Transgenic Inhibitors Identify Two Roles for Protein Kinase A in Drosophila Development

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ABSTRACT

We have initiated an analysis of protein kinase A (PKA) in Drosophila using transgenic techniques to modulate PKA activity in specific tissues during development. We have constructed *GAL4/UAS*-regulated transgenes in active and mutant forms that encode PKAc, the catalytic subunit of PKA, and PKI(1-31), a competitive inhibitor of PKAc. We present evidence that the wild-type transgenes are active and summarize the phenotypes produced by a number of *GAL4* enhancer-detector strains. We compare the effects of transgenes encoding PKI(1-31) with those encoding PKAr*, a mutant regulatory subunit that constitutively inhibits PKAc because of its inability to bind cyclic AMP. Both inhibitors block larval growth, but only PKAr* alters pattern formation by activating the Hedgehog signaling pathway. Therefore, transgenic PKI(1-31) should provide a tool to investigate the role of PKAc in larval growth regulation without concomitant changes in pattern formation. The different effects of PKI(1-31) and PKAr* suggest two distinct roles, cytoplasmic and nuclear, for PKAc in Hedgehog signal transduction. Alternatively, PKAr* may target proteins other than PKAc, suggesting a role for free PKAr in signal transduction, a role inhibited by PKAc in reversal of the classical relationship of these subunits.

CYCLIC AMP and its target protein kinase A (PKA) are central elements of a ubiquitous signaling pathway important in the cell cycle, cellular communication, memory formation, and behavior. In Drosophila, genetic manipulation of cAMP levels is possible through mutations of the *dunce* gene, which encodes a cAMP-specific phosphodiesterase, and mutations of the *rutabaga* gene, which encodes a calcium-dependent form of adenylyl cyclase. Zygotes homozygous for *dunce* null mutations are retarded in growth but develop into morphologically normal flies. These adult flies contain up to six times the normal levels of cAMP (Davis and Kiger 1981), and they exhibit defects in the nervous system (behaviors and memory formation) and in oogenesis (Byers *et al.* 1981; Bellen and Kiger 1987; Levine *et al.* 1994). Mutant *rutabaga* flies appear to be completely normal except for defective memory formation. Measurements of cAMP in early embryos produced by *dunce* and *rutabaga* double-mutant females demonstrate that *dunce* plays a major role (modulated by *rutabaga*) in maternal regulation of embryonic cAMP content (Whitehouse-Hills *et al.* 1992), which in turn can produce a wide spectrum of developmental defects (Bellen *et al.* 1987; Bellen and Kiger 1988).

PKA mediates most of the known effects of cAMP in a wide range of eukaryotic species and is thought to be a major target of cAMP in Drosophila. PKA consists of a cAMP-binding regulatory moiety (PKAr) and a catalytic moiety (PKAc). It is generally described as a heterotetrameric complex, R_2C_2, consisting of a dimer of two identical regulatory subunits (R_2 = PKAr), with each subunit bound to a monomeric catalytic subunit (C = PKAc). Upon binding cAMP, the tetramer dissociates to $R_2 + 2C$, freeing the active site of the catalytic subunit from inhibition (Taylor *et al.* 1990). In Drosophila, PKAc is encoded by the *DC0* gene. Hypomorphic mutants of *DC0* show effects suggesting that PKAc is required at a number of stages for normal growth and development (Lane and Kalderon 1993), learning (Skoulakis *et al.* 1993), and behavior (Levine *et al.* 1994).

A role for PKAc in Hedgehog signaling during development has been inferred from experiments designed to reduce the level, or inhibit the activity, of PKAc. Reduction in PKAc level has been achieved in Drosophila by producing mitotic clones of cells homozygous for lethal alleles of *DC0* (Jiang and Struhl 1995; Lepage *et al.* 1995; Li *et al.* 1995; Pan and Rubin 1995; Strutt *et al.* 1995) or by use of homozygous-viable recessive mutations of *DC0* (Lepage *et al.* 1995). Inhibition of PKAc activity has been achieved by ectopic expression, under *GAL4/UAS* control, of a mutant Drosophila PKA regulatory subunit type I, PKAr*, which is defective in its ability to bind cAMP (Li *et al.* 1995). Both techniques elicit abnormal development that mimics that caused by stimulation of the Hedgehog signaling pathway, *i.e.*, induction of *decapentaplegic* (*dpp*), *wingless* (*wg*), and *patched* (*ptc*) expression. In the wing imaginal disc, for

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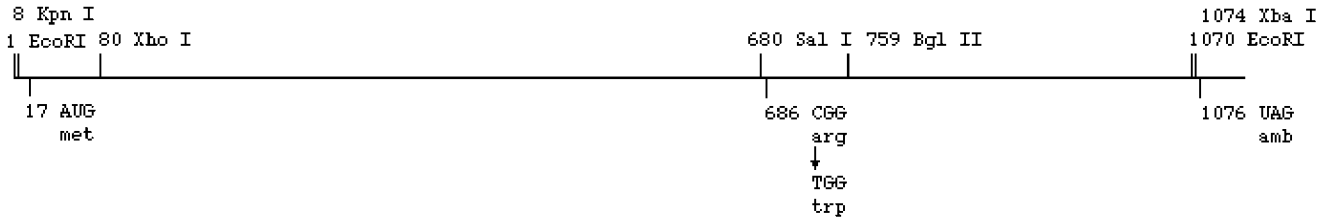

Figure 1.—Structure of the *DC0* cDNA in plasmid pUAST-DC08. Restriction sites are shown above the line with numbering beginning at the first base of the 5′ *Eco*RI site. Sites of coding interest are shown below the line: the AUG initiation codon, the UAG termination codon, the R^{224} mutant codon, and its wild-type equivalent.

example, stimulation of the Hedgehog pathway in the wing margin of the anterior compartment leads to duplications of anterior wing patterns. In interpreting these experiments, it is assumed that free PKAc activity represses the Hedgehog signaling pathway, and that reduction (by *DC0* mutation) or inhibition (by PKAr*) of free PKAc activity stimulates the pathway. How an extracellular Hedgehog signal might normally affect PKAc activity is not clear. Studies carried out in Drosophila cell culture suggest that PKAc repression of Hedgehog signal transduction may occur by direct phosphorylation of the cytoplasmic form of transcription factor Ci, encoded by *cubitus interruptus*, leading to its conversion by proteolysis to a repressor of Hedgehog target genes (Aza-Blanc *et al.* 1997; Chen *et al.* 1998). Conversely, inhibition of proteolysis leads to an accumulation of full-size Ci, its translocation to the nucleus, and activation of Hedgehog target genes.

