginal discs are normal in size and morphology. Furthermore, we see normal patterns of *engrailed* and *distalless* expression in *crol*^{4418} mutant leg discs dissected from late third instar larvae, indicating that there are no apparent defects in disc patterning (data not shown).

Lethal phase analysis revealed that homozygous *crol*^{4418} mutants survive through embryogenesis and hatch to form first instar larvae (see Materials and methods). Furthermore, collections of *crol*^{4418} first and third instar larvae progress normally through prepupal development, but die before adult eclosion (Fig. 1). An identical lethal phase can be seen in animals that carry *crol*^{4418} in combination with *Df(2L)esc*^{10} (Fig. 1), a 380 kb deficiency that removes the *crol* locus (from 33A1-33B2, Frei et al., 1985). This observation suggests that *crol*^{4418} represents a null allele for this locus, a conclusion that is consistent with the absence of *crol* transcription in this mutant (Fig. 6B). Genetic and molecular analysis of *crol*^{6470} and *crol*^{k08217} demonstrated that these are also null alleles with a lethal phase essentially identical to that of *crol*^{4418} (data not shown).

crol mutants die during two stages of pupal development. The first lethal phase occurs at the beginning of pupal development, 14-16 hours after puparium formation, corresponding to stage P5 of Bainbridge and Bownes (1981). These mutants display severe defects in head eversion and leg elongation (Fig. 2B). The remaining 40-50% of the mutants die at the end of pupal development, stage P14, with a microcephalic phenotype and malformed legs (Fig. 2D). While the penetrance of the microcephalic phenotype is variable, the 'crooked legs' phenotype is highly penetrant and is similar in all animals examined. This can be easily seen in the third pair of legs, which are smaller than wild type and distorted (Fig. 3B). We also see a kink near the middle of the femur (Fig. 3B) and a few missing bristles (Fig. 3C). The other two *crol* alleles display lethal phenotypes that are indistinguishable from those seen in *crol*^{4418} mutants (data not shown). Finally, excision of the P element in *crol*^{4418} results in a complete rescue of lethality, indicating that these lethal phenotypes are due to the P-element insertion (data not shown).

The malformed leg phenotype is indicative of defects during leg disc elongation (Kiss et al., 1988; von Kalm et al., 1995).

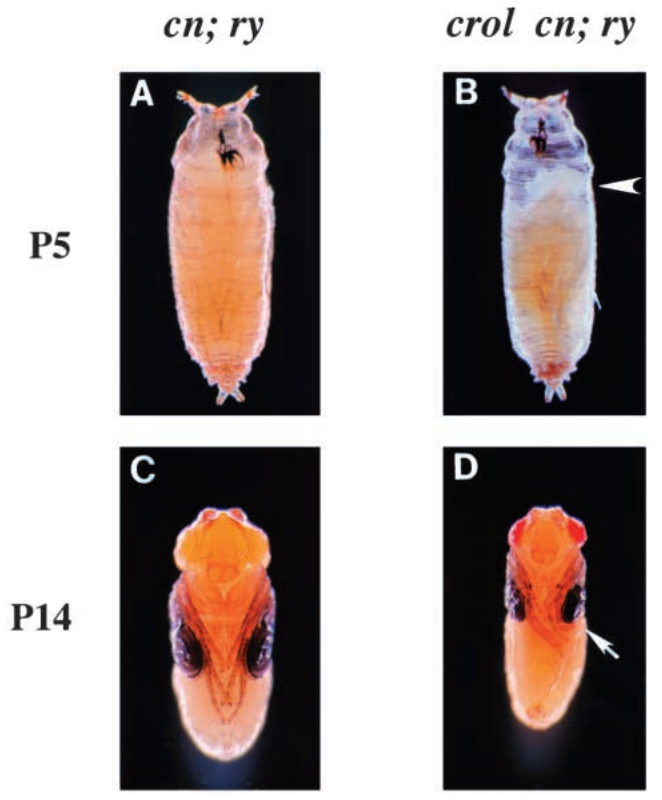

Fig. 2. Lethal phenotypes of *crol*^{4418} mutants. Both wild-type *cn*; *ry* (A,C) and homozygous mutant *crol*^{4418} *cn*; *ry* animals (B,D) are shown at the two major *crol* lethal phases. (A) A wild-type stage 5 pupa. (B) A *crol*^{4418} homozygous mutant arrested at stage P5 with severe defects in both head eversion and leg elongation. The anterior end of the pupa is empty, with the partially everted head marked by an arrowhead. (C) A wild-type stage 14 pupa. (D) A dead *crol*^{4418} homozygous mutant at stage 14. Mutants at this lethal phase display a variable microcephalic phenotype and severely distorted legs, with a third leg often curved around the wing (arrow). The red eyes in D are due to the presence of the *crol*^{4418} *ry*$^{+}$ P element.

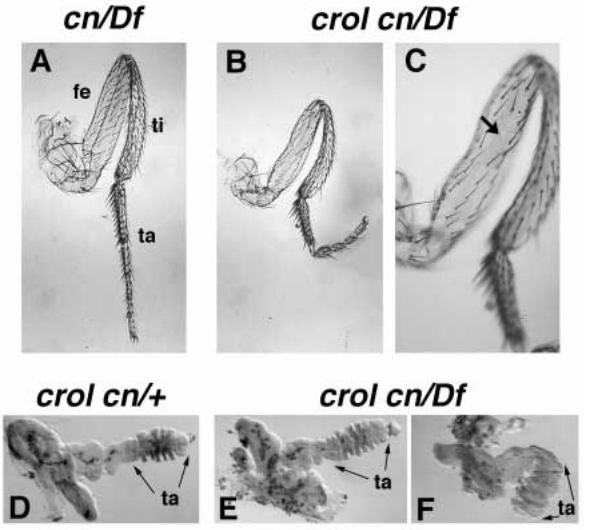

Fig. 3. Legs and leg imaginal discs dissected from *crol* mutants are malformed. (A-C) Third legs dissected 4.5 days after puparium formation from *y; cn/Df(2L)esc*^{10} control pupae (A) or *yw; crol*^{4418} *cn/Df(2L)esc*^{10} hemizygous mutants (B), mounted and photographed at the same magnification. (C) A higher magnification of B shows that some bristles (arrow) are missing on the femur of mutant animals. The femur (fe), tibia (ti), and tarsal segments (ta) of the leg are marked. (D) A leg imaginal disc dissected from *yw; crol*^{4418} *cn/+* control 6 hour prepupae shows normal disc eversion and elongation. Leg discs dissected from *yw; crol*^{4418} *cn/Df(2L)esc*^{10} mutants show a range of defects from partial elongation (E) to severe malformation (F). The presumptive tarsal segments are marked by arrows in D-F.

