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Krüppel-homolog, a Stage-Specific Modulator of the Prepupal Ecdysone Response, Is Essential for *Drosophila* Metamorphosis

Frédéric Pecasse,[1] Yannick Beck, Claude Ruiz, and Geoff Richards[2]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, B.P. 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

We have characterised a P-element-induced prepupal mutant of *Drosophila melanogaster* which after an apparently normal embryonic and larval development fails to complete head eversion, an essential step in metamorphosis. The P-element insertion disrupts an ecdysone-regulated transcript which, although expressed during embryonic and larval stages, appears critical for preparing the late prepupal response to ecdysone. By a combination of molecular and genetic studies, in which we recovered new alleles, we show that the locus is complex, containing at least two distinct promoters. Its transcripts contain a short region described previously by R. Schüh *et al.* (1986, *Cell* 47, 1025–1032), who screened for homologues of the *Krüppel* gene. Our studies on the corresponding gene, named *Krüppel-homolog* (*Kr-h*), add to a growing body of evidence that specific isoforms of a number of key genes are implicated in both embryogenesis and metamorphosis.

Key Words: **metamorphosis; ecdysone; Zn fingers; *Drosophila;* head development; prepupal stage.**

INTRODUCTION

Holometabolous insects establish two distinct body plans. The first, determined during embryogenesis, overtly gives rise to the larval form. The second, elaborated at metamorphosis, involves the destruction of certain larval tissues, the transformation of others, and the differentiation of adult tissues, most notably from the imaginal discs. In *Drosophila* the initiation of metamorphosis becomes evident at puparium formation in which the larva stops wandering and adopts a barrel-like form. Both tanning of the larval cuticle, which forms the puparium, and apolysis, which separates the underlying hypoderm, are initiated shortly thereafter. The majority of the hypoderm is detached by 3 to 4 h after pupariation and at this stage a gas bubble is evident in the middle of the prepupa. Without dissection, few changes can be observed for the next 5 to 6 h although imaginal disc differentiation, movement, and fusion progress. Renewed activity becomes apparent in the last hours of prepupal development which are marked by muscle contractions and movements of the gas bubble which terminate at 12 h after puparium formation with the eversion of the head, which takes up the adult position (see Bainbridge and Bownes, 1981, for a detailed description of events during this period).

Metamorphosis is orchestrated by the steroid hormone ecdysone, which exerts its effects through members of the nuclear hormone receptor superfamily, key molecules of a highly conserved regulatory mechanism found in both invertebrates and vertebrates (Mangelsdorf *et al.*, 1995; Thummel, 1996; Richards, 1997 for reviews). Unlike vertebrates, in which several families of receptors and their corresponding ligands exist, the situation in insects is more intriguing in that there is a relative paucity of hormones and development appears to be regulated mainly by the interplay of ecdysone and juvenile hormones (Riddiford, 1993). In addition to initiating metamorphosis, ecdysone also regulates insect moulting between larval instars and is involved in adult fertility, and the question arises as to how ecdysone can induce stage- and tissue-specific changes in gene expression.

A combination of genetic and molecular studies has started to provide insights into this problem in *Drosophila melanogaster.* We now know that ecdysone exerts its

[1] Present address: IRIBHN–Campus Erasme, C4-124, 808, Route de Lennik, 1070 Brussels, Belgium.

[2] To whom correspondence should be addressed. Fax: (33) 3 88 65 32 03. E-mail: richards@igbmc.u-strasbg.fr.

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function by binding to a heterodimer formed by the ecdysone receptor (EcR) and Ultraspiracle proteins (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993), in which the EcR partner is any one of three isoforms which show stage and tissue specificity (Talbot *et al.*, 1993). Equally the early genes induced by an increase in ecdysone titre are complex transcription units consisting of a number of isoforms. Consistent with the tissue coordination model of Hogness (Burtis *et al.*, 1990; Thummel *et al.*, 1990) the combination of isoforms and their level of expression is stage and tissue specific (Huet *et al.*, 1993), suggesting that the cell is in some way preset to respond to hormone. This state, or competence, changes from tissue to tissue and throughout development (Richards *et al.*, 1999) and thus ensures that the hormone elicits stage- and tissue-specific effects.

The most detailed molecular studies of the response to ecdysone have used larval and prepupal salivary glands. These have extended the concepts first suggested by studies on the puffing patterns of the giant polytene chromosomes and have led to detailed descriptions of the genes activated during the late larval and late prepupal responses to hormone (Russel and Ashburner, 1996; Thummel, 1996; Richards, 1997 for reviews). Studies on the transition between these two responses have shown that the expression of several members of the nuclear receptor superfamily contributes to competence in late prepupal salivary glands. *EcR* transcripts, which decline during the late larval response to ecdysone, are dramatically induced in 6-h prepupal glands just prior to the late prepupal response (Huet *et al.*, 1993, 1995). Stage specificity appears to derive from the expression of the *FTZ-F1* gene in the midprepupal period (Broadus *et al.*, 1999). The corresponding protein is localised on prepupal ecdysone-sensitive puff loci, suggesting that its presence is necessary for regulation by ecdysone in late prepupae (Lavorgna *et al.*, 1993). By expressing a *FTZ-F1* transgene in late larvae, Woodard *et al.* (1994) were able to induce *E93*, a gene normally induced by ecdysone in late prepupal salivary glands (Baehrecke and Thummel, 1995), in larval glands. The prepupal-specific activation of *FTZ-F1* requires the prior interactions of DHR3 and E75B (White *et al.*, 1997; Lam *et al.*, 1997, 1999), orphan receptors which are active at the end of the late larval response to ecdysone.

With the aim of characterising further genes that intervene in the stage-specific response to hormone, we have studied a P-element-induced mutant which after an apparently normal larval life and pupariation dies during the prepupal period and fails to complete the late prepupal response to ecdysone and pupation. We used this initial mutant to screen a collection of P-element-induced mutants and recovered further alleles of the locus showing lethality at the same stage. In the course of the molecular characterisation of the transcript disrupted by the P element insertion we found that a short sequence from the locus had been isolated previously by homology with the *Krüppel* (*Kr*) gene (Schuh *et al.*, 1986) and named *Krüppel-homolog* (*Kr-h*) although in the absence of mutants the earlier study was not pursued. By convention we have kept the original designation for this locus. We find that the gene has at least two distinct promoters and observe multiple *Kr-h* transcripts during development. Among these, a 4.5-kb transcript, interrupted by the initial P-element insertion, is indispensable for the normal progression to metamorphosis. Studies with late larval mutants (ecd^1, dor^{22}, and npr^6) and with *in vitro*-cultured salivary glands show that this transcript is itself ecdysone regulated. As initially predicted, the expression of other genes regulated by ecdysone in prepupae is altered in the *Kr-h* mutant.

MATERIAL AND METHODS

Drosophila Stocks

The Oregon-R wild-type strain maintained at 25°C on a standard agar medium was used throughout as a standard. The $Kr\text{-}h^1$ stock carries the ry^+ marked Carnegie 20-derived P element (Rubin and Spradling, 1983) harbouring a *Sgs-3* gene with its intron deleted (denoted ΔI:3 in Mettling *et al.*, 1987). The stock is maintained as a second chromosome balanced lethal ($P[ry^+lethal]/CyO;\ ry^{506}/ry^{506}$). To mobilise the transposon, $Kr\text{-}h^1$ females were crossed to $P[ry^+lethal]/CyO;\ ry^{506}Sb^1e\ P(\Delta 2\text{-}3)ry^+99B/ry^{506}$ males. In all, 76 of 765 offspring lacked either the *Cy* (= $P[ry^+lethal]*/P[ry^+lethal]$) or ry^+ (= $P[ry^+lethal]*/Cy$) marker and 38 independent events were recovered and analysed molecularly (see Results). The $Kr\text{-}h^7$ transposon was also mobilised using a similar approach.

To facilitate selection of homozygotes, $Kr\text{-}h^1$ males were crossed to $y\ w;\ CyO\ P[y^+]/noc^{Sco}$ females. F1 males $y\ w;\ P[ry^+lethal]/CyO\ P[y^+]$ were backcrossed to females $y\ w;\ CyO\ P[y^+]/noc^{Sco}$ and thereafter the $y\ w;\ P[ry^+lethal]/CyO\ P[y^+]$ stock, in which lethal homozygotes have brown mouthparts, was established.

The *Kr-h* alleles isolated from the collection of Török *et al.* (1993), provided by Bernard Mechler, were originally designated l(2)61/34–$Kr\text{-}h^2$, l(2)168/2–$Kr\text{-}h^3$, l(2)124/9A–$Kr\text{-}h^4$, l(2)124/9B–$Kr\text{-}h^5$, l(2)59/5–$Kr\text{-}h^6$, l(2)44/11–$Kr\text{-}h^7$.