Reciprocally, the effects of ectopic PKAc expression have been studied using a transgene encoding a mutant mouse PKAc (PKAc*) that is defective in its ability to bind PKAr. This transgene shows no effect on patterning of the wing imaginal disc, but it produces blistered wings in adult flies (Jiang and Struhl 1995; Li *et al.* 1995). In embryos, on the other hand, PKAc* is reported to induce expression of *wg* and *ptc*, genes that are also activated in embryos by PKAr* (Ohlmeyer and Kalderon 1997).

To dissect the various roles of the cAMP signaling pathway in Drosophila, we have sought to control PKAc activity using the *GAL4/UAS* transgenic system developed by Brand and Perrimon (1993). We have created *UAS* transgenic strains to express synthetic genes encoding the N-terminal domain (residues 1–31) of the rabbit skeletal muscle PKAc-inhibitor protein, PKI, in active [$Arg^{19,20}$] and inactive [$Gly^{19,20}$] forms (Taylor *et al.* 1990). These two Arg residues play an essential role in forming a highly specific pseudosubstrate binding site recognized by PKAc. The N-terminal domain of PKI is a highly specific competitive inhibitor of mammalian PKAc, and it has been shown to inhibit Drosophila PKAc *in vitro* (Drain *et al.* 1991). We have also created *UAS* transgenic strains to express wild-type Drosophila PKAc and a mutant PKAc in which Arg replaces Trp at position 224. We use these strains to show that transgenic PKI(1-31) expression inhibits both transgenic and endogenous PKAc activities, and we compare its effects with those of PKAr*. We find that PKI(1-31) and PKAr* have similar effects on larval growth but differ in their ability to induce ectopic Hedgehog signaling in embryos and imaginal discs, thus identifying distinct roles for PKA in growth and pattern formation.

MATERIALS AND METHODS

***PKAc* transgene construction:** A *DC0* cDNA clone modified to contain *Eco*RI and *Kpn*I sites immediately upstream of the PKAc initiation codon (Lane and Kalderon 1993) was provided by Dr. Daniel Kalderon. The entire coding sequence was excised by first cutting with *Xba*I (to preserve the overlapping *Eco*RI site at the termination codon) and then cutting with *Eco*RI. This fragment was cloned into pUAST (Brand and Perrimon 1993) cut with *Eco*RI and *Xba*I to form plasmid pUAST-DC08 (Figure 1). A gene encoding a FLAG epitope appended to the C terminus of PKAc was constructed from pUAST-DC08 by PCR using an upstream primer overlapping the *Bgl*II site [CCGATCCAGATCTATGAG] and a downstream primer that removes the overlapping *Eco*RI and *Xba*I sites and adds nucleotides encoding the epitope (underlined), followed by two termination codons and an *Xba*I site [GCATTCTA GACTATTACTTATCGTCATCGTCCTTGTAATCAAACTCAG CAAACTCCTTGG]. The PCR product was cut with *Bgl*II and *Xba*I and inserted into pUAST-DC08 cut with these enzymes to create pUAST-DC0F1. The integrity of this substitution was verified by sequencing from the *Bgl*II site to the new *Xba*I site. Fly strains carrying the two transgenes were created by embryo injection of pUAST-DC08 and pUAST-DC0F1 plasmids with a helper plasmid, phsπΔ2-3 (Misra and Rio 1990).

Automated sequencing of the entire *DC0* cDNA in pUAST-DC08 revealed a T-to-C substitution at nucleotide 2793 (Kalderon and Rubin 1988), which produces a Trp-to-Arg substitution at amino acid 224 of PKAc. This position corresponds to amino acid 221 of mammalian PKAc, a highly conserved site. The adjacent Asp^{220} is nearly invariant in protein kinases, and it contributes directly to stabilization of the catalytic loop and the PKI peptide binding site (Knighton *et al.* 1991). The wild-type *DC0* sequences published by Foster *et al.* (1988) and Kalderon and Rubin (1988) are in agreement on T at this position, suggesting that the cDNA mutation is a product of reverse transcription.

Vectors with the wild-type *DC0* sequence were produced using a genomic *Xho*I-*Xba*I fragment of *DC0* cloned in plasmid p-XX, which was provided by Dr. F. Rob Jackson. The relevant region of p-XX was sequenced to verify that it had T at position 2793, and the *Sal*I-*Bgl*II fragment containing the wild-type sequence was excised. Because the pUAST vector contains

TABLE 1

Progeny produced by crossing *GAL4* and *UAS-PKIF* or *UAS-PKAr strains**

		Ratio of *GAL4* to *CyO* or *TM3* Progeny			
Homozygous *UAS*	Heterozygous *GAL4*:	*GAL4-KO5/CyO*	*GAL4-JW1/CyO*	*GAL4-PP3/TM3, Sb*	*GAL4-RK5/TM3, Sb*
UAS-PKIG^{19,20}F a-1		70/52	99/77	58/60	63/73
UAS-PKIG^{19,20}F a-2		88/53	76/105	106/102	81/70
UAS-PKIF 1-1		1/102	1/83	0/106	4/105
UAS-PKIF 5-1		9/281	0/206	0/109	4/77
UAS-PKIF 10-2		0/99	0/90	0/62	3/73
UAS-PKIF 12-2		27/123	1/59	0/71	3/58
UAS-PKAr BDK 33*		22/79	0/74	1/128	14/117
UAS-PKAr BDK 35*		4/84	0/98	0/187	0/163

*Sal*I sites, it was necessary to subclone the *Kpn*I-*Xba*I fragment of pUAST-DC08 into Bluescript to give pBS-DC08. The mutant *Sal*I-*Bgl*II fragment of pBS-DC08 was excised and replaced with the wild-type *Sal*I-*Bgl*II fragment of p-XX to give pBS-DC0A. The *Xho*I-*Bgl*II fragment of pBS-DC0A was then used to replace the mutant *Xho*I-*Bgl*II fragments of pUAST-DC08 and pUAST-DC0F1, giving vectors pUAST-DC0A and pUAST-DC0FA, which were used to produce transgenic strains as described above. The strains used here are designated as *UAS-PKAc*, *UAS-PKAcF*, and *UAS-PKAcR^{224}F*.