Accordingly, we examined the morphology of *crol*^{4418} mutant leg discs 6 hours after puparium formation, when they should have completed their eversion and elongation (Fristrom and Fristrom, 1993). Legs dissected from control 6 hour prepupae showed a similar size and shape, indicative of complete eversion and elongation (Fig. 3D). In contrast, a range of defects are evident in legs dissected from *crol*^{4418} prepupae (Fig. 3E,F). Of nine mutant animals examined, three contained legs with less severe defects. The 2nd-5th tarsal segments of these legs are slightly more expanded than wild type and the leg is not fully elongated (Fig. 3E). The remaining six *crol*^{4418} mutants had legs that were severely distorted and reduced in length (Fig. 3F). It is possible that these legs arise from the class of *crol* mutants that die early in pupal development with more severe leg defects (Fig. 2B). These observations indicate that at least part of the crooked legs phenotype is due to defects in leg disc elongation.

lacZ is widely expressed from the *crol*^{4418} P-element insertion and is induced at puparium formation

The *crol*^{4418} allele was derived from a P-*lacZ* enhancer trap mutagenesis, and thus carries a *lacZ* reporter gene that should provide an indication of the temporal and spatial patterns of *crol* expression (Karpen and Spradling, 1992). As shown in Fig. 5, *lacZ* is induced in leg imaginal discs, salivary glands and the central nervous system (CNS) isolated from *crol*^{4418}/+ late third instar larvae and prepupae. This induction of *lacZ* expression is coincident with the high titer ecdysone pulse that triggers puparium formation suggesting that *crol* expression is regulated by ecdysone.

Expression of *lacZ* can be detected initially in leg imaginal discs isolated from late third instar larvae (−4 hours) and is restricted to the precursors of the tarsal segments. This expression expands in early prepupal leg discs to the precursors of the femur and tibia. Other imaginal discs in *crol*^{4418} animals do not express *lacZ* (data not shown).

Expression of *lacZ* in the salivary glands is induced at puparium formation, slightly later than *lacZ* induction in leg discs (Fig. 4). An identical pattern of expression is present in both fat bodies and trachea (data not shown). In contrast, β-galactosidase can be detected in the ventral ganglion and presumptive optic lobes of the CNS in mid-third instar larvae. Expression in these cell types increases noticeably at puparium formation, in apparent synchrony with *lacZ* expression in the salivary gland. Interestingly, *lacZ* is also expressed specifically in the corpus allatum of the ring gland at all stages examined. The corpus allatum is the endocrine organ responsible for releasing juvenile hormone (Riddiford, 1993). A possible function for *crol* in this cell type is, however, difficult to predict since the role of juvenile hormone is not well understood during pre-adult *Drosophila* development.

crol is required for proper ecdysone-regulated gene expression during prepupal development

The *BR-C* and *E74* both manifest their effects on metamorphosis through the activation of genetic regulatory hierarchies. In order to determine if *crol* also functions in these hierarchies, we examined the temporal patterns of transcription for a number of ecdysone primary- and secondary-response genes in *crol* mutant animals. RNA was isolated from staged third instar larvae and prepupae hemizygous for the *crol*^{4418} allele (*y w; crol*^{4418}*cn / Df(2L)esc*^{10}) or for the *crol*^{4418} parental chromosome (*y; cn/Df(2L)esc*^{10}) and analyzed by northern blot hybridization (Fig. 5). Identical results were obtained using RNA samples isolated from an independent time course, as well as from staged *crol*^{6470} homozygotes. Only one difference was noticed between the two alleles, affecting the levels of *βFTZ-F1* transcription (see below). The transcription patterns observed in the parental control stock (*cn/Df*) are essentially

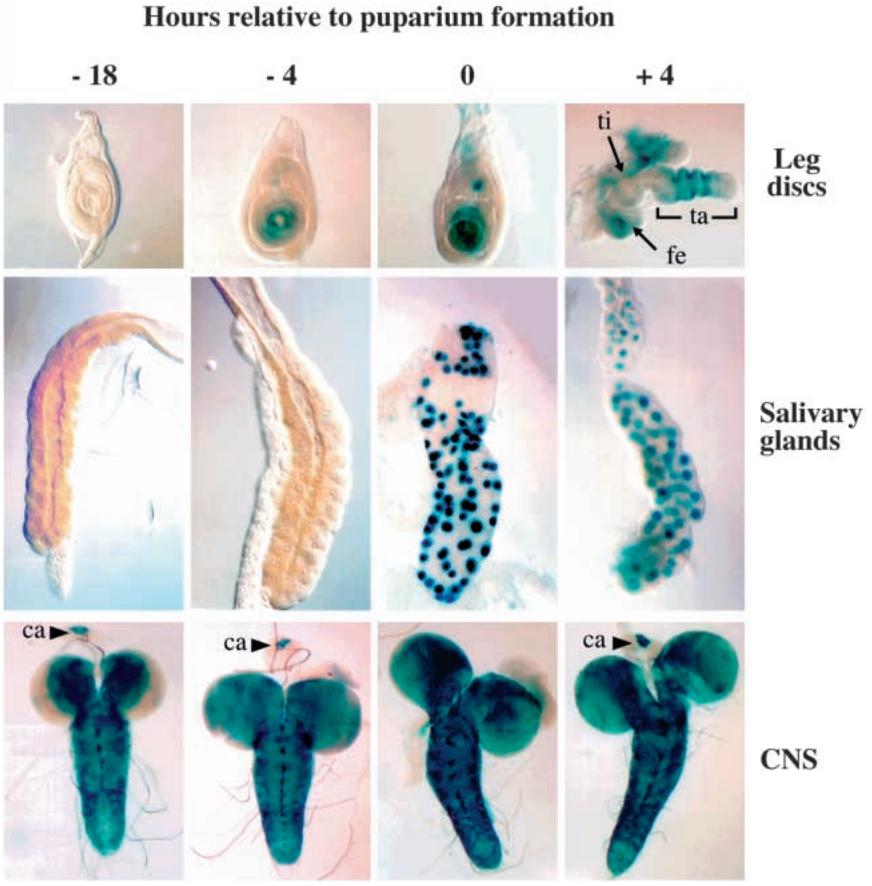

Fig. 4. *lacZ* expression in *crol*^{4418}/+ animals is induced at puparium formation. Leg imaginal discs, salivary glands and central nervous systems (CNS) were dissected from staged *crol*^{4418}/CyO y^{+} larvae and prepupae and stained to detect expression of *lacZ*. Mid- and late-third instar larvae were staged as described (Andres and Thummel, 1994) at 18 or 4 hours prior to puparium formation, respectively, while 0 hour and +4 hour prepupae were staged from puparium formation. The femur (fe), tibia (ti) and tarsal segments (ta) of the leg are marked, as well as the corpus allatum (ca) of the ring gland.

identical to those reported in wild-type *Canton S* animals (Andres et al., 1993).