Larvae from the temperature-sensitive ecd^1 strain (Garen *et al.*, 1977) were transferred to 29°C at the beginning of the third larval instar while control larvae were maintained at 20°C. At 29°C these animals eventually leave the food and then remain blocked in an extended wandering phase. Females from the X-chromosome 2B mutant strains bearing either the dor^{22} $[y\ l(1)t^{187}/FM6\ l\text{-}69j/Dp(1)y^2Y67g]$ or the *BR-C* npr^6 $[y\ l(1)t^{435}/FM6\ l\text{-}69j/Dp(1)y^2Y67g]$ mutation were crossed with Oregon-R males to obtain hemizygous males, which die as late larvae. *FM6 l-69j*-carrying males die before the third instar, whereas both classes of females are viable and serve as internal controls.

Developmental Staging, Dissections, and Observations

For embryonic and larval development animals were staged at egg laying. For salivary glands during the late larval ecdysone response, the contralateral lobe was puff staged, while for prepupal and pupal stages animals were selected as white prepupae and aged on moist filter paper at 25°C. When necessary, several tissues from a single animal were dissected and frozen at −80°C in RNA extraction buffer (see Huet *et al.*, 1993). For photography, prepupae or pupae were placed in a depression slide covered with Insect Ringer and positioned with the aid of a coverslip.

Salivary Gland Culture

Individual salivary glands were incubated in 25 μl of culture medium in glass depression slides, covered with grease-sealed coverslips, and maintained at 25°C. Contralateral lobes were either cultured in parallel ($\pm 1.8 \times 10^{-6}$ M ecdysone) or served as 0-h time points. Culture medium was modified Grace's medium (50:9:1—Grace's medium (Gibco):distilled water:ethanol). 20-OH ecdysone (Simes, Milan) was added in the alcohol fraction as appropriate.

Nucleic Acid Techniques

Standard procedures were used unless otherwise stated. The Oregon wild-type genomic library in λEMBL4 was a gift from Vince Pirrotta. The embryonic cDNA libraries (3–12 and 12–24 h) were a gift from Tom Kornberg and the late larval and prepupal cDNA libraries (both oligo(dT) and random primed) were a gift from Pat Hurban and Carl Thummel. Sequencing was essentially performed on a Perkin–Elmer automated sequencer using double-stranded DNA templates. Sequences were later consolidated with the genomic sequences from the Berkeley *Drosophila* Genome Project (unpublished).

Total cDNA Probe

One microgram of poly(A)$^+$ RNA from third-instar larvae was coprecipitated with 10 μg of random hexanucleotides, 0.5 mM each dNTP (except dCTP), 0.05 mM dCTP supplemented with 3.3 μmol [α-^{32}P]dCTP (3000 Ci/mmol), 10 mM DTT, 35 units of RNasin and then reverse transcribed with 200 units of M-MLV reverse transcriptase (Life Technologies) in the reaction buffer supplied. Reverse transcription was performed 1 h at 37°C and stopped by the addition of 20 mM EDTA and 0.4 N NaOH, treated 30 min at 65°C, and neutralised by 1 M HCl, 100 mM Tris–HCl, pH 8.3. Free nucleotides were eliminated on a Sephadex G50 column and this total cDNA probe was then hybridised to Southern blots of digested λEMBL phage from the chromosomal walk.

Polymerase Chain Reactions

For genotyping, DNA was prepared by crushing one fly in 50 μl of 10 mM Tris–HCl, pH 8.2, 1 mM EDTA, 25 mM NaCl, and 200 μg/ml proteinase K and incubating 30 min at 37°C (Gloor and Engels, 1992). The proteinase K was then inactivated by heating 3 min at 95°C. One microliter of the extract is sufficient for the PCR assay. Alternatively, to distinguish between larvae homozygous or heterozygous for the P-element insertions, the contaminant DNA present in RNA preparations of salivary glands can be used as template. In this case 1 μl from the initial 12 μl extract (see below) is sufficient. DNA was denatured 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Primers and internal probes were 20-mers chosen so that GC content was as close to 50% as possible. Two primers (denoted A and B) hybridising either side of the insertion site and a third primer (C) hybridising to the extremities of the P element were used in PCR to genotype the animals. If the animal is homozygous for the insertion, only the A–C and B–C fragments are amplified as the A–B separation (>10 kb) is too great for amplification under standard PCR conditions. A similar strategy was adapted for the analysis of events following P-element mobilisations.

RT-PCR using RNA from individual salivary glands (or other tissues) were performed as described in Huet *et al.* (1995). The microextract of RNA is taken up in 12 μl of distilled water and diluted fivefold before aliquoting. One microliter of the initial 12 μl is used to monitor RNA extraction and thereafter 1 μl of the final dilution is used in RT-PCR. Oligonucleotides and probes were as previously published (Huet *et al.*, 1993, 1995) or as follows. *E93* primers were TGGCCGACTTCAATCTGATC and ATCTAGCTTGTTGGCCACCA and the internal hybridisation probe CGCCAACTACCTCAGAAGTA. *Kr-hα* primers were TATACGTCGCTACGATCGCT and TTCAAGTTCTGAGGGAGTCG and the probe CGCACTAGCCAATGACCAAT. The sequences of primers used to amplify and label the *Kr-h* exon-specific probes of Figs. 3 and 5 are available on request. For *diap1* primers were TTCAGAGGAAAGGAGCCAGA and TCGGCACATTGTTGGTAGTG with ATAACAACACGAACGCGACC as probe. For *diap 2*, GCTTTCTCTACGTTGGACGA and ATATTCGCCACTGCTTCAGC, with ATTGAACCATGTCAGGCCAC as probe. For *rpr*, GGCAGTGGCATTCTACATAC and TCATTGCGATGGCTTGCGAT, with CGCCAGTACACTTCATGTCA as probe. For *hid*, ATGACGTAGCGTCGAGTTCA and TACATCCTGACCCACTCGTA, with AAGTGCTATACGCCCTCTAC as probe.

RESULTS

Kr-h^1—A P-Element-Induced Mutant That Fails to Complete the Prepupal Stage

The initial P-element-induced mutation, localised to the 26B/C border by *in situ* hybridisation, was obtained by Mettling *et al.* (1987) and maintained as a heterozygous balanced stock. Emerging cultures are characterised by the presence of dark brown pupal cases, corresponding to homozygous mutant animals. On closer examination one observes developmental aberrations from the late prepupal period (c. 12 h after pupariation) onwards, most clearly seen by the failure of the legs to complete evagination and take up their pupal position. Strikingly, mutant animals dissected 24 to 36 h after pupariation have intact larval salivary glands. As these glands are normally histolysed during the late prepupal response to ecdysone (10 to 14 h after pupariation), this suggested that the lesion occurred prior to or during this response.

In the stock, here renamed *Kr-h^1*, 25% of animals (the *Cy* homozygotes) die at the end of embryogenesis or as early first-instar larvae. As *Kr-h^1* mutant larvae represent a third of the first group of third-instar larvae that leave the food, the mutation has no effect on embryonic and larval development. Gas bubble formation, some 3 to 4 h after pupariation, is on schedule. However the displacement of the bubble appears abnormal and animals fail to complete head eversion (Figs. 1A–1F). Random bubble displacement continues for several hours and internal pulsions can be detected for several days, indicating that death is not immediate. A few animals develop further and show a striking cryptocephalic phenotype in which eye pigmentation is evident within the thorax (Figs. 1H and 1J and see below). The larval mouthparts remain attached to the imaginal head structures. In these animals, although the abdominal cuticle is formed at pupation, segment differentiation, as

FIG. 1. Heterozygous and homozygous prepupae and pupae from the *Kr-h*^{1} line. (A–C) *Kr-h*^{1} heterozygotes (*Kr-h*^{1}/Cy) and (D–F) *Kr-h*^{1} homozygotes at (A, D) 10 h after pupariation (ap), (B, E) 13 h ap, (C, F) 2 days ap. (G, I) A *Kr-h*^{1} heterozygote at 4 days ap compared with (H, J) the minority *Kr-h*^{1} late pupal homozygous lethal phenotype, both dissected from their pupal cases. A, B, D, E, G, H—dorsal view; C, F, I, J—ventral view. ab—abdominal bristles, ep—eye pigmentation, mp—larval mouthparts.