***PKIF* transgene construction:** Plasmids containing synthetic genes encoding the N-terminal domain of rabbit skeletal muscle protein kinase A inhibitor protein in active and inactive forms, PKI(1-31) and mutant [Gly^{18,19}]PKI(1-31), were provided by Dr. Joseph Avruch (Grove *et al.* 1987). Genes encoding PKI(1-31) with an appended C-terminal FLAG epitope were produced by PCR with an upstream primer containing a *Bam*HI site [TGCAGGGATCCCACCATG] and a downstream primer that adds nucleotides encoding the epitope (underlined), followed by two termination codons and an *Xba*I site [CGTATCTAGACTATTACTTATCGTCATCGTCCTTGTAATCAGAGGCAGAGGAGACC]. The PCR products were cloned into Bluescript to give plasmids pBS-PKIF and pBS-PKIG^{19,20}F, whose structures were verified by sequencing. *Bam*HI-*Xba*I fragments from each of these were subcloned into pUAST cut with *Bgl*II and *Xba*I to give vectors pUAST-PKIF and pUAST-PKIG^{19,20}F, which were used to produce transgenic strains designated *UAS-PKIF* and *UAS-PKIG^{19,20}F*, as described above.

Fly strains and crosses: The enhancer detector vector pGawB (Brand and Perrimon 1993), carried in the strain *w GAL4 106-1*, was jumped by crossing females to males of strain *w ; Dr/TMS, Sb Δ2-3* (Lindsley and Zimm 1992). Independent jumps to autosomes of the same strain used for embryo injections were mapped by standard procedures to chromosomes and established in balanced stocks (chromosomes II and III only) by students in the undergraduate genetics laboratory course at UC Davis as part of their instruction in Mendelian genetics. *GAL4* stocks are designated with each student's initials and strain number, *e.g.*, *GAL4-RK5*.

Strains carrying *UAS* transgenes were created from injected embryos of a *y w* strain, mapped to a chromosome, and established in balanced stocks by standard procedures, taking care that all insertions recovered on a particular chromosome are independent.

*UAS-PKAr** strains and *GAL4-E22C* were generously supplied by Dan Kalderon (Li *et al.* 1995; Ohlmeyer and Kalderon 1997). The *UAS-GFP* strain is described by Yeh *et al.* (1995). All other mutations are described in Lindsley and Zimm (1992). All crosses were carried out at 25°.

Immunostaining and microscopy: Heads of wandering third instar larvae were everted in modified Robb's medium and fixed for 1 hr at room temperature in 0.05 m sodium phosphate, pH 7.6, 4% formaldehyde, and 1% NP-40, followed by dehydration in absolute methanol and storage at −20°. They were rehydrated by washing three times in PT (Patel 1994) and blocked for 1 hr at room temperature in 0.1 m sodium phosphate, pH 7.6, 0.1% Tween 20, 2% BSA, and 2% serum. Anti-FLAG M2 monoclonal antibody (Eastman Kodak, Rochester, NY) at a final dilution of 1:500 and anti-DC0 rabbit polyclonal antibody (Lane and Kalderon 1993) at a final dilution of 1:400 were incubated with larval heads in fresh block for 1–2 hr at room temperature followed by storage at 4° overnight. Antibodies were removed, and heads were washed repeatedly in PBT (Patel 1994) and blocked for 30 min at room temperature. Secondary antibodies labeled with FITC or Texas red (Vector Laboratories, Burlingame, CA) were added directly to the heads at a final dilution of 1:100 and incubated at room temperature for 3–4 hr. Antibodies were removed and heads were washed repeatedly in PBT, rinsed once in PBS, and equilibrated in 70% glycerol, 2.5% DABCO (Patel 1994). Wing discs were dissected and mounted in the same solution and viewed with a Leica TCS-NT confocal microscope. Fluorescence was analyzed quantitatively using the Leica instrumentation and Scion Image software.

Wings were dissected from bodies in 95% ethanol, dehydrated in 100% ethanol, equilibrated in toluene, mounted in cedarwood oil (Sigma, St. Louis), and photographed with a dissecting microscope (Zeiss, Jena, Germany). Eggs were collected on grape juice agar (without added yeast; Ashburner 1989), and larvae were mounted in Hoyer's medium (Ashburner 1989), cleared at 60°, and photographed with a Zeiss Axioplan microscope using phase contrast.

RESULTS

Effects of ectopic PKI(1-31): The activity of *UAS-PKIF* transgenes has been established by identifying *GAL4* strains that produce phenotypic effects in conjunction with the transgene. Approximately 220 new *GAL4* insertions on chromosomes II and III were established in balanced stocks and crossed to a strain carrying two transgenes on chromosome I, *UAS-PKIF 10-2* and *12-2*. Twenty-one *GAL4* strains were found to significantly affect the recovery of adult progeny. Table 1 shows the progeny recovered from four such randomly chosen *GAL4* strains (heterozygous with balancer chromosomes *CyO* or *TM3*) when crossed to four strains with

TABLE 2

Progeny produced by crossing *GAL4* and *UAS-PKAc* strains

Females / males	Males / females:	Ratio of *GAL4* to *CyO* or *TM3* Progeny			
		GAL4-KO5	*GAL4-JW1*	*GAL4-PP3*	*GAL4-RK5*
UAS-PKAcF 1.1/CyO		0/161	0/107	0/95[a]	0/175
UAS-PKAcF 1.3/TM3, Sb		0/56	0/46	0/15[a]	0/89
UAS-PKAcF 5.2/TM3, Sb		0/74	0/58	0/88[a]	1/63
UAS-PKAcF 5.5/CyO		0/83	0/69	0/25	10/81
UAS-PKAcF 5.9/TM3, Sb		0/96	0/70	0/40[a]	52/48
UAS-PKAc 13.1/CyO		0/106[a]		0/116[a]	3/46
UAS-PKAc 14.3/TM3, Sb		0/130[a]		0/100[a]	0/67
UAS-PKAc 14.4/CyO		0/125[a]		0/145[a]	6/50
UAS-PKAc 15.1/TM3, Sb		0/82[a]		0/39[a]	0/36
UAS-PKAc 15.3/CyO		24/129[a]		0/54[a]	35/47
UAS-PKAc 16.5/TM3, Sb		0/90[a]		0/53[a]	59/51
UAS-PKAcR^{224}F 1-2a		36/33[a]	69/53[a]	124/82[a]	
UAS-PKAcR^{224}F 1-3		60/50[a]	109/96[a]	119/84[a]	
UAS-PKAcR^{224}F 4-1		111/68[a]	71/56[a]	110/83[a]	
UAS-PKAcR^{224}F 5-1		67/57[a]	47/33[a]	79/79[a]	

[a] In these crosses, the *GAL4* strain carried the balancer chromosome.

an active *PKIF* transgene and to two strains with an inactive *PKIG^{19,20}F* transgene. Note that progeny carrying a *GAL4* chromosome and an inactive transgene are recovered with about the same frequency as sibs carrying a balancer chromosome and an inactive transgene, while progeny carrying a *GAL4* chromosome and an active transgene are recovered at much lower frequencies than sibs carrying a balancer chromosome. The high degree of lethality exhibited by the four *PKIF* transgenes demonstrates that they are expressed and biologically active.