We examined the expression patterns of several genes that encode key transcription factors in the ecdysone cascades. These include the *EcR* ecdysone receptor gene as well as the *BR-C*, *E74A*, *E74B*, *E75A*, *E75B*, *DHR3* and *βFTZ-F1*. *E75A* and *E75B* are two isoforms of the *E75* early puff gene that encode orphan members of the nuclear receptor superfamily (Segraves and Hogness, 1990). *DHR3* and *βFTZ-F1* encode distinct orphan receptors, with *DHR3* functioning as an inducer of *βFTZ-F1* expression in mid-prepupae (Lavorgna et al., 1993; Lam et al., 1997; White et al., 1997). *E75B* inhibits this *DHR3* activation function through direct heterodimerization (White et al., 1997). *βFTZ-F1*, in turn, appears to function as a competence factor that facilitates the reinduction of the early genes by ecdysone in late prepupae (Woodard et al., 1994).

DHR3 is specifically expressed in early prepupae and is unaffected by *crol* mutations (data not shown). In contrast, the other genes are all expressed at later stages and, interestingly, their transcription is selectively reduced in mid- and late $crol^{4418}$ mutant prepupae. *EcR* and *E74B* are both submaximally transcribed in $crol^{4418}$ mid-prepupae (Fig. 5). Similarly, the peak of *BR-C*, *E74A*, *E75A* and *E75B* transcription in response to the prepupal ecdysone pulse is significantly reduced, while the earlier induction of these genes in response to the late larval ecdysone pulse is unaffected (Fig. 5). Consistent with the stage-specificity of this mutant phenotype, we also see a significant reduction in the transcription of the stage-specific early gene *E93* (Baehrecke and Thummel, 1995). The timing of these transcriptional responses confirms that *crol* mutations have no effect on the duration of larval and prepupal development, but rather indicates that *crol* is required for the proper magnitude of ecdysone-induced gene expression in prepupae. The level of *βFTZ-F1* mRNA is also reduced in $crol^{4418}/Df$ mutants (Fig. 5). However, $crol^{6470}$ homozygotes show only an approximate two-fold reduction in *βFTZ-F1* mRNA levels, yet the reduction in early gene transcription in these mutants is indistinguishable from that seen in $crol^{4418}$ mutants (data not shown). This observation suggests that *crol* works independently of *βFTZ-F1* to regulate the prepupal genetic response to ecdysone.

We also examined several ecdysone-inducible genes that are expressed specifically in the imaginal discs and salivary glands. The expression of four salivary gland-specific secondary-response genes is unaffected by *crol* mutations: the *Sgs-3* and *Sgs-5* glue genes (Meyerowitz and Hogness, 1982; Guild and Shore, 1984), and the *L71-1* and *L71-6* late puff genes (Wright et al., 1996) (data not shown). This is consistent with the wild-type expression of known regulators of these secondary-response genes, the *BR-C* and *E74A*, in *crol* mutant late third instar larvae (Fig. 5). Similarly, expression of the fat body specific gene *Fbp-1* (Lepesant et al., 1978) is unaffected by *crol* mutations (data not shown). Because of the leg phenotypes in *crol* mutants, we also examined four genes that appear to play a role in imaginal disc morphogenesis. These included the *Stubble* gene (*Sb*), which encodes an apparent integral membrane serine protease required for disc eversion (Appel et al., 1993), the *Brg-P9* secondary-response gene, which encodes an apparent protease inhibitor (Emery, 1995), *IMP-E1*, which is induced directly by ecdysone and may play a role in epithelial movements during disc eversion (Natzle et al., 1988), and the *EDG-84A* pupal cuticle gene (Fechtel et al., 1988). None of these genes are significantly affected in *crol* mutants, with the exception of *Brg-P9*, which appears to be expressed for a longer time and at higher levels in *crol* mutant prepupae (Fig. 5).

The *crol* gene encodes at least three related C_2H_2 zinc finger proteins

The P element in $crol^{4418}$ maps to a region of the genome that is covered by the chromosomal walk of Frei et al. (1985). Southern blot hybridization using genomic probes rescued