FIG. 2. Mutant phenotypes from the *Kr-h*^{2} to *Kr-h*^{7} series. (A) *Kr-h*^{5}, 5 days after pupariation (ap). (B) *Kr-h*^{3}, 4 days ap. (C) *Kr-h*^{6}, 3 days ap. (D) *Kr-h*^{5}, 6 days ap. (E) *Kr-h*^{3}, 3 days ap. (F) *Kr-h*^{5}, 6 days ap. (G) *Kr-h*^{7}, 3 days ap. (H) *Kr-h*^{7}*/Df(2L)Kr-h7.1*, 3 days ap. Phenotypes A and B are relatively common while phenotypes C to F are found at a low frequency. G and H are essentially found in *Kr-h*^{7}, see text. Necroses are indicated by white arrows. The aberrant position of the anterior spiracles in G and H is indicated by white dots.

seen by bristle formation, does not extend beyond the proximal abdominal segments (Fig. 1H).

We mobilised the transposon by crossing in a source of transposase (see Material and Methods) and established 38 lines selected for the loss of the *ry*$^{+}$ phenotype. Viability was restored in 26 cases, confirming that the original lethality was caused by the insertion of the transposon. Using primers flanking the insertion site (obtained from the initial cloning studies—see below), we detected deletions of up to 50 bp by PCR although viability was equally restored when up to 800 bp of transposon sequence remained. In 12 cases the reversions to *ry* were due to internal deletions of the P element and the lethal phenotype was unchanged (data not shown).

Identification of the Effective Lethal Phases of Further Kr-h Alleles

We characterised further alleles by crossing *Kr-h*^{1} to some 500 strains selected as second chromosome lethals from the P-element collection of Török *et al.* (1993). Six isolates failed to complement and gave rise to lethal prepupae when intercrossed both with *Kr-h*^{1} and with each other (see Material and Methods for the original designation of these strains). We determined the effective lethal phase of each *Kr-h* allele (Tables 1 and 2). If we add the c. 10% mortality seen in the *Oregon* and *ry*^{506}*C-S* controls to that of *Cy* homozygotes, we expect 32.5% mortality (25% *Cy* homozygotes plus 10% lethality of the remaining 75%) prior to pupariation, if there is no lethality due to the *Kr-h* mutation before pupariation. For *Kr-h*^{1} to *Kr-h*^{5} this is the case (Table 1), and after pupariation, for these alleles, lethal *Kr-h* homozygotes represent approximately 25% of the total embryos and one-third of the prepupae or pupae. For *Kr-h*^{6} (54.9% lethality prior to pupariation) and *Kr-h*^{7} (61.7% lethality) there are clearly embryonic and/or larval losses (Table 1), suggesting that *Kr-h* has a role earlier in development (see below), and consequently postpupariation lethality is only 10 and 18%, respectively. By eclosion the combined corrected lethalities are close to 50% (*Cy* homozygotes plus *Kr-h* homozygotes) for all alleles except *Kr-h*^{7} (70% lethality).

To determine the time of death, the development of the newly formed prepupae, scored in Table 1, was followed

TABLE 1
Lethal Phases of *Kr-h* Mutations

Strains	Total embryos	% dead animals bp	% dead animals ap	% total/ correction[a]
Oregon R	127	11.0	1.6	12.6
ry^{506}*C-S*	95	10.5	2.1	12.6
Kr-h^{1}	200	34.5	25.0	59.5/50.1
Kr-h^{2}	204	33.8	26.0	59.8/50.4
Kr-h^{3}	382	34.3	23.8	58.1/48.7
Kr-h^{4}	198	38.4	27.3	65.7/56.3
Kr-h^{5}	194	30.9	30.4	61.3/51.9
Kr-h^{6}	191	54.9	10.0	64.9/55.5
Kr-h^{7}	188	61.7	18.1	79.8/70.4

Note. Embryos from the *Kr-h* balanced lethal stocks were counted and transferred to regular medium for larval development. Those that reached pupariation were counted, transferred to fresh tubes, and observed until adult eclosion. Lethality, calculated with respect to the number of embryos, was estimated both before and after pupariation (bp and ap, respectively) and the total corrected ([a]) taking into account losses in the control lines *Oregon-R* and *ry*^{506} *C-S* due to culture conditions. 75% of the zygotes were exposed to this factor (*Cy* homozygotes are excluded).

using the morphological markers of Bainbridge and Bownes (1981). In each of the control lines two animals died as late pupae, after the onset of wing coloration. For all *Kr-h* alleles, the vast majority of homozygous animals show a lethal phenotype before head sac eversion at P4(ii) (as for *Kr-h*^{1}, Figs. 1E and 1F) and, except for *Kr-h*^{6}, represent a third of the prepupal population (Table 2). However, in the case of *Kr-h*^{7} the majority of the prepupal lethals were heterozygotes (see below). Minority phenotypes are shown in Fig. 2. In intercrosses, heterozygotes between *Kr-h*^{1} and the newly recovered alleles showed similar levels of post-pupariation lethality as in *Kr-h*^{1}. In the intercross between *Kr-h*^{6} and *Kr-h*^{7}, many heterozygotes survived to pupariation, suggesting that this combination of alleles complements for early development giving rise to fewer losses than those seen in the homozygotes of the parental strains (Table 3 and data not shown).

When developmental arrest occurs soon after gas bubble formation, the bubble remains visible in the middle of prepupa (Fig. 2A). At 1 to 4 days after pupariation, these animals are separated from the puparium (Fig. 2B). In animals arrested later in the prepupal stage, the gas bubble may remain in the anterior or posterior region or indeed laterally. Although head eversion does not occur, legs and wings are visible and elongated and the abdomen and thorax appear similar to those of wild-type pupae of the same age (Fig. 2C). Exceptionally, animals that had undergone head eversion died with the legs and wings apparent and translucent but with hypertrophied eye structures (Fig. 2D). In a few cases, 5 to 6 days after pupariation, a red eye coloration is visible within an abnormal head sac of animals which otherwise resemble late prepupae (Figs. 2E and 2F). In most cases necroses appear at one-third body length and increase with time (Figs. 2B, 2C, and 2D). We observed deformed homozygous *Kr-h*^{7} prepupae in which the anterior spiracles were close to the midline (Fig. 2G). Following the molecular analysis (see below), we mobilised the transposon in *Kr-h*^{7} to obtain *Df(2L)Kr-h7.1*, a deletion of *Kr-h* which results in lethality in embryonic and early larval stages (Beck *et al.*, manuscript in preparation). When the *Kr-h*^{1} to *Kr-h*^{5} alleles were crossed to *Df(2L)Kr-h7.1*, we observed the same phenotypes as seen in the parent strains with similar frequencies of prepupal lethals (Table 3 and data not shown). For *Kr-h*^{6} prepupal lethality was lower, while when *Kr-h*^{7} was crossed to the deletion, a more extreme form of the deformed phenotype was observed in all *Kr-h*^{7}*/Df(2L)Kr-h7.1* heterozygotes that reached pupariation (Fig. 2H).

TABLE 2
Postpupariation Lethality in *Kr-h* Strains

Pupal stage: Marker:	P1–P2 White puparium; bubble prepupa		P4(ii) Head sac eversion	P8 Yellow eyes	P11(i) Thoracic bristles	P12(ii) Black wings	P14 Green meconium	P15(ii) Eclosed adult
Oregon R	113	—	(0%)	—	—	2	—	—
ry^{506}*C-S*	85	—	(0%)	—	—	—	2	—
Kr-h^{1}	131	40	(30%)	—	8	—	—	2
Kr-h^{2}	135	49	(36%)	2	—	—	2	—
Kr-h^{3}	249	82	(33%)	1	—	3	5	—
Kr-h^{4}	122	48	(39%)	—	—	—	6	—
Kr-h^{5}	134	48	(35%)	—	2	—	9	—
Kr-h^{6}	86	16	(18%)	1	—	—	2	—
Kr-h^{7}	72	27	(37%)	—	—	—	7	—

Note. Prepupae were collected between P1 (white puparium) and P2 (bubble prepupa). Thereafter numbers refer to the animals whose development was arrested between two morphologically defined stages (Bainbridge and Bownes, 1981). Percentage lethality prior to head sac eversion is calculated with respect to the number of prepupae collected.

TABLE 3
Postpupariation Lethality in Crosses between *Kr-h* Strains

	Kr-h^1	*Kr-h*^{5}	*Kr-h*^{6}	*Kr-h*^{7}	*Df(2L)Kr-h7.1*
Kr-h^1	*n* = 809 **33.3%**	519 **32.6%**	537 **36.7%**	245 **36.3%**	458 **31.2%**
Kr-h^5		514 **27.6%**	202 **29.7%**	289 **24.2%**	224 **32.6%**
Kr-h^6			342 **17.0%**	500 **34.2%**	373 **29.8%**
Kr-h^7				669 **17.6%**	615 **23.9%**

Note. Combinations of *Kr-h* alleles were obtained by crossing heterozygous adults (*Kr-h*^{x}/*Cy*). Prepupal lethality is expressed as a percentage (bold) of the number of prepupae counted (*n*). *Kr-h*^{1} and *Kr-h*^{5} were chosen as representative of the alleles *Kr-h*^{1} to *Kr-h*^{5} (see text).