From the results obtained with these and other *GAL4* strains, we find that most lethality caused by PKIF occurs at hatching or during the larval stages. PKIF expression driven by *GAL4-PP3* causes larvae to die just before or soon after hatching from the egg. Other *GAL4* strains permit development to second or third instars before death, most larvae being abnormally small and, near death, misshapen. Some larvae continue to feed for many days after pupation would normally have occurred and never reach normal size. Of the few larvae that eventually pupate, some produce small but morphologically normal adult flies, some as small as half size. These small flies emerge from small pupal cases, indicating that their size is a result of retarded larval development rather than failure to carry out the final mitotic division during imaginal disc development. This growth retardation and failure to pupate resembles that described for hypomorphic *DC0* mutants (Lane and Kalderon 1993), suggesting that PKIF is inhibiting endogenous PKAc as intended. The particular phenotype produced depends upon the particular *GAL4* strain, and must be caused by differences in the tissue specificity, time of expression during development, and strength of expression of either the *GAL4* or *PKIF* transgene.

The GAL4 expression pattern of each of these strains has been examined using a *UAS-GFP* transgene. Green fluorescent protein (GFP) expression in first instar larvae of *GAL4-PP3* and *GAL4-JW1* is much more intense than in first instar larvae of *GAL4-KO5* and *GAL4-RK5*. *GAL4-PP3* and *GAL4-JW1* have in common the expression of GFP in the central and peripheral nervous systems, muscles, tracheae, and proventriculus. *GAL4-KO5* and *GAL4-RK5* show more restricted expression in first instar larvae, but by third instar, GFP expression in both is evident in the central nervous system, tracheae, proventriculus, and fat body. Further study of particular *PKIF* transgene expression will be required to establish which tissue is the focus of a particular phenotype.

Effects of ectopic PKAc: The activities of *PKAc* transgenes with and without *FLAG* epitope, as well as the mutant *PKAcR^{224}* with *FLAG* epitope, have been assayed with the same set of *GAL4* strains as shown in Table 2. The *UAS-PKAcF* and *UAS-PKAc* transgenes are clearly expressed and biologically active. It would appear that the C-terminal FLAG epitope does not affect PKAc activity. The *UAS-PKAcR^{224}F* mutant transgene appears to be completely inactive. The nature and degree of lethality observed in the crosses shown in Table 2 depends both upon the *GAL4* strain and the *UAS* strain used. The particular phenotype must depend upon the factors discussed previously, especially the strength of expression of the *UAS* transgene. The *UAS-PKAcF 1.1* transgene is one of the strongest; combined with each of the four *GAL4* strains, all larvae die well before pupation. Slightly weaker transgenes show more heterogeneity in combination with different *GAL4* strains. For example, *UAS-PKAcF 1.3* is like *PKAcF 1.1*, except that with *GAL4-KO5*, many individuals die as blackened pharate

TABLE 3

Progeny produced by crossing homozygous *GAL4-RK5* females to *UAS* males

Males	Non-*UAS* chromosomes	*UAS-PKIF*	*UAS-PKAcF*	*UAS-PKIF, UAS-PKAcF*
UAS-PKIF 1-1 / *Sp* , *UAS-PKAcF 5.9* / *TM3, Sb*	18	2	15	40
UAS-PKIF 7-1 / *CyO* , *UAS-PKAcF 5.9* / *TM3, Sb*	28	10	5	35
UAS-PKIF 5-1, UAS-PKAcF 5.9 / *TM3, Sb*	76			83
UAS-PKIF 5-1 / *TM3, Sb*	63	2		
UAS-PKAcF 5-9 / *TM3, Sb*	45		11	
UAS-PKIF 5-1, UAS-PKAcF 1.3 / *TM3, Sb*	57			18
UAS-PKAcF 1.3 / *TM3, Sb*	80		0	

adults before eclosion. Two of the weakest transgenes are *UAS-PKAcF 5.9* and *UAS-PKAc 15.3*; combined with *GAL4-RK5*, some individuals die as blackened pharate adults, but adult progeny also eclose exhibiting a variety of overlapping phenotypes. Some are as small as half size; some have warped or collapsed wings, flecks on the surface of the eye, bristles glued to the thorax, and combinations of these defects; others are normal. Combined with *GAL4-KO5*, *UAS-PKAcF 5.9* produces only dead and blackened pharate adults, while *UAS-PKAc 15.3* also produces some eclosed adults with collapsed wings.

Comparison of the effects of ectopic PKI(1-31) and PKAc: *GAL4* strains that produce effects with *PKAc* transgenes are recovered more frequently than those that produce effects with *PKIF* transgenes, indicating that the number (or target size) of tissues that can be affected is greater for PKAc than PKI(1-31). A screen of an additional 96 new *GAL4* strains identified 37 strains that are lethal in conjunction with *PKAcF 5.2* and 5 that produce abnormal adults. As indicated above, only 21 out of 220 new *GAL4* strains were lethal with *PKIF* transgenes. When a group of 28 randomly chosen *GAL4* strains were crossed to *PKAcF 1.1*, 19 were lethal (at larval or pupal stages) or produced reduced numbers of normal and abnormal adult progeny. When these 19 *GAL4* strains were crossed to *PKIF 5-1*, 1 was completely lethal and 7 produced reduced numbers of morphologically normal adult progeny (3 of these produced small but normal adults). No abnormal effects were observed with the remainder of the *GAL4* strains.