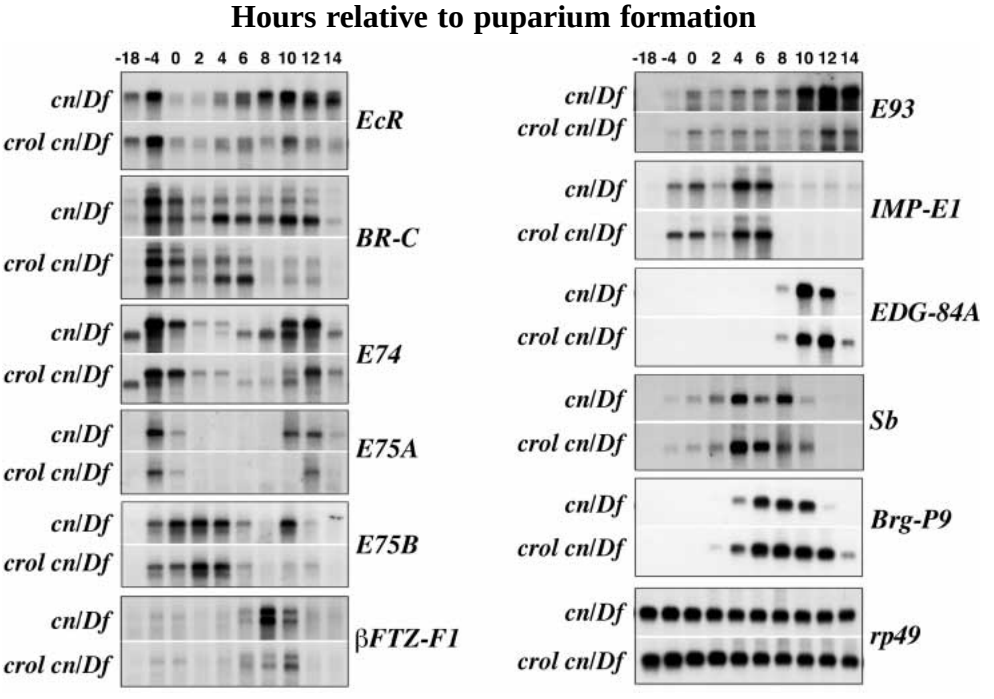

Fig. 5. *crol* mutations affect ecdysone-regulated gene expression in prepupae. Total RNA isolated from *y; cn/Df(2L)esc*^{10} control animals (*cn/Df*) and *yw; crol*^{4418} *cn/Df(2L)esc*^{10} hemizygous mutants (*crol cn/Df*) was fractionated by formaldehyde agarose gel electrophoresis and analyzed by northern blot hybridization. Four sets of control and mutant blots were prepared and individual sets were hybridized with radiolabeled probes directed against each ecdysone-regulated gene shown on the right. Developmental times are given in hours relative to puparium formation (see Materials and methods for staging). Each blot was hybridized to detect *rp49* mRNA (O'Connell and Rosbash, 1984) as a control for equivalent loading and transfer in each lane. The upper band in the *E74* panels corresponds to *E74A* mRNA and the lower band corresponds to *E74B*.

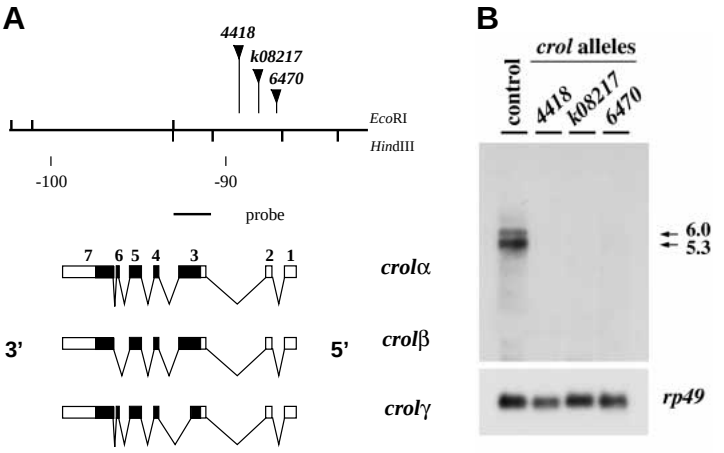

Fig. 6. Genomic organization of the *crol* locus. (A) *Eco*RI and *Hin*dIII restriction maps of genomic DNA encompassing the *crol* gene are shown at the top, with the positions of the three P-element insertions marked (*4418*, *k08217* and *6470*). The coordinates are from Frei et al. (1985). The three *crol* mRNA isoforms (α, β and γ) and open reading frames (black boxes) are depicted at the bottom. (B) The P-element insertions prevent accumulation of *crol* transcripts. RNA was extracted from white prepupae homozygous for each of the three *crol* mutations indicated on the top. As a control, total RNA was extracted from *y; cn*^{1}*; ry*^{506} white prepupae, the parental strain for *crol*^{4418} and *crol*^{6470} (Karpen and Spradling, 1992). Equal amounts of total RNA were fractionated by formaldehyde agarose gel electrophoresis and analyzed by northern blot hybridization to detect *crol* transcription. Hybridization to detect *rp49* mRNA (O'Connell and Rosbash, 1984) was used to confirm equivalent loading and transfer in each lane.

from the P-element insertions in *crol*^{4418}, *crol*^{k08217} and *crol*^{6470} demonstrated that these P elements lie within several kilobase pairs of one another at coordinate –90 on the genomic walk. This corresponds to position 33A6-7 in the polytene chromosomes and positions the P-element insertions approximately 15-20 kb from the *esc* gene at 33B1-2 (Fig. 6A). Two transcripts, 5.3 and 6.0 kb in length, were detected by northern blot hybridization using probes from a 7 kb region surrounding the *crol*^{4418} P-element insertion site (Fig. 6B). These RNAs were not detectable in the three *crol* mutant backgrounds, strongly suggesting that these transcripts correspond to the *crol* gene (Fig. 6B).

84 cDNA clones were isolated from screens of seven cDNA libraries, and the six longest overlapping clones were sequenced on both strands. Introns were mapped by genomic DNA sequencing, Southern blot hybridization and PCR amplification. These studies defined two mRNA isoforms that we have called *crolβ* and *crolγ*. Two independent cDNA clones contain the complete *crolβ* coding region, and one cDNA contains a complete *crolγ* coding region. The 5′ sequences of the *crolβ* and *crolγ* cDNAs overlap an independent clone that contains the 5′ non-coding exons 1 and 2, leading to the proposed gene structure shown in Fig. 6A. The *crolß* and *crolγ* isoforms are generated by differential splicing such that *crolβ* lacks the sixth exon of *crolγ*, and a splice donor site within the third exon of *crolβ* is used to generate the *crolγ* mRNA. The existence of these mRNA isoforms was confirmed by RT-PCR analysis of RNA isolated from white prepupae (data not shown). A third mRNA isoform was also identified by RT-PCR, designated *crolα*, which utilizes the distal exon 3 splice site of *crolβ* and carries the sixth exon of *crolγ*. The *crol* gene thus encodes at least three different mRNA isoforms: *crolα* is 6263 nucleotides in length, *crolβ* is 6050 nucleotides in length, and *crolγ* is 5645 nucleotides in length. It is unclear, however, how these isoforms relate to the 6.0 and 5.3 kb RNAs detected by northern blot hybridization (Fig. 6B). Only the 6.0 kb RNA corresponds in length to a *crol* mRNA isoform – *crolβ*. Furthermore, probes derived from exons 1 and 2, the 3′ end of exon 7, or 3′ sequences of exon 3, which are specific to *crolα* and *crolβ*, all detect both 6.0 and 5.3 kb RNA size classes on northern blots. This observation suggests that the *crol* mRNA isoforms may utilize more than one promoter and/or 3′ polyadenylation signal. It seems likely that additional *crol* mRNA isoforms will be identified by further molecular characterization of this locus.