Late lethalities were observed in all *Kr-h* alleles in which animals had undergone head eversion and died between P12 and P15 (Table 2). All were distinct from the minority homozygous phenotypes shown in Figs. 1 and 2. By using the *y* mouthpart marker, we found that heterozygotes are included in this class (data not shown). As their frequency is higher than in the control lines, this may indicate that although these *Kr-h* mutations essentially prevent head eversion, absolute levels of *Kr-h* products may be critical in late metamorphosis. Overall, heterozygote lethality was highest in *Kr-h*^{7} when prepupal lethality was included (see Discussion).

Characterisation of Transcripts at the Kr-h Locus

We obtained a molecular probe by using inverse PCR on genomic DNA from *Kr-h*^{1} digested with *Hin*dIII and circularised by ligation. Using primers positioned at the ends of the P-element fragment we obtained a 375-bp fragment including 320 bp of DNA flanking the P-element insertion which was labelled and used as a probe to obtain the corresponding wild-type genomic region from an Oregon-R library (Fig. 3). Attempts to detect transcripts using this probe on Northern blots of total RNA were unsuccessful. We then labelled cDNA using poly(A)$^+$ RNA from late third-instar larvae and used this as a probe on digests of the phage of the first step of the genomic walk. We detected a weak signal on the 1.9-kb *Eco*RI fragment which includes the insertion site of the P element (data not shown). The nucleotide sequence of this fragment did not reveal an open reading frame or obvious transcriptional regulatory signals. However, we confirmed the presence of a transcribed region and delimited its position within the 1.9-kb fragment by using pairs of oligonucleotides sequentially in RT-PCR using RNAs from late third-instar larvae. By using, alternatively, only one primer for the reverse transcription reaction for each pair, we were able to define the direction of transcription. We obtained the 5′ extremity of the transcript by RACE (Frohman *et al.*, 1988) and confirmed the start site by primer extension (data not shown). Probes from this region were then used to screen larval and prepupal cDNA libraries. The cDNA clones obtained were used to position the corresponding transcript on the genomic region. In all, three steps of hybridisation were necessary to obtain overlapping phage covering the region of the cDNAs (Fig. 3).

The complete sequence (Fig. 4), compiled from overlapping cDNAs, revealed a 4.5-kb transcript which corresponds to the transcript seen by Northern analysis of larval poly(A)$^+$ RNA (Fig. 3). After an initiation codon in a consensus setting (Cavener and Ray, 1991) there is an ORF of 791 amino acids. This includes the 260-bp sequence encoding three zinc fingers, previously described by Schuh *et al.* (1986). In fact the 4.5-kb transcript, hereafter denoted *Kr-hα*, encodes a central domain with eight putative C_2H_2 zinc fingers (Zn1 to Zn8) which is preceded by a glutamine-rich domain and followed by a serine/threonine-rich domain, both characteristic of transcription factors. The latter is also rich in proline and glutamic acid residues and contains a number of potential PEST sequences (Rechsteiner and Rogers, 1996). Zn1 is separated by a 57-amino-acid-long spacer from the remaining seven fingers. Note that Zn6 starts with a CX_4C motif. The *Kr-h*^{1} insertion is localised in the 5′ untranslated region which explains the fact that we obtained revertants even with imperfect excisions of the transposon leaving up to 800 bp in place (see above).

Schuh *et al.* (1986) detected several transcripts in embryos and isolated a 3.8-kb cDNA, but did not describe this in detail. We screened embryonic cDNA libraries to recover the 3.8-kb species, *Kr-hβ*, which proved to have a distinct first exon which derives from an embryonic-specific promoter (Figs. 3 and 4). The start site of this transcript was determined by primer extension (data not shown). This transcript contains an open reading frame that would encode an additional 54 N-terminal amino acids compared to *Kr-hα*, although unlike *Kr-hα*, the initiation codon is not preceded by a consensus upstream sequence. In the longest embryonic cDNA we recovered, the two short introns, previously characterised with *Kr-hα* (Fig. 3), were present. If the first of these is not spliced it would give rise to a truncated protein with a single zinc finger. However, RT-PCR with primers spanning these introns using either embryonic or late larval RNA as templates suggests that this is a premessenger with delayed processing as RNA species with both introns are relatively abundant in both embryonic and larval RNAs (data not shown). We have not been able to completely define the structure of the minority

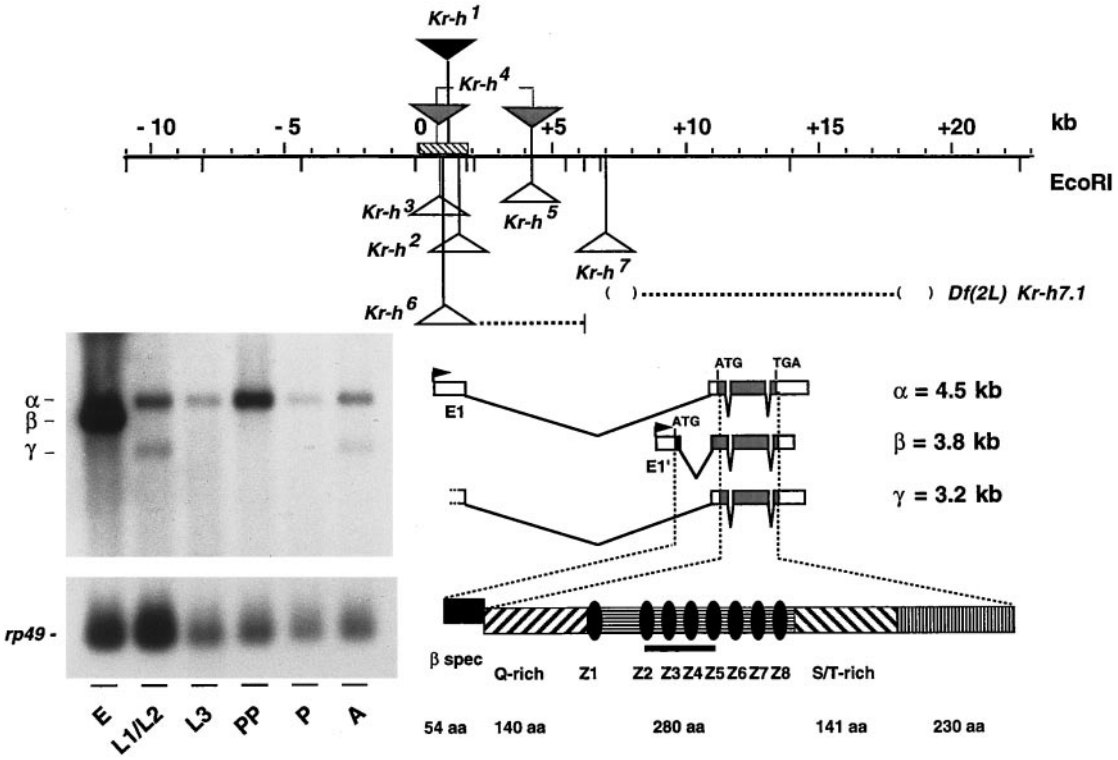


FIG. 3. The molecular map of the *Kr-h* locus. The collection of λEMBL phage covering 35 kb of genomic sequence is summarised with their *Eco*RI restriction sites. The initial 1.9-kb *Eco*RI fragment referred to in the text is shown as a hatched box. The sites of P-element insertions are indicated: *Kr-h*^{1} (black triangle), 5 single *P-lacW* insertions (open triangles), and 1 double *P-lacW* insertion (grey triangles). The *P-lacW* insertions are denoted *Kr-h*^{2} to *Kr-h*^{7}. Note that the *Kr-h*^{6} insertion is associated with a deletion of c. 6 kb as indicated. *Df(2L)Kr-h7.1* was recovered by the mobilisation of the *Kr-h*^{7} transposon, parentheses denote the limits of the 3′ end of the deletion. The three principal *Kr-h* transcripts, α, β, and γ, as seen in Northern analyses, are positioned with respect to the genomic map, together with their structure and size in kb. The *Kr-h*γ 5′ extremity has not been determined (see text). For Northern analyses 10 μg of poly(A)$^+$ RNA from whole animals was fractionated for each stage as indicated E, embryos; L1/L2, L3, 1st-, 2nd-, and 3rd-instar larvae; PP, prepupae; P, pupae; A, adults. The filter was hybridised with a probe from the common region of the *Kr-h* transcripts as well as a *rp49* probe to monitor loading. Start sites are denoted by arrows, exons by box sections with the translation start (ATG) and termination (TGA) codons marked. A grey box section indicates the Zn finger domain (see Fig. 4 and text). The region described by Schuh *et al.* (1986), Zn2–Zn5, is shown by a thick gray line.