In several screens of *GAL4* strains using *PKAcF* transgenes, many *GAL4* strains have been found that produce larval death, death of pharate adults before eclosion, or living adults exhibiting wing and other defects. In the cases of larval death caused by PKAc, the larvae die without lingering to feed for a long period as do larvae affected by PKI(1-31). Moreover, in contrast to the effects of PKAc, we have yet to find a *GAL4* strain that produces morphologically abnormal adult flies as a consequence of PKI(1-31) expression.

Interaction between ectopic PKI(1-31) and PKAc: Evidence that the lethality produced by ectopic PKI(1-31) is caused by inhibition of endogenous PKAc activity is provided by the failure of PKIG^{19,20}F to produce any effect (Table 1). Additional evidence for this is provided by coexpression of *PKIF* and *PKAcF* transgenes in Table 3. Transgenic *PKAcF 5.9* expression rescues some individuals from lethality caused by three different *PKIF* transgenes expressed by *GAL4-RK5*. Indeed, *PKIF* and *PKAcF* transgenes appear to be mutual suppressors since transgenic *PKAcF* expression alone (particularly by *PKAcF 1.3*) can be lethal. In contrast, coexpression of *UAS-PKAcR^{224}F* and *UAS-PKIF* using several different *GAL4* strains does not titrate PKIF activity, further substantiating the inactivity of the R^{224} mutant protein (data not shown).

Comparison of the effects of ectopic PKI(1-31) and PKAr*: As mentioned above, we have yet to find a *GAL4* strain that produces morphologically abnormal adult flies as a consequence of PKI(1-31) expression. This striking observation contrasts with effects of *UAS-PKAr** transgenes that mimic ectopic Hedgehog expression (Li *et al.* 1995; Wolfgang *et al.* 1996). Therefore, we have closely compared the effects of *PKAr** and *PKIF* transgenes expressed by the same *GAL4* strains.

A particularly illuminating strain is *GAL4-RZ4*. The combination of *UAS-PKAr* BDK 35* and *GAL4-RZ4* pro-

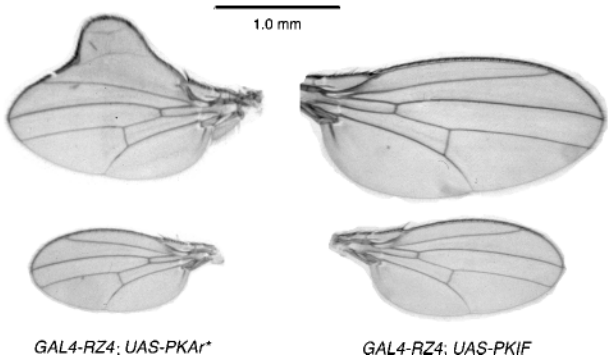

Figure 2.—Effects on wing morphology and size of PKAr* and PKIF expressed under the control of *GAL4-RZ4*. (Top) Wings from flies of approximately average size produced by crossing *GAL4-RZ4*/*CyO* flies to *UAS-PKAr* BDK 35* and *UAS-PKIF 5-1* flies. (Bottom) Wings are from late-emerging siblings produced in the same crosses. Left wings are of genotype *GAL4-RZ4*; *UAS-PKAr* BDK 35*; right wings are of genotype *GAL4-RZ4*; *UAS-PKIF 5-1*.

duces adult flies with 51% having anterior wing duplications (62/122 have at least one wing affected in some degree) characteristic of ectopic Hedgehog signaling. Those flies with normal wings are often reduced in size because of retarded larval growth (Figure 2). The combination of *UAS-PKIF 5-1* and *GAL4-RZ4*, on the other hand, produces adult flies that vary in size as a result of retarded larval growth, but always have normal morphology (Figure 2).

Figure 3 compares the effects of expressing *PKIF 5-1* and *PKAr* BDK 35* (A and C) with the effect of expressing *PKAcF 5.9* (B). The anterior margin of the wing is clearly sensitive to ectopic expression of either *PKAr** or *PKAcF*, producing quite different results, but not to ectopic expression of *PKIF*. Virtually the same fraction of adult flies that show wing duplications caused by PKAr* (51%) show a notched anterior wing margin produced by PKAcF (49/94 or 52%). The effect of PKAcF on the wing suggests a reduction of *wg* expression in the cells forming the wing margin (Couso *et al.* 1994). Coexpression of *PKAcF* with either *PKIF* or *PKAr** (Figure 3, D and E) produces normal development, proving that both PKIF and PKAr* are inhibitors of ectopic PKAcF activity. Furthermore, this demonstrates that PKIF is indeed active in cells producing the anterior wing margin. Nevertheless, expression of *PKIF* alone has no effect on wing development (Figure 3A), while *PKAr** alone elicits Hedgehog signaling and wing duplication (Figure 3C). Moreover, coexpression of *PKAcF* and *PKAr** blocks ectopic Hedgehog signaling produced by *PKAr** alone (Figure 3E), as would be expected if both are expressed at comparable levels. We have been unable similarly to titrate PKAr* activity by coexpression of PKAcR^{224}F, suggesting that the R^{224} mutant protein binds neither PKAr nor PKIF (see above and data not shown).

We have also compared the effects of PKIF and PKAr* using *GAL4-1J3*. With this strain, PKAr* is severely detrimental; in our hands, most adults fail to emerge from the pupal cases and exhibit severely truncated legs with multiple ectopic bristle columns, as described by Li *et al.* (1995). On the other hand, PKIF causes no effect on viability with this *GAL4* strain, and adults are normal (data not shown).