The locations of the P elements in *crol*^{4418}, *crol*^{k08217} and *crol*^{6470} are consistent with a complete elimination of *crol* transcription in these mutant animals (Fig. 6B). The *crol*^{6470} mutation arises from a P-element insertion in the first intron of the *crol* gene, and *crol*^{4418} and *crol*^{k08217} are due to P-element insertions in the second intron (Fig. 6A). Furthermore, the promoters for all *crol* mRNA isoforms must lie upstream from, or near, the *crol*^{6470} insertion site, since no *crol* transcripts can be detected in this mutant background (Fig. 6B).

Sequence analysis revealed that the *crol* mRNAs contain an unusually long 5′ leader sequence of at least 1418 nucleotides. Furthermore, this 5′ leader contains 13 AUG triplets upstream from the long open reading frame that encodes the *crol* protein products. Most of these 5′ open reading frames are short (1-22 codons in length) and the AUG triplets are in a poor context for optimal translational initiation (Kozak, 1986; Cavener and Ray, 1991). There are two exceptions to this rule: the tenth open reading frame, six codons in length, has only a one nucleotide mismatch from the optimal AUG sequence context. More interestingly, the first open reading frame in the *crol* mRNAs shows a similarly favorable sequence context for translational initiation, and it is the longest open reading frame of the 5′ leader – encoding a 37 amino acid arginine- and serine-rich polypeptide. It thus seems likely that this short open reading frame is normally translated in vivo, although its possible function remains unknown. Long 5′ leader sequences seem to be a characteristic of only a few *Drosophila* regulatory genes, including *Antennapedia* and the ecdysone-inducible *E74A* early gene (Burtis et al., 1990; Oh et al., 1992). Both of these genes use internal ribosome entry to allow translation of their long open reading frame, suggesting that *crol* mRNAs may also contain sequences that facilitate internal ribosome entry (Oh et al., 1992; Boyd, 1993). These observations also raise the interesting possibility that *crol* expression may be regulated at the translational level.

The three *crol* mRNA isoforms have identical 5′ and 3′ sequences flanking a variable internal region that is generated by differential splicing (Fig. 6A). This sequence arrangement leads to the synthesis of three distinct CROL protein isoforms that have identical amino- and carboxy-terminal regions

A

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                                                            ZF1
CROLα  NGGGQNVALAPDGTPIATGTHVCDICGKMFQFRYQLIVHRRYHSERKPFMCQVCGQGFTTSQDLTRHGKIHIGGPMFTCIVCFNVFANNTSLERHMKRHS
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CROLα  MQHVSAASSVPSVVTPVVTTGGTTITLGGPPPLPKSEHKEDGKPPHGIEMYKVNIEDISQLFTYHEVFGKIHGDVVNHQLAAAHGGQLPPPPPLPPQVTS  100
CROLβ  ....................................................................................................  100
CROLγ  ....................................................................................................  100

CROLα  HAASAAAAAAAASTNNAAVAAVMASANAAAAAAAAAASAGGGLPPATSGNGGQQVTVTTTSSSTSSGGSTTSGGTTTTAGELLMPKMEGGIHGVDGSGNGG  200
CROLβ  ....................................................................................................  200
CROLγ  ..............................................................................|                       178

                             ZF1                          ZF2                         ZF3
CROLα  NGGGQNVALAPDGTPIATGTHVCDICGKMFQFRYQLIVHRRYHSERKPFMCQVCGQGFTTSQDLTRHGKIHIGGPMFTCIVCFNVFANNTSLERHMKRHS  300
CROLβ  ....................................................................................................  300
CROLγ

                  ZF4                        ZF5                        ZF6
CROLα  TDKPFACTICQKTFARKEHLDNHFRSHTGETPFRCQYCAKTFTRKEHMVNHVRKHTGETPHRCDICKKSFTRKEHYVNHYMWHTGQTPHQCDVCGKKYTR  400
CROLβ  ....................................................................................................  400
CROLγ                                                                                      |................  194

ZF7                         ZF8                        ZF9                         ZF10
CROLα  KEHLANHMRSHTNETPFRCEICGKSFSRKEHFTNHILWHTGETPHRCDFCSKTFTRKEHLLNHVRQHTGESPHRCSYCMKTFTRKEHLVNHIRQHTGETP  500
CROLβ  ....................................................................................................  500
CROLγ  ....................................................................................................  294

                 ZF11                         ZF12                        ZF13                        ZF14
CROLα  FKCTYCTKAFTRKDHMVNHVRQHTGESPHKCTYCTKTFTRKEHLTNHVRQHTGDSPHRCSYCKKTFTRKEHLTNHVRLHTGDSPHKCEYCQKTFTRKEHL  600
CROLβ  ....................................................................................................  600
CROLγ  ....................................................................................................  394

                             ZF15                         ZF16                        ZF17
CROLα  NNHMRQHSSDNPHCCNVCNKPFTRKEHLINHMSRCHTGDRPFTCETCGKSFPLKGNLLFHQRSHTKGQEMERPFACEKCPKNFICKGHLVSHMRSHSGEK  700
CROLβ  ......................................................................................|               686
CROLγ  ....................................................................................................  494

           ZF18
CROLα  PHACTLCSKAFVERGNLKRHMKMNHPDAMMPPPPVHPHPQIPAGVLTQVKQEVKPIIIPHHSATTTMHTIQQITAGAAGGAGAVQLTPGLVPLVTSTLIS  800
CROLβ                                                           V..........................................  729
CROLγ  ....................................................................................................  594

CROLα  HNAAAQQQSQKQQAAAAAAAQQQAAAAAAAQQQAAQQQAAAAHQQHQQQVAAQHQQQAAVAAHQQQQQQLQQQQQLLQLSIQQAAHHHQQEQHRQQQQQQ  900
CROLβ  ....................................................................................................  829
CROLγ  ....................................................................................................  694

CROLα  HQQQQQQQHHQQQQQGHPQAPPPQQQQQPPPIALISDPSALARAAIQLQHLPANVEQHPVVY                                        962
CROLβ  ..............................................................                                        891
CROLγ  ..............................................................                                        756
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B