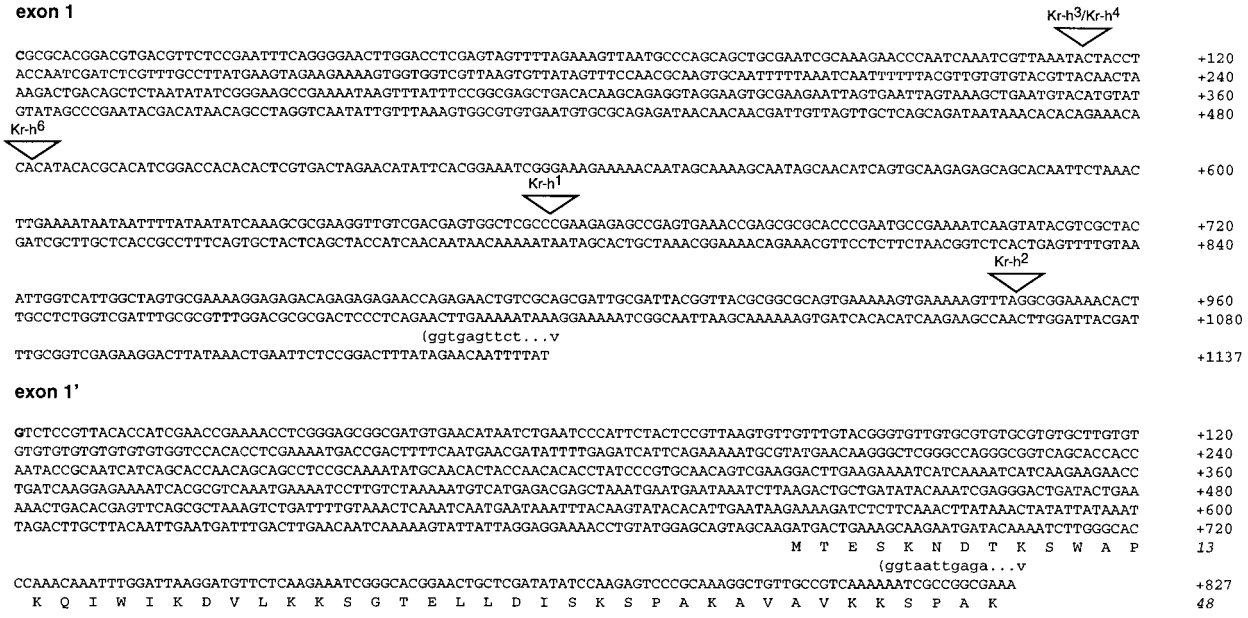
*Kr-h*γ transcript, which is recognised by probes from the 3′ region of exon 1, but not by probes 5′ to the P element of *Kr-h*^{1}.

We also undertook plasmid rescue with the *P-lacW* strains so as to localise the corresponding P-element insertions (Bier *et al.*, 1989). In each case the event was localised to the first exon of *Kr-h*α or the first intron (Fig. 3), showing that the region corresponding to the primary transcript is a hot spot for such insertions. *Kr-h*^{4} and *Kr-h*^{5} are related but distinct in that the former contains two insertions in the locus, only one of which is found in *Kr-h*^{5}. In the case of *Kr-h*^{6} the insertion was associated with a deletion of a large part of the first intron of *Kr-h*α, 5′ of the embryonic promoter. As the P-element insertions are all upstream of the embryonic promoter (Figs. 3 and 4) we presume that the embryonic transcript is not structurally altered. However, while *Kr-h*^{1} to *Kr-h*^{5} are essentially prepupal lethals, both *Kr-h*^{6} and *Kr-h*^{7} present a percentage of earlier lethalities (Table 1) which may reflect suboptimal levels of the *Kr-h*α or β transcripts or in the case of *Kr-h*^{7} reflect a partially dominant effect of a novel transcript (see below).

The Stage- and Tissue-Specific Expression of Kr-h Transcripts

*Kr-h*β transcripts are essentially limited to embryogenesis and are detected at low levels in 0- to 4-h embryos. Thereafter they are abundant in midembryogenesis and then decline (Fig. 5A, see also Fig. 3). Lower levels of *Kr-h*α transcripts appear during midembryogenesis (Fig. 5A) and then again in first-instar larvae (not shown). A major period of expression starts during the second instar and is main-

exon 1



exon 1'

common region

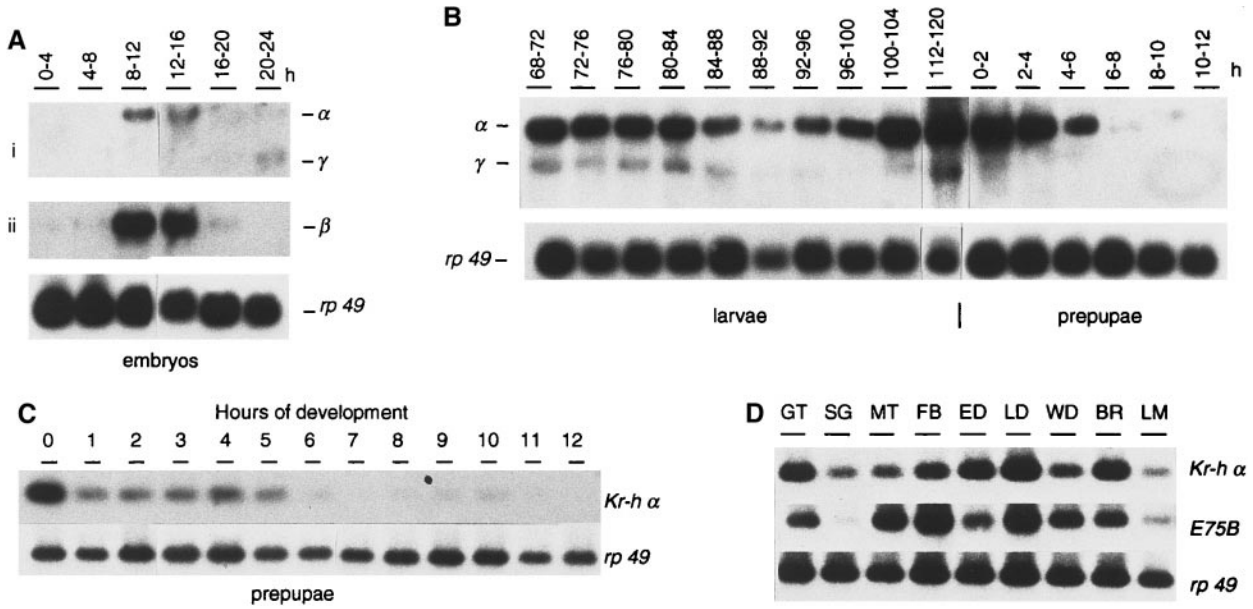


FIG. 5. *Kr-h* expression during embryonic, larval, and prepupal development. For each stage 10 μg of poly(A)$^+$ RNA extracted from whole animals was fractionated, transferred to nylon filters, and hybridised with (A) (i) a probe specific to exon 1 common to *Kr-h*α and γ and (ii) a probe specific to exon 1′ of *Kr-h*β (see Fig. 3) and (B) a probe from exon 1 of *Kr-h*α and γ. Staging is in hours after egg laying until pupariation (120 h) when animals were resynchronised as 0-h prepupae. Filters were also hybridised to a *rp49* probe to monitor loading. (C) *Kr-h*α and *rp49* expression analysed by RT-PCR in the same RNA extract from individual staged prepupal salivary glands. (D) *Kr-h*α, *E75B*, and *rp49* transcripts were assayed by RT-PCR in different tissues of a 0-h prepupa. GT, gut; SG, salivary glands; MT, Malpighian tubules; FB, fat body; ED, eye discs; LD, leg discs; WD, wing discs; BR, larval brain; LM, larval muscles.

tained until 6 to 8 h after pupariation (Fig. 5B). *Kr-h*γ transcripts appear between 20 and 24 h of embryogenesis (Fig. 5A) and thereafter *Kr-h*γ shows a developmental profile similar to that of *Kr-h*α although transcripts are some 10-fold less abundant when detected with a probe from the common region (Fig. 3). Both *Kr-h*α and *Kr-h*γ are also present in adults (Fig. 3).