To understand the actions of PKIF and PKAr*, it is

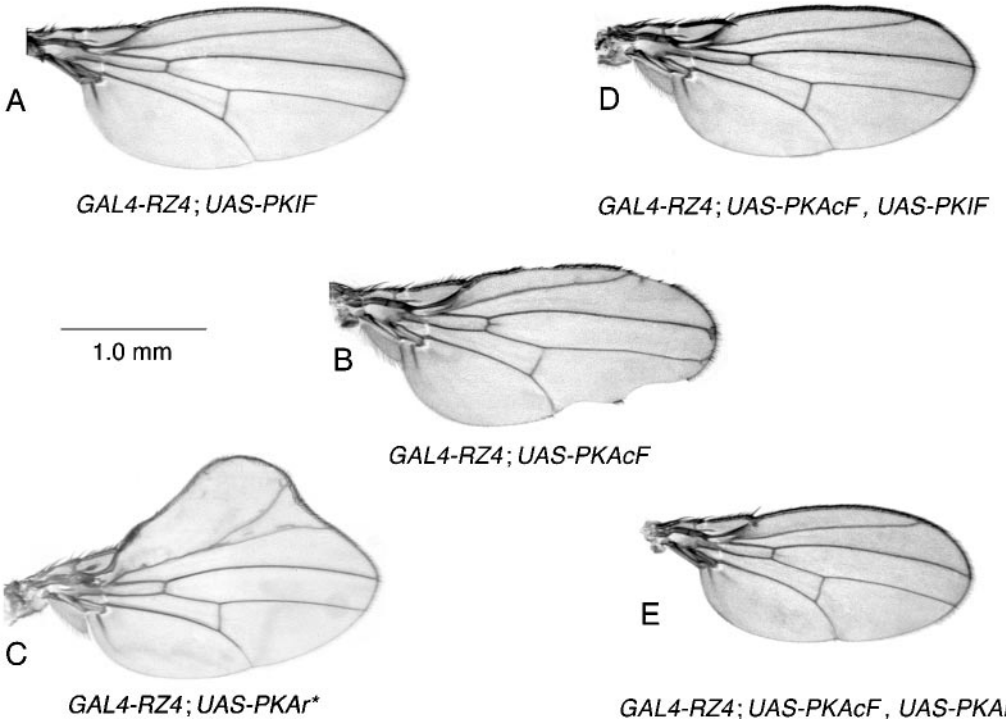

Figure 3.—Effects on wing morphology of expressing PKIF, PKAcF, and PKAr*, alone or in combination, under the control of *GAL4-RZ4*. The *UAS* transgenes used are *PKIF 5-1*, *PKAr* BDK 35*, and *PKAcF 5.9*. See text for discussion.

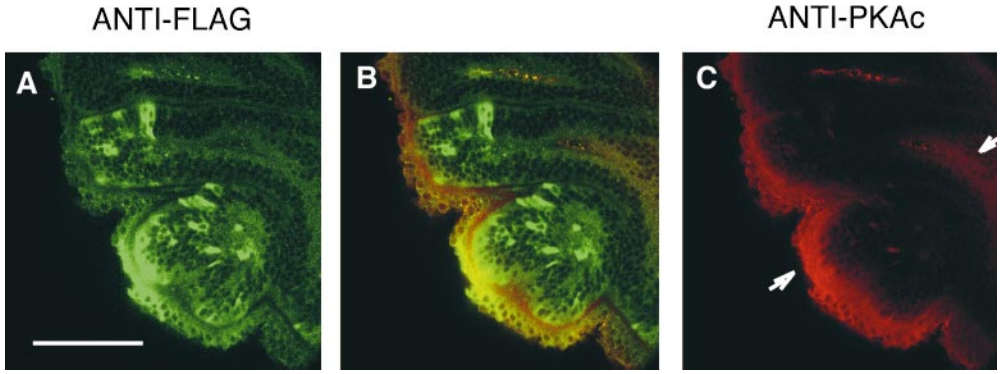

Figure 4.—Confocal section of a wing imaginal disc from a larva of genotype *GAL4-RZ4*; *UAS-PKAcF 5.9*. (A) Ectopic PKAcF is detected in green using an anti-FLAG antibody. (B) Area of highest ectopic expression appears yellow in the merged image. (C) Both ectopic PKAcF and endogenous PKAc are detected in red using an antibody to the *DC0* gene product. The bar in A is 50 μm. See text for discussion.

important to assess their levels relative to that of endogenous PKAc. We have done this indirectly by quantitating the level of ectopic PKAcF expression that is suppressed by these inhibitors. Ectopic PKAcF can be detected by an antibody against the FLAG epitope, and both endogenous PKAc and ectopic PKAcF can be detected using an antibody against PKAc (Lane and Kalderon 1993). Figure 4 shows a confocal section of a third instar wing disc stained with both antibodies. Regions of ectopic PKAcF expression can be identified using the anti-FLAG antibody (green). In Figure 4A, the extent of the tissue is shown by low, nonspecific, background fluorescence, while the region of highest ectopic PKAcF expression (yellow in the merged image in Figure 4B) is overexposed. Total PKAc level throughout the tissue can be assessed using the anti-PKAc antibody (red). In Figure 4C, the fluorescence level has been adjusted so that it is within the linear range of the pixels to obtain a quantitative image. Measures of pixel values show the difference between the two regions marked with arrows to be approximately threefold (196/64, full scale = 256). Because it is not evident at what low level of fluorescence staining becomes nonspecific, threefold is a *minimum* estimate of the ratio of ectopic PKAcF to endogenous PKAc expression. Both *PKIF* and *PKAr** inhibit the phenotypic effects of this ectopic PKAcF, indicating that these inhibitory transgenes must be expressed at levels comparable to that of *PKAcF 5.9* (assuming that inhibition involves stoichiometric association of either inhibitory protein with PKAcF). Yet, expressed alone in the presence of significantly lower levels of endogenous PKAc, these two inhibitors do not produce equivalent phenotypic effects. PKIF has no effect, whereas PKAr* activates Hedgehog signaling.

The results of crossing *UAS-PKAr** strains with the *GAL4* strains used to characterize *UAS-PKIF* strains are shown in Table 1. In general, death occurs earlier for larvae expressing PKAr* than for those expressing PKIF. With *GAL4-PP3* and *GAL4-JW1*, a significant portion of embryos die and turn brown before hatching, while the rest die as young first instars. With *GAL4-RK5*, small larvae feed and linger for up to 3 wk before dying. With *GAL4-KO5* and *GAL4-RK5*, the adults that emerge are all abnormal: the cuticle of the thorax and abdomen has an abnormal sheen; there is melanization of thorax and wings, as well as warped wings; there are crossed scutellar bristles, an indication of abnormal wing disc development. The surviving adults expressing PKAr* are not abnormally small like those morphologically normal survivors expressing PKIF.