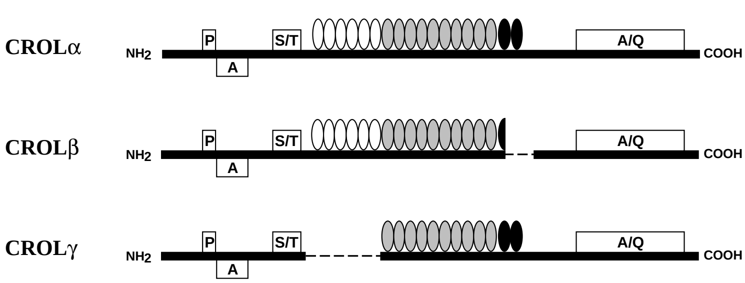

Fig. 7. *crol* encodes at least three C_2H_2 zinc finger protein isoforms. (A) The deduced amino acid sequence of the longest CROL protein isoform, CROLα, is depicted. The dots represent identical amino acids in the other two isoforms, CROLβ and CROLγ, and vertical lines (|) mark regions that are missing from these proteins by alternative splicing. The amino acids are numbered on the right, and each zinc finger is underlined. DNA sequences can be obtained from GenBank. The accession numbers are: *crolα*, AF020347; *crolβ*, AF020348; *crolγ*, AF020349. (B) Schematic representation of the three CROL isoforms. The proteins are shown in an amino-terminal to carboxy-terminal orientation. Sequences rich in proline (P), alanine (A), serine and threonine (S/T), or alanine and glutamine (A/Q) are marked. Zinc fingers common to all three CROL isoforms are shown in grey, those found only in CROLα and CROLβ are shown in white, and those found only in CROLα and CROLγ are shown in black. The sequences in CROLα that are missing from the other two isoforms are marked by a dotted line.

flanking a variable number of internal C_2H_2 zinc fingers. Translation of the three CROL protein isoforms initiates with the same AUG triplet, which lies in a near ideal context for initiation. Conceptual translation of the CROL protein products is depicted in Fig. 7A. The longest isoform, CROLα, is 962 amino acids in length and contains 18 zinc fingers while the other two isoforms, CROLβ and CROLγ, are 891 and 756 amino acids in length and contain 16 and 12 zinc fingers, respectively. CROLβ is missing one and a half of the C-terminal zinc fingers in CROLα, while CROLγ is missing the first six zinc fingers of CROLα (Fig. 7B).

crol transcription is induced by ecdysone during the onset of metamorphosis

The expression of β-galactosidase in *crol*[4418] animals suggested that the gene marked by this enhancer trap insertion might be regulated by the steroid hormone ecdysone (Fig. 4). As an initial test of this hypothesis, we examined the temporal profile of *crol* transcription in staged late third instar larvae and prepupae. The developmental northern blots shown in Fig. 5 were hybridized with a radiolabelled probe derived from exon 3 of the *crol* gene, sequences that are shared by all three mRNA isoforms. As expected, hybridization of the northern blot

prepared from *crol*^{4418} mutants showed no detectable *crol* transcription (data not shown). In contrast, both the 6.0 and 5.3 kb *crol* mRNAs were detected in control animals, and their levels fluctuated in parallel with the changes in ecdysone titer (Fig. 8A). *crol* mRNA can be detected in mid-third instar larvae, consistent with the expression of β-galactosidase in the CNS of *crol*^{4418} animals at this stage in development (Fig. 4). The level of *crol* mRNA then increases in late third instar larvae, in parallel with the high titer ecdysone pulse that triggers puparium formation (Fig. 8A). The levels of *crol* transcription decrease to low levels in mid-prepupae and then rise significantly in 12 hour prepupae, following the ecdysone pulse that triggers head eversion. This correspondence between the rises in ecdysone titer and the induction of *crol* transcription are consistent with *crol* being an ecdysone-inducible gene. To test this hypothesis more directly, salivary glands were dissected from mid-third instar larvae and cultured for 4 hours in the absence or presence of ecdysone. RNA was then isolated and *crol* transcription was analyzed by northern blot hybridization (Fig. 8B). This study revealed that *crol* mRNA levels are induced approximately two-fold by ecdysone, similar to the level of induction seen in vivo in late third instar larvae. A similar induction of *crol* transcription was seen in cultures of mixed larval organs treated with ecdysone (data not shown). These observations support the hypothesis that *crol* transcription is inducible by ecdysone, but the relatively low level of induction suggests that other factors may contribute to this regulation.

DISCUSSION

Drosophila metamorphosis represents a spectacular biological transformation in both form and function. Most organs and tissues that are necessary for larval growth and viability are destroyed during metamorphosis as new tissues and structures are assembled into the adult fly (Robertson, 1936; Bodenstein, 1965). An integral aspect of this remodeling is the external structures of the adult head and thorax that develop from imaginal discs. The discs are determined during embryogenesis and proliferate during larval development as they undergo pattern formation (Cohen, 1993). The patterned imaginal disc, however, is itself a static structure. Its growth, morphogenesis and terminal differentiation are dependent on pulses of ecdysone during metamorphosis (Fristrom and Fristrom, 1993; von Kalm et al., 1995). We would like to understand how ecdysone directs the imaginal discs to assume their appropriate morphological and functional properties during these final stages of their development. To achieve this goal, we need a means of identifying ecdysone-inducible regulatory genes that are required for appropriate disc morphogenesis and differentiation. The isolation and characterization of the *crooked legs* gene provides a first step toward this goal. Below, we describe the *crol* mutant phenotypes and present a model for *crol* function during the early stages of metamorphosis.

crol is required for adult head eversion and leg morphogenesis during metamorphosis

Mutations in the *crol* locus lead to lethality during pupal development with defects in adult head eversion and leg development. Although no earlier lethality is evident in these mutants, *crol* mRNA is maternally provided and thus *crol* may have essential functions during early stages of development (data not shown). Experiments to examine *crol* mutant embryos derived from germline clones are currently underway.

Although at least part of the leg phenotype associated with *crol* mutations can be attributed to defects in leg elongation during early prepupal development, *crol* also appears to exert functions in the leg during later stages of development. One manifestation of this is a kink near the middle of the femur (Fig. 3B). The femur and tibia are initially fused at 18 hours after puparium formation and are divided into distinct segments between 21 and 36 hours after puparium formation (Fristrom and Fristrom, 1993). It is possible that the kink in *crol* mutant femurs is due to a defect in this morphogenetic process. In addition, some bristles are occasionally missing from *crol* mutant legs, indicating defects in the final stages of leg differentiation (Fig. 3C).