Our initial interest was in the expression of the gene during metamorphosis when *Kr-h*α is dominant (Fig. 5B). To study this period in more detail, we used RT-PCR analyses of staged wild-type prepupal salivary glands. These show that *Kr-h*α is expressed in young prepupae and that transcripts decline between 4 and 6 h after pupariation (Fig. 5C), a few hours before the gross morphological abnormalities appear. In white prepupae, *Kr-h*α transcripts are present at similar levels in all of the tissues tested (Fig. 5D), consistent with a general role in the hierarchies of gene expression leading to metamorphosis. Note that *E75B* activity in these same samples is more variable, reflecting timing differences in its expression in different tissues (Huet *et al.*, 1993).

To determine the nature of the *Kr-h* alleles we undertook Northern analyses of *Kr-h* transcripts in *Kr-h*^{1} and *Kr-h*^{7} heterozygous and homozygous late larvae (not shown) and 0- to 4-h prepupae (Fig. 6). Results were similar at both stages. In the case of *Kr-h*^{1} homozygotes, in which the P element is inserted in the first exon, *Kr-h*α transcripts were absent but the probe detected low levels of two transcripts,

FIG. 4. The nucleic acid sequence of the two major *Kr-h* transcripts and their predicted amino acid sequences. Start sites, determined by primer extension, are denoted in bold. Nucleotide numbering for the α- and β-specific (see Fig. 3) and common exons are independent. The predicted amino acid sequence is numbered for the longer β isoform. Exon–intron boundaries are indicated with intron extremities in lowercase. The three principle domains, glutamine rich (Q), zinc finger (Zn), and serine/threonine rich (S/T) are boxed. Within the zinc finger domain the eight fingers (Z1 to Z8) are indicated. The polyadenylation site of *Kr-h*α and the putative polyadenylation site of *Kr-h*β (as deduced from the longest cDNA clone) are underlined. The sites of P-element insertions in the exons are shown. The second insertion of *Kr-h*^{4} and the remaining insertions are localised in the first intron upstream of the promoter of the *Kr-h*β transcript (see Fig. 3). Sequences are deposited in the EMBL database—Accession Nos. AJ005440 and AJ005441. Our different cDNA and genomic sequences were consolidated with those of the Berkeley *Drosophila* Genome Project (unpublished).

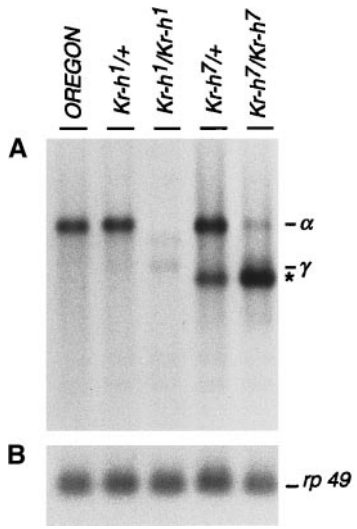


FIG. 6. Expression of *Kr-h* transcripts in homozygous or heterozygous $Kr\text{-}h^1$ and $Kr\text{-}h^7$ prepupae. For each lane 10 μg of poly(A)$^+$ RNA extracted from whole animals (0- to 4-h prepupae) was fractionated, transferred to nylon filters, and hybridised with (A) a mixture of probes corresponding to exons 1 to 4 (Fig. 3) and (B) a *rp49* probe to monitor loading. $Kr\text{-}h^1$ and $Kr\text{-}h^7$ animals were selected as *y* (homozygous) or y^+ (heterozygous) larvae and collected after pupariation. In (A) a novel transcript in $Kr\text{-}h^7$ heterozygotes and homozygotes, migrating faster than *Kr-hγ*, is marked by an asterisk.

which migrated at the level of the *Kr-hβ* and *Kr-hγ* transcripts, respectively. In the case of $Kr\text{-}h^7$ homozygotes, *Kr-hα* transcripts were reduced some 20-fold in the homozygous mutant pupae, suggesting that the presence of the P element in the first intron gives rise to a hypomorphic allele. In addition the $Kr\text{-}h^7$ allele gives rise to a novel transcript migrating somewhat faster than the *Kr-hγ* transcript (asterisk, Fig. 6).

The Prepupal Ecdysone Response in Salivary Glands Is Disrupted in $Kr\text{-}h^1$ Homozygotes

Both the initial morphological studies and the expression of *Kr-hα* suggested that we should detect the molecular consequences of the mutation during the prepupal period. We collected several series of homozygous $Kr\text{-}h^1$ prepupae and their heterozygous sibs aged from 0 to 12 h after pupariation and examined key transcripts of the late larval and prepupal ecdysone responses in salivary glands by RT-PCR. *Kr-h* transcripts were absent or severely reduced in homozygous glands (not shown, see Fig. 6 for whole-animal analyses) and in heterozygotes presented the same profile as in wild-type glands (Fig. 5C). In the first hours following pupariation the most notable differences were the relative persistence of *DHR3* and *E75B* in homozygotes and the premature induction of *EcR B* transcripts, which appeared some 2 h earlier (Fig. 7). While profiles of *βFTZ-F1* transcripts were similar, overall levels were somewhat higher in glands from homozygotes. From 4 to 8 h, the midprepupal transcript *E74B* (as well as *E75C* and *DHR39*, not shown) was induced prematurely in homozygotes. Thereafter the late prepupal transcripts such as *E74A*, *E75B*, or *E93* (as well as *E75A* not shown), normally induced between 9 and 10 h, were either delayed or failed to reach a full induction (Fig. 7). Overall, the most striking consequence of these disruptions was the appearance of a mid-prepupal rift in the normal orderly progression of gene expression leading to pupation.

Ecdysone Regulation of Kr-hα Transcript Levels

The above results suggest a role for *Kr-h* in modulating the stage-specific response to ecdysone. To investigate whether *Kr-h* itself is regulated by hormone we undertook a

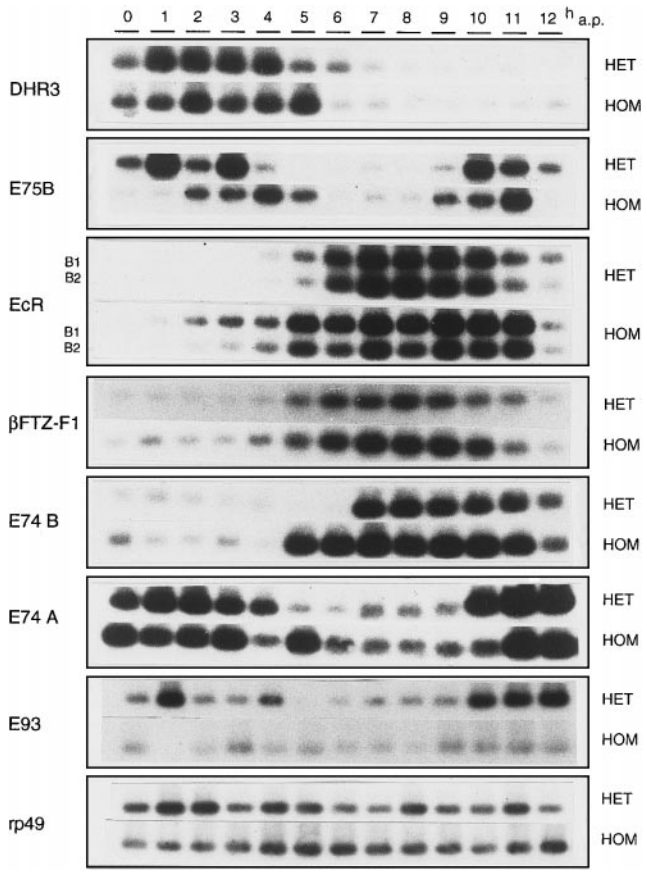


FIG. 7. Expression of ecdysone-regulated transcripts in salivary glands from *Kr-h* homozygous or heterozygous prepupae. *DHR3*, *E75B*, *EcR B1* and *B2*, *E74B*, *E74A*, and *E93* transcripts were analysed by RT-PCR in the same RNA extract from individual salivary glands from homozygous (HOM) or heterozygous (HET) prepupae aged from 0 to 12 h after pupariation (a.p.). *rp49* was used to control RNA extracts.