Ohlmeyer and Kalderon (1997) have used *GAL4-E22C* to express *UAS-PKAr** in the embryonic ectoderm, and they found changes in gene expression and larval morphology consistent with the induction of Hedgehog signaling. We have used this strain to express *UAS-PKAr** *BDK 35* and have found that most larvae hatch, and all die, within a few hours. Many of these larvae exhibit an absence of thoracic denticle belts, as well as the change in the pattern of abdominal denticle belts described by Ohlmeyer and Kalderon (1997) (Figure 5, left). These pattern alterations are consistent with a mild overexpression of the *wg* gene (Noordermeer *et al.* 1992). In contrast, larvae expressing *UAS-PKIF* hatch and exhibit normal morphology (Figure 5, right). Depending upon the particular *UAS-PKIF* strain, the larvae may die soon after hatching, or may linger for many days with little growth before dying (some may molt to second instars), or may grow slowly, with some survivors emerging as normal adults. Coexpression of *UAS-PKAr** *BDK 35* and *UAS-PKAcF 5.9* restores normal pattern and morphology to abdominal denticle belts and partially restores thoracic denticle belts (Figure 5, middle). Embryos expressing the strongest *UAS-PKAc* transgenes usually fail to hatch, and the majority develop normal larval denticle belts (data not shown). In contrast, denticle belts produced by *UAS-PKAc** expression are described as similar to those produced by *UAS-PKAr** expression (Ohlmeyer and Kalderon 1997).

DISCUSSION

The screens of *GAL4* strains presented here suggest that many tissues are sensitive to ectopic PKAc expression, which is not surprising. Significantly, fewer tissues appear to be sensitive to inhibition of endogenous PKAc activity by PKI(1-31), suggesting that a subset of tissues

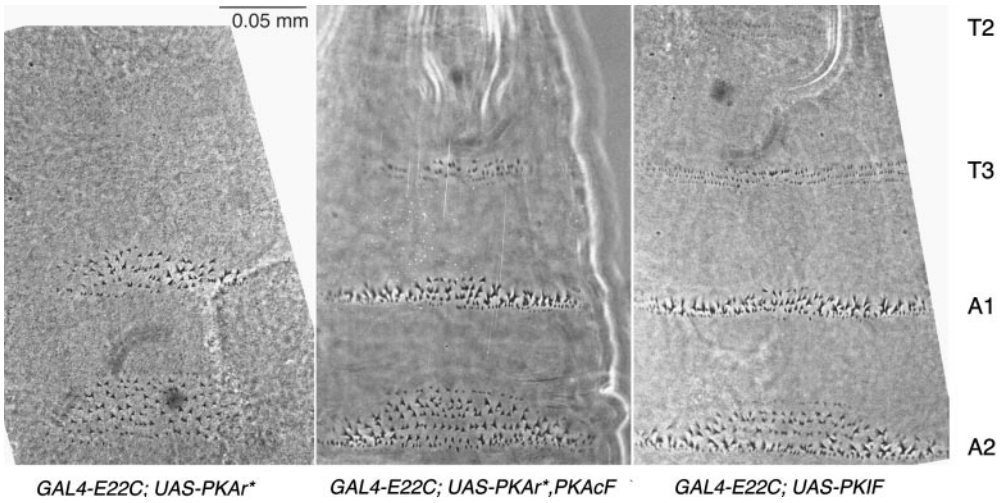

Figure 5.—Effects of expressing PKAr* and PKIF on larval ventral denticle belts. The normal pattern and morphology is seen at right (*GAL4-E22C*; *UAS-PKIF*), where the second (T2) and third (T3) thoracic segment belts and the first (A1) and second (A2) abdominal segment belts are labeled. PKAr* expression removes the thoracic belts and changes the morphology and pattern of the abdominal belts, as seen on the left (*GAL4-E22C*; *UAS-PKAr**). Coexpression of PKAr* and PKAcF suppresses the effect of PKAr* on the pattern and morphology of the abdominal belts and partially restores the thoracic belts, as seen in the middle (*GAL4-E22C*; *UAS-PKAr**, *PKAcF*). The *UAS* transgenes used are *PKIF 5-1*, *PKAr* BDK 35*, and *PKAcF 5.9*.

does not require PKAc activity. The only developmental phenotype produced by both PKAc and PKI(1-31) is the small, morphologically normal adult observed in some crosses. This is reminiscent of the identical learning defects exhibited by *dunce* and *rutabaga* mutants, where in certain neurons, disturbance, up or down, of ambient cAMP levels produces the same learning defect (Feany 1990). Indeed, the production of small adults appears to mimic the phenotype of *NF1* mutants (The *et al.* 1997). NF1 regulates the rutabaga adenylyl cyclase (Guo *et al.* 1997), suggesting that PKI(1-31) acts downstream of NF1 to inhibit cAMP-activated PKAc kinase activity.

Phenotypes produced by PKI(1-31) and PKAr* are surprisingly different. The phenotypic effects of PKI(1-31) appear to represent a subset of those of PKAr*. Both retard or block larval growth. PKAr* alone affects patterning in embryos and imaginal discs by activating Hedgehog signaling, and it alone causes abnormal differentiation in imaginal discs (which may reflect minor aberrations in patterning). The origin of this difference might reside in some fundamental difference in the biological properties of PKI(1-31) and PKAr* or perhaps in their relative stabilities in different cell types. However, PKI(1-31) is demonstrably active in wing imaginal discs (Figure 3D) and in other tissues (Table 3) since it is capable of inhibiting ectopic PKAcF. Regardless of the origin of the difference, it would appear that PKI(1-31) specifically targets larval growth.

Newly hatched larvae consist of two cell types: (1) mitotic cells composing the imaginal discs, gonad, and some neuroblasts, and (2) endoreplicating cells making up the exclusively larval tissues. These latter cells do not divide after hatching, but they increase in size as the larva grows, being maintained by cycles of DNA replication without nuclear division. These two cell types are regulated in fundamentally different ways, as demonstrated by their responses to nutritional deprivation (Britton and Edgar 1998). It would appear that mitotic cells are not sensitive to expression of PKI(1-31), but only to expression of PKAr*, whereas endoreplicating cells are sensitive to both. It is interesting to note that *UAS-PKIF, GAL4-E22C* larvae die within a few hours of hatching if kept on grape juice agar lacking amino acids, but they can feed for many days before dying or pupating if kept on regular food. Normal larvae can survive for days without amino acids (Britton and Edgar 1998), suggesting that PKI(1-31) sensitizes larvae to starvation.