One goal of our screen was to identify new regulatory genes that contribute to the complex developmental pathways controlled by ecdysone during metamorphosis. Consistent with this goal, the *crol* mutant phenotypes resemble those associated with some heteroallelic combinations of *BR-C* alleles and with

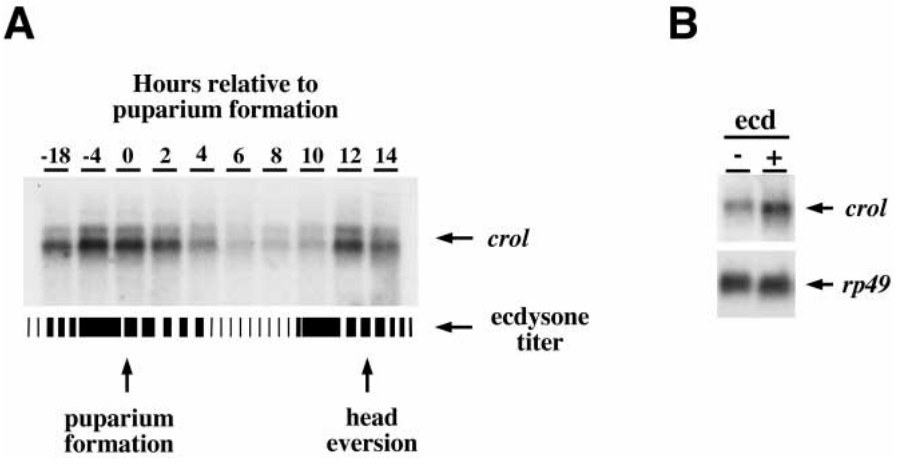

Fig. 8. *crol* transcription is regulated by ecdysone during the onset of metamorphosis. (A) The *cn*/*Df* control northern blot described in Fig. 5 was probed to detect the 5.3 and 6.0 kb *crol* mRNAs. Developmental times are given in hours relative to puparium formation (see Materials and methods for staging). A schematic representation of the ecdysone titer is shown at the bottom, showing the hormone peaks that trigger puparium formation and head eversion. (B) Ten pairs of salivary glands were dissected from wild-type mid-third instar larvae and incubated at 25°C for 4 hours in 100 μl of Grace medium (Gibco) containing either 5×10^{-6} M 20-hydroxyecdysone (+ecd) or an identical amount of ethanol, the solvent for the hormone solution (–ecd). Total RNA was extracted and *crol* and *rp49* transcription were analyzed by northern blot hybridization.

a null *E74B* mutation. Legs dissected from $br^1/npr1^3$ *BR-C* mutant pupae are malformed and significantly shorter than those of wild-type animals (Kiss et al., 1988), resembling those seen in *crol* mutant pupae. Furthermore, this phenotype can be attributed, at least in part, to defects in leg elongation during prepupal development (Fristrom et al., 1987).

The *E74B* lethal phenotypes also resemble those seen in *crol* mutant pupae. *E74B* mutants die during prepupal and early pupal development with defects in head eversion and leg elongation (Fletcher et al., 1995). These *E74B* phenotypes, however, are distinct from those seen in *crol* mutants. The *E74B* cryptocephalic phenotype is associated with a failure in gas bubble translocation from the middle of the prepupa to its anterior end. In wild-type animals, this process creates a space at the anterior end of the prepupa into which the adult head can later evert (Chadfield and Sparrow, 1985). The gas bubble in *crol* mutant prepupae, however, appears to translocate properly. Similarly, the leg defects of *E74B* mutants are distinct from those associated with *crol* mutations. *E74B* mutant legs are smaller than wild type and are missing distal segments of the tarsus (Fletcher et al., 1995). These similar, but nonetheless distinguishable mutant phenotypes suggest that *crol* and *E74B* have unique and perhaps overlapping functions in adult head and leg development. These observations also support the hypothesis that these complex developmental responses involve interactions among a number of ecdysone-inducible regulatory genes and identify *crol* as a critical member of these developmental pathways.

crol encodes at least three related zinc finger proteins

Structural characterization of the *crol* locus revealed that this is a complex gene encoding at least three related protein isoforms. These proteins have identical N-terminal and C-terminal sequences flanking a variable central region that encodes tandem repeats of 12-18 C_2H_2 zinc fingers. The presence of zinc fingers within the CROL proteins suggests a molecular mechanism for *crol* function, through the direct regulation of gene expression. Furthermore, the N- and C-terminal sequences shared by the three CROL protein isoforms are rich in proline, alanine, glutamine, serine and threonine residues. Similar stretches of homopolymeric amino acids are associated with a number of transcription factors, further supporting a role for *crol* in gene regulation (Mitchell and Tjian, 1989). Consistent with this possibility, *crol* mutations result in defects in ecdysone-regulated gene expression during prepupal and early pupal development (Fig. 5). It remains unclear, however, at what level CROL exerts its regulatory functions since, unlike most transcription factors, at least some zinc finger proteins are also capable of binding RNA (El-Baradi and Pieler, 1991).

crol transcription is induced by ecdysone at the onset of metamorphosis

The pattern of *lacZ* staining in $crol^{4418}/+$ larvae and prepupae combined with the temporal pattern of *crol* transcription during the onset of metamorphosis provide evidence in support of a role for ecdysone in inducing *crol* expression. Furthermore, *crol* mRNA levels increase approximately two-fold in salivary glands cultured in the presence of ecdysone, similar to the level of *crol* induction seen in vivo in late third instar larvae (Fig. 8B). Although these observations indicate that *crol* transcription is induced by ecdysone, they do not allow us to determine if this is a primary- or secondary-response to the hormone. An attempt to address this issue by using cycloheximide in cultured salivary glands was complicated by the ability of this drug to stabilize *crol* mRNA levels (data not shown).

The pattern of *crol* transcription in early pupae is consistent with *crol* induction as an 'early-late' response to ecdysone, similar to the response of the *DHR3* and *E78B* orphan receptor genes (Koelle et al., 1992; Stone and Thummel, 1993). The levels of *crol* mRNA increase 12 hours after puparium formation, 2 hours after the initial induction of *E74A* and *E75A* transcription and thus 2 hours after the peak in ecdysone titer (Fig. 5). This delay in *crol* induction is similar to the delay seen in *DHR3* and *E78B* induction relative to the late larval ecdysone pulse. This regulation has been attributed to a dual requirement for both the ecdysone-receptor complex and the synthesis of ecdysone-induced proteins (Ashburner and Richards, 1976; Stone and Thummel, 1993; Horner et al., 1995). Further studies of *crol* regulation should provide insights into its mode of regulation by ecdysone.