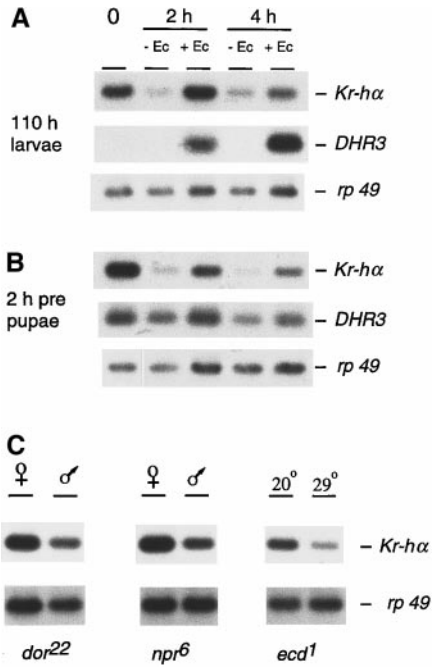


FIG. 8. Ecdysone regulation of *Kr-hα* transcripts in wild-type salivary glands *in vitro* and in mutant strains. Salivary glands from Oregon-R (A) 110-h larvae and (B) 2-h prepupae were cultured for 2 or 4 h in the absence (− Ec) or presence (+ Ec) of 1.8×10^{-6} M ecdysone. *Kr-hα, DHR3,* and *rp49* transcripts were analysed by RT-PCR in the same RNA extracts from five individual salivary glands and the products then pooled. In (C) *Kr-hα* and *rp49* transcripts were assayed in RNA extracts from glands of female (control) and male (mutant) dor^{22} and npr^{6} late third-instar larvae. For ecd^{1}, control glands are from larvae maintained at 20°C while mutant glands are from larvae maintained at 29°C.

series of experiments with salivary glands from larvae and early prepupae (Fig. 8). When glands from 110-h wild-type wandering larvae were cultured with or without hormone for 2 or 4 h, the existing *Kr-hα* transcript levels (see Fig. 5) showed a slight increase at 2 h with hormone but decreased in the absence of hormone (Fig. 8A). This effect was clearly more marked than that seen with *rp49,* which otherwise showed a similar response, confirming and extending the observations of Andres and Cherbas (1992) that these transcripts show developmental variation and at certain periods are not the best loading control. In contrast *DHR3* transcripts were induced by 2 and 4 h of hormonal treatment in these same glands (Fig. 8A). We then assayed the same transcripts in a similar experiment using glands from 2-h prepupae (Fig. 8B) as we had observed similarities in the time course of disappearance of *Kr-hα* and *DHR3* transcripts in prepupae. *Kr-hα* transcript levels remained higher in the presence of hormone, at a time when the decreasing hormone titre *in vivo* would normally lead to their disappearance (Fig. 5C). The hormonal effect was more marked for *Kr-hα* than for *DHR3* transcripts while *rp49* transcripts once more showed a modest response to hormone (Fig. 8B).

The behaviour of *Kr-hα* in larval glands is reminiscent of that of *hsp27* whose transcript levels increase prior to the late larval increase in ecdysone titre (Huet *et al.,* 1996) but are nonetheless sensitive to that increase. We tested this hypothesis by studying *Kr-hα* transcripts in three late larval mutant strains, dor^{22}, npr^{6}, and ecd^{1}, that affect the expression of ecdysone-induced genes to varying degrees (suggesting differences in the underlying regulatory pathways—see Huet *et al.,* 1996; Richards *et al.,* 1999). *dor* encodes a protein with a zinc-finger-like motif (Shestopal *et al.,* 1997), the npr^{6} mutant is a "long" allele of the *Broad-Complex* which encodes a family of proteins differing in their "zinc-finger" motifs (DiBello *et al.,* 1991), while ecd^{1} mutants are deficient in ecdysone (Garen *et al.,* 1978). In each case, *Kr-hα* transcript levels were lower in mutant animals, which are developmentally arrested in an extended wandering stage, than in the control siblings, the effect being more pronounced for npr^{6} and ecd^{1} than dor^{22} (Fig. 8C).

We finally studied the response to hormone in glands, from both wild-type and $Kr\text{-}h^{1}$ 6-h prepupae (Fig. 9A). The results are similar to those observed *in vivo* (Fig. 7) in that while *EcR* transcripts are repressed by 4 h of treatment in wild-type glands, in the mutant glands their disappearance is more gradual. *E93* transcripts are clearly induced in the wild-type glands while in the mutant glands they show a higher basal level which does not increase dramatically with hormone. Thus the mutant glands are disrupted in their capacity to respond to exogenous hormone and show an attenuation of the normally sharp transcriptional response to hormone seen in late prepupal glands. In the same extracts we also examined transcripts of a number of genes known to be active in late prepupal salivary glands (Jiang *et al.,* 1997), which have been shown to play a role in apoptosis in *Drosophila.* Transcripts of *diap2* were present but at lower levels than in wild-type glands, while those of *hid* were induced in *Kr-h* glands cultured with hormone while they were repressed in wild-type glands (see also Jiang *et al.,* 1997). Transcripts of *rpr* were more abundant in *Kr-h* glands than in wild-type glands while for *diap1* the effect was reversed. While these results suggest disruption of the ecdysone regulation of these apoptosis genes in the *Kr-h* mutant, it should be noted that *diap2, hid,* and *rpr* transcripts are abundant in mutant glands 24 h after pupariation, at a time when the glands of heterozygous sibs have histolysed (Fig. 9B).

DISCUSSION

Metamorphosis in *Drosophila* provides a unique opportunity to study the hormonal regulation of animal development. Initiated with the visual support of puffs in the giant polytene chromosomes of the salivary gland, these studies have been extended into many other tissues, including both larval and presumptive adult tissues. They provide a detailed description of the molecular consequences of the increases in ecdysone titre associated with pupariation and pupation. Advances in molecular analyses now encourage

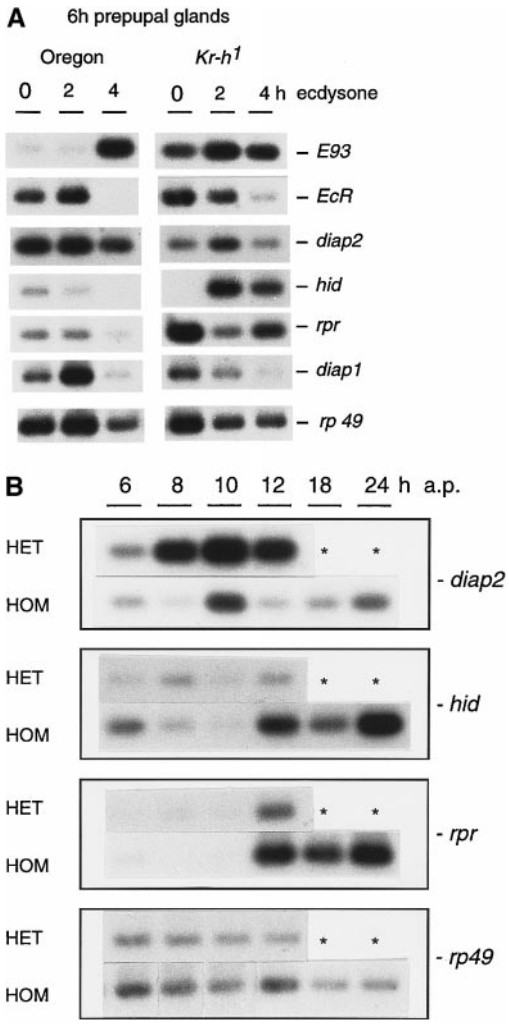


FIG. 9. The ecdysone response in 6-h prepupal *Kr-h*^{1} salivary glands and the expression of genes involved in apoptosis. (A) Salivary glands from 6-h prepupae of Oregon-R and *Kr-h*^{1} homozygotes were cultured for 2 or 4 h in the presence of 1.8×10^{-6} M ecdysone. *E93*, *EcR*, *diap2*, *hid*, *rpr*, *diap1*, and *rp49* transcripts were analysed by RT-PCR in the same RNA extracts from five individual salivary glands and the products then pooled. (B) Salivary glands were dissected from heterozygous (HET) or homozygous (HOM) *Kr-h*^{1} prepupae ages from 6 to 24 h after pupariation (a.p.). *diap2*, *hid*, *rpr*, and *rp49* transcripts were analysed by RT-PCR in the same RNA extracts as those presented in Fig. 7.

approaches based on the morphological characterisation of mutants of metamorphosis and in this study we have used such an approach to isolate *Kr-h*, a novel modulator of the ecdysone response.