Both proteins are effective inhibitors of the catalytic site of PKAc, possessing a pseudosubstrate binding site with a pair of adjacent Arg residues that interact with the catalytic site (Taylor *et al.* 1990). PKAr, which is larger than PKI(1-31) or full-length PKI(1-77), makes additional contacts with PKAc that make the PKAr:PKAc complex more stable than the PKI(1-77):PKAc complex (Herberg and Taylor 1993). PKI(5-24) and PKI(1-77) bind to PKAc with the same affinity (Lew *et al.* 1997), and PKI(1-31) is probably no different. The C terminus of PKI(5-24) is not involved in binding to PKAc, so the FLAG epitope at the C terminus of PKI(1-31) should not interfere with its inhibitory function (Knighton *et al.* 1991). Free PKAc and PKI(1-77) are small enough to enter the nucleus by diffusion (Harootunian *et al.* 1993). PKI(1-77) possesses a nuclear export signal (residues 35–49) that is hidden until PKAc is bound, whereupon the PKI(1-77):PKAc complex is extruded from the nucleus (Wen *et al.* 1995a). Moreover, the expression

and intracellular distribution of PKI(1-77) is regulated during the cell cycle and is necessary for its progression (Wen *et al.* 1995b). PKI(1-31) would be expected to inhibit both nuclear and cytoplasmic PKAc. In addition, it could compete with a Drosophila homologue of PKI(1-77) for nuclear PKAc and block its export. PKAr, on the other hand, is cytoplasmic whether or not it is complexed with PKAc because it is too large to enter the nucleus (Harootunian *et al.* 1993). In addition, anchoring proteins are known that bind PKAr to the membrane or cytoskeleton (Pawson and Scott 1997). PKAr* would be expected to inhibit cytoplasmic PKAc and to deplete nuclear PKAc by forming a cytoplasmic sink for PKAc that diffuses from the nucleus.

With regard to Hedgehog signaling, a possible target of PKAr* and PKI(1-31) in the cytoplasm would be the complex responsible for the proteolysis of the transcription factor Ci (Aza-Blanc *et al.* 1997), where they would inhibit phosphorylation of the PKA sites necessary for proteolysis of Ci^{155} to the repressor form Ci^{75} (Chen *et al.* 1998). The ability of PKAr* to interact with anchoring or other proteins might give it greater access to this complex than PKI(1-31), accounting for the failure of the latter to activate Hedgehog target genes.

Another possible explanation of the different actions of PKAr* and PKIF(1-31) is that free PKAr* (and by implication free PKAr) has a target other than PKAc through which it activates Hedgehog signaling. Precedent for such a role exists. In Dictyostelium, free PKAr binds and activates a cAMP-specific phosphodiesterase that is postulated to have functional homology to the cAMP-specific phosphodiesterase encoded by *dunce.* The Dictyostelium phosphodiesterase is also activated by bovine $PKAr^{1}_{a}$, and a synthetic monomeric form of this regulatory subunit is a more potent activator than the dimeric form (Shaulsky *et al.* 1998). (The Dictyostelium PKAr protein lacks a dimerization domain, and its PKA exists as a heterodimer.) In this scenario, in the absence of a cAMP signal, PKAc would bind to PKAr, inhibiting this novel activity. Reduction in the level of PKAc, *e.g.*, in a mitotic clone of cells homozygous for a lethal allele of *DC0*, would lead to free PKAr that would activate Hedgehog signaling.

In an alternative scenario, the effect of PKAr* on Hedgehog target genes could be caused by its ability to deplete nuclear PKAc, a role that cannot be fulfilled by PKI(1-31). Since the normal role of PKI(1-77) is not only to inhibit, but to export, nuclear PKAc, it is possible that PKAc plays another critical role in the nucleus in addition to its catalytic role in phosphorylation. For example, PKAc might function as a corepressor with Ci^{75} to block transcription of Hedgehog target genes. Consistent with this hypothesis, Chen *et al.* (1998) have shown that PKI(1-60) activates Ci-mediated chloramphenicol acetyltransferase transcription from a model Gli enhancer in Drosophila Kc cells, a finding they attribute solely to inhibition of proteolysis of cytoplasmic Ci^{155}. It may be that PKAc can function as a corepressor even if its catalytic site is occupied by PKI(1-31). Corepression by PKI(1-31):PKAc and Ci^{75} might block transcription of target genes, even in the presence of Ci^{155} produced by concommitant inhibition of Ci^{155} proteolysis in the cytoplasm. Small changes in the ratio of Ci^{155} and Ci^{75} are believed to be critical for activation of Hedgehog target genes (Aza-Blanc *et al.* 1997). In addition, Day *et al.* (1989) have pointed out that PKI(1-77) may differ from PKI(1-31) because only the former reduces basal transcription from cAMP-stimulated promoters. If PKAc has such an additional role, then the R^{224} mutant must have lost this function, as well as its ability to bind PKAr* and PKIF, since *PKAc*R^{224}*F* produces no abnormal phenotypes (data not shown) and has no effect on viability (Table 2). On the other hand, the hypothesized nuclear role of PKAc might be catalytic if nuclear PKAc is in some way inaccessible to nuclear PKI(1-31).

These considerations suggest that normal Hedgehog signal transduction may require both inhibition of cytoplasmic PKAc activity and export of nuclear PKAc. A Drosophila homologue of PKI(1-77) would be a good candidate for carrying out these functions. The fact that PKI(1-77) seems to play some role in regulating the cell cycle (Wen *et al.* 1995b) may help to explain why PKI(1-31) has different effects on endoreplicating cells and mitotic cells. Resolving the nature of the roles played by PKAc in the cytoplasm and in the nucleus may lead to simultaneous understanding of the effects seen here on pattern formation and on cell growth.

Direct comparisons of the effects of PKI(1-31) and of PKI(1-77) are needed to provide more insight into how different PKAc inhibitors are functioning. *PKAc* transgenes with specific catalytic site mutations should provide evidence for or against a noncatalytic nuclear role for PKAc. *PKAr** transgenes with domain-specific mutations should provide insight into the role of *PKAR** in Hedgehog signaling. Identification of a Drosophila homologue of PKI(1-77) and study of its regulation will be important to achieve a clear understanding of the roles of PKAc. From a practical standpoint, PKI(1-31) transgenes should provide a useful tool for investigating the role of PKA in larval growth regulation, independent of its effects on pattern formation. Mutations that permit larvae to survive the effect of PKI(1-31) and develop to adults should help to identify elements controlling larval growth. Conversely, mutations that sensitize adults or embryos to PKI(1-31) may reveal elements important for pattern formation.

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