Models for *crol* function during prepupal and early pupal development

The changes in ecdysone-regulated gene expression seen in *crol* mutant prepupae provide a framework for understanding the molecular basis of the *crol* mutant phenotypes (Fig. 9). Four genes were examined that are expressed in imaginal discs and that appear to play a role in imaginal disc development: *IMP-E1*, *Sb*, *Brg-P9* and *EDG-84A*. Of these genes, only *Brg-P9* was affected in *crol* mutants – *Brg-P9* is expressed at higher levels, and for a longer duration, in *crol* mutant prepupae, suggesting that *crol* may normally repress this gene (Fig. 5). Interestingly, *Brg-P9* encodes a protein with sequence similarity to the kunitz class of serine protease inhibitors (Emery, 1995). Several studies predict an important function for proteases in imaginal disc morphogenesis during prepupal development (Poodry and Schneiderman, 1971; Fekete et al., 1975; Pino-Heiss and Schubiger, 1989). In addition, mutations in the *Stubble-stubbloid* gene (*Sb*), which encodes an apparent transmembrane serine protease, interact with the *BR-C* to regulate appendage elongation (Beaton et al., 1988; Appel et al., 1993). Some heteroallelic combinations of *Sb* alleles lead to defects in leg elongation that are similar to those seen in *crol* mutants and proper *Sb* leg elongation can be restored by simply culturing the mutant leg discs in the presence of trypsin (Appel et al., 1993). It is possible that increased levels of *Brg-P9* expression, and perhaps other serine protease inhibitors, could block the activity of *Sb* and other serine proteases in the leg discs, and thus prevent proper leg elongation during prepupal development (Fig. 9A). It is also likely that *crol* regulates other, as yet unidentified, target genes that function during leg elongation. The identification of other secondary-response genes that are regulated by *crol* and expressed in leg imaginal discs should provide a better understanding of *crol* function in this tissue.

crol mutations lead to stage-specific effects on ecdysone-induced regulatory gene expression during the onset of metamorphosis. The levels of *EcR* and *E74B* transcription are reduced in 6-10 hour *crol* mutant prepupae, and the *BR-C*,

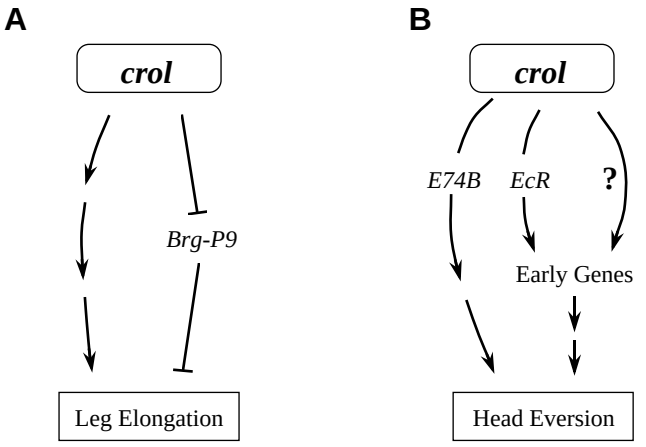

Fig. 9. Models for *crol* function during prepupal and early pupal development. (A) The targets of *crol* activity in the prepupal leg disc remain largely undefined. The *crol* locus does, however, appear to repress *Brg-P9* transcription, consistent with the ability of serine protease inhibitors to block leg elongation (see text). (B) The stage-specific effects of *crol* mutations on *E74B* and *EcR* transcription could indirectly regulate adult head eversion in early pupae. The question mark indicates that *crol* may also be a direct regulator of early gene expression.

E74A, *E75A*, *E75B* and *E93* early genes are submaximally induced in response to the ecdysone pulse in 10 hour prepupae (Fig. 5). These effects on gene expression provide a molecular basis for understanding the defects in adult head eversion seen in *crol* mutants (Fig. 9B). As mentioned above, *E74B* has been shown to be required for head eversion, although the mechanism(s) by which *E74B* regulates this response remain unknown (Fletcher et al., 1995). The reduced expression of *E74B* in *crol* mutant prepupae thus provides one means of interpreting the effect of *crol* mutations on adult head development (Fig. 9B). Alternatively, reduced levels of *EcR* expression in *crol* mutants could attenuate early gene induction by ecdysone and thereby indirectly affect head eversion (Fig. 9B). Finally, it is possible that *crol* directly regulates early gene expression in prepupae (Fig. 9B). In this regard, it is interesting to note that preliminary studies have shown that *crol* is expressed normally in *BR-C* and *E74* mutants, confirming that *crol* functions either upstream from, or in parallel with, these regulatory genes (data not shown). Further molecular and genetic studies of the *crol* locus should provide insights into how this gene can exert its multiple effects on the ecdysone regulatory hierarchies during metamorphosis.

A genetic screen for regulators of adult tissue development during metamorphosis

The screening strategy used to identify the *crol* locus provides a new avenue for studies of ecdysone function, by providing a genetic approach to the identification of regulatory genes that function during insect metamorphosis. All known regulatory genes in the ecdysone hierarchies have been isolated based on their cytogenetic location or membership in the nuclear receptor superfamily (Russell and Ashburner, 1996; Thummel, 1996). *crol* represents the first gene isolated independently of these criteria, based solely on its mutant phenotypes. In fact, *crol* would not have been identified based on its cytogenetic location since it does not map to an ecdysone-inducible puff in the salivary gland polytene chromosomes (Ashburner, 1972). Given the successful identification of this gene, we have expanded our screen to a collection of 1300 single P-element-induced lethal mutations on the second chromosome. From this screen, we have identified a dozen genes that appear to be regulated by ecdysone and play an essential role in imaginal disc development during metamorphosis (J. Gates and C. S. T., unpublished results). It seems likely that this approach will provide new opportunities to unravel the molecular mechanisms of ecdysone action during *Drosophila* development. In addition, molecular and genetic characterization of these loci should provide new insights into the mechanisms by which ecdysone triggers the remarkable transformation from a patterned imaginal disc to a differentiated adult structure.

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