Kr-hα Mutants Display Developmental Defects during the Prepupal Stage

Metamorphosis involves the extensive remodelling of the *Drosophila* body plan. While certain larval tissues are destined for histolysis, others are reprogrammed. In parallel the imaginal discs differentiate into adult tissues. These events are not simultaneous in all tissues and a precisely timed series of events is initiated by the rise in ecdysone titre at the end of the third larval instar. Its progression requires the subsequent fluctuations of hormone titre in mid- and late prepupae. In the prepupal *Kr-h* lethal alleles the early phase appears to progress normally, at least until gas bubble formation at 3 to 4 h after pupariation, and it is only in midprepupae that the underlying genetic programmes show the first signs of disturbances which become morphologically apparent in late prepupae. We followed gas bubble displacement in individual prepupae over several hours. By successive movements in both the anterior and the posterior parts, the gas bubble plays a role in separating cuticles and the animal finally separates completely from the puparium at head eversion, which marks pupation. In *Kr-hα* lethal alleles, movements of the bubble appear random as though the neuromuscular system is uncoordinated. Equally, an aberration in the ecdysone response is seen by the persistence of the salivary gland which normally histolyses some 14 h after pupariation. Similar observations have been reported for *EcR* (Bender *et al.*, 1997), *βFTZ-F1* (Broadus *et al.*, 1998), and, most recently, *DHR3* mutants dying in the prepupal or early pupal period (Lam *et al.*, 1999).

The differentiation of the adult head, thorax, and abdomen appear to a certain extent independent. While all but a few animals fail to achieve head eversion, the leg and wing discs in some individuals (Fig. 1B) fuse to form a thorax which most closely resembles the adult structure. In certain cases one can observe head characteristics, notably eye pigmentation, without head eversion. Perhaps significantly a similar aberrant differentiation producing a cryptocephalic phenotype has also been described for compound mutants of two key genes of the ecdysone response, *E74* and *BR-C* (Fletcher and Thummel, 1995). This suggests that *Kr-h* also acts in this regulatory pathway. The cuticle of the abdomen (derived from nests of histoblasts) forms, but with the exception of bristle formation in the anterior segments it looks more like larval or pupal cuticle, as though metamorphosis had not taken place in this part of the body. Such a differential response of body compartments, including a gradient of response in the abdomen, has been observed in classical approaches, notably the response to topical applications of juvenile hormone in late larvae or prepupae (see for example, Postlethwait, 1974).

Kr-h Transcripts Have Stage-Specific Functions

Like many genes involved in the ecdysone response (Thummel, 1996; Richards, 1997, for reviews), *Kr-h* has alternate promoters that give rise to transcripts which may encode at least two protein isoforms which contain different domains characteristic of transcription factors. We cannot exclude that *Kr-hγ* will also produce a novel isoform, but to date we have not succeeded in characterising

this minor transcript. Similarly we cannot formally exclude the possibility that transcripts that retain the short introns are in fact translated into truncated proteins (see Results). We have obtained an antibody against a region common to the two major isoforms which detects Kr-h at a restricted number of chromosomal sites in larval and prepupal salivary glands (Y. Beck, unpublished). Further antibodies will be necessary to distinguish the proteins that are synthesised and to determine their stage and tissue distribution. Such diversity when integrated with combinatorial possibilities of the other genes, notably by direct protein–protein interactions, may be fundamental to the stage- and tissue-specific response to ecdysone.

This study was initiated by our long-standing interest in metamorphosis. The important transcriptional activity in embryos (Schuh *et al.*, 1986) and the molecular structure of the locus suggested both that *Kr-h* might be necessary for embryogenesis and that we might obtain earlier lethal alleles by deleting the β promoter and/or the common region of the α and β transcripts. We have recently generated embryonic and early larval lethal alleles by the mobilisation of the P element in $Kr\text{-}h^{7}$ which fulfil this prediction [*Df(2L)Kr-h7.1*, Fig. 3 and Table 3, and unpublished results]. These strains will provide valuable tools for investigating the role of *Kr-h* during earlier stages of development. Our present interpretation of the genetic (Tables 1 to 3) and molecular (Figs. 3 and 6) analyses is that $Kr\text{-}h^{1}$ to $Kr\text{-}h^{5}$ homozygotes survive embryogenesis and larval development as *Kr-h* transcript levels are adequate. In $Kr\text{-}h^{6}$ and $Kr\text{-}h^{7}$, embryonic or early larval lethalities are important and in the case of *Df(2L)Kr-h7.1* homozygotes, the oldest larvae observed do not survive to the end of the second instar.

Kr-hα Transcript Levels Are Ecdysone Regulated

Kr-hα levels are sensitive to ecdysone levels and rise as metamorphosis approaches, reaching a maximum in late larval and early prepupal glands. In the late larval mutants ecd^{1}, dor^{22}, and npr^{6}, transcripts remain at intermoult levels (see Richards *et al.*, 1999, for similar experiments with other ecdysone-regulated transcripts). In addition to a requirement for hormone (as seen with ecd^{1} and dor^{22}), the reduced levels in the *Broad-Complex* mutant npr^{6} may indicate cross-regulation from within the established salivary gland hierarchy during the late larval response to hormone.

Kr-hα Modulates the Prepupal Ecdysone Response in Salivary Glands

The absence of *Kr-hα* transcripts in the $Kr\text{-}h^{1}$ mutant has modest effects on the level of expression of key genes of the ecdysone regulatory hierarchies but, perhaps more importantly, causes a shift in their time of expression, both *in vivo* and in cultured glands. This disturbs the fine balance in regulatory factors necessary for the successful progression of the hormonal response as a midprepupal rift appears at the time when the salivary gland is preparing for the late prepupal response to ecdysone (Fig. 6 and unpublished results). As this coincides with the time of disappearance of *Kr-h* transcripts in wild-type glands, it suggests that while the high level of *Kr-h* transcripts found in larvae and early prepupae is not vital, as larval development proceeds normally in $Kr\text{-}h^{1}$, maintaining activity for the first 5 to 6 h of the prepupal period is essential. As there is premature induction of *EcR* transcripts in the mutant, this may indicate that *Kr-h* has a role in delaying the appearance of the ecdysone receptor. In this respect the fact that the profile of *Kr-h* transcripts in prepupal glands resembles that of *DHR3*, shown to be necessary for the progression to the prepupal ecdysone response (White *et al.*, 1997; Lam *et al.*, 1997), and the similarities in their response to hormone treatment of 2-h prepupal glands, may reflect a shared regulatory mechanism. However, while the molecular consequences for the ecdysone regulatory hierarchy in *Kr-h* mutants are more subtle than those seen in *DHR3* mutants (Lam *et al.*, 1999), the lethal phenotype appears more temporally restricted. Up to 90% of *Kr-h* mutants die as prepupae (Table 2), whereas only 25% of DHR3 mutants die at this stage, the others dying later in pupal development (Lam *et al.*, 1999).

Initially, because of the failure of the salivary gland to histolyse, we suspected that the late prepupal response to hormone did not occur. However, it is now clear that this response is initiated, as *E74A*, *E75A*, *E75B*, and *E93* transcripts are induced although the induction of the latter is clearly suboptimal. Note that our RT-PCR assay detects basal levels of *E93* prior to the late prepupal induction from 10 h onwards, which were not apparent in the Northern analyses of Baehrecke and Thummel (1995). As it has been suggested that *E93* is a key regulator of programmed cell death (Jiang *et al.*, 1997), it is possible that the gland survives up to 36 h after pupariation because apoptosis of the salivary gland is blocked in *Kr-h*. However, although the regulation of a certain number of genes known to be involved in apoptosis is modified, the fact that their transcripts are abundant in mutant glands that escape histolysis suggests that other factors are involved (Fig. 9).

Kr-h and the Regulatory Networks of Metamorphosis and Embryogenesis

It is clear that our approach can be used to isolate novel regulators of metamorphosis. Together with a similar study undertaken by D'Avino and Thummel (1998) this opens new perspectives for extending our knowledge of the regulatory networks that translate a hormonal signal into a complex stage- and tissue-specific programme during animal development. In this respect it is important to note that using whole animal extracts we observed relatively minor differences in transcript profiles between homozygous and heterozygous prepupae (data not shown) as the temporal differences in ecdysone-regulated gene expression between

tissues (Huet *et al.*, 1993) obscure the details of events occurring in individual tissues.

A particularly interesting possibility is that many of the genes characterised for their role in metamorphosis may be first used in similar regulatory networks during embryogenesis. Encouraging results in this respect are the demonstrations that both *DHR3* (Carney *et al.*, 1997) and *EcR* (Bender *et al.*, 1997) are required during embryogenesis. Although we have respected the *Drosophila* nomenclature convention in retaining the original designation of *Kr-h*, to date there is no evidence of a functional relationship between *Kr* and *Kr-h*, and our current studies are directed at examining *Kr-h*'s role both in embryogenesis and during metamorphosis.

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