Drosophila Costal1 Mutations Are Alleles of Protein Kinase A That Modulate Hedgehog Signaling

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Manuscript received December 18, 2003
Accepted for publication March 1, 2004

ABSTRACT

Hedgehog (Hh) signaling is crucial for the development of many tissues, and altered Hh signal transduction can result in cancer. The Drosophila Costal1 (Cos1) and costal2 (cos2) genes have been implicated in Hh signaling. cos2 encodes a kinesin-related molecule, one component of a cytoplasmic complex of Hh signal transducers. Mutations in Cos1 enhance loss-of-function cos2 mutations, but the molecular nature of Cos1 has been unknown. We found that previously identified alleles of Cos1 actually map to two separate loci. Four alleles of Cos1 appear to be dominant-negative mutations of a catalytic subunit of protein kinase A (pka-C1) and the fifth allele, Cos1A1, is a gain-of-function allele of the PKA regulatory subunit pka-RII. PKA-RII protein levels are higher in Cos1A1 mutants than in wild type. Overexpression of wild-type pka-RII phenocopies Cos1 mutants. PKA activity is aberrant in Cos1A1 mutants. PKA-RII is uniformly overproduced in the wing imaginal disc in Cos1A1 mutants, but only certain cells respond by activating the transcription factor Ci and Hh target gene transcription. This work shows that overexpression of a wild-type regulatory subunit of PKA is sufficient to activate Hh target gene transcription.

The secreted signaling molecule Hedgehog (Hh) was originally discovered as a gene required for proper patterning of the Drosophila larval cuticle and is now known to function in many different tissues at many different stages of Drosophila development. Hh signaling has been highly conserved during evolution and is employed in the development of many mammalian tissues and organs as well. Human cancers, including basal cell carcinoma, medulloblastoma, and digestive tract tumors, occur when the Hh signal is overproduced or restraining influences on Hh signal transduction are damaged (Hahn et al. 1996; Johnson et al. 1996; Goodrich et al. 1997; Oro et al. 1997; Raffel et al. 1997; Taipale and Beachy 2001; Berman et al. 2003; Thayer et al. 2003). During Drosophila larval development, Hh is expressed and secreted by cells in the posterior (P) of developing imaginal discs. Anterior (A) cells respond to Hh by activating transcription of target genes in cells just anterior to the A/P compartment boundary. In the wing imaginal disc these transcriptional targets include patched (ptc), en (engrailed), and transforming growth factor β (TGFβ) family member decapentaplegic (dpp). Basler and Struhl 1994; Capdevila and Guerrero 1994). Dpp itself is a secreted signaling molecule that functions as a morphogen to pattern the wing (Zecca et al. 1995; Nellen et al. 1996). When too little Hh signaling occurs in the wing and dpp expression is attenuated, structures at the A/P boundary are lost (Johnson et al. 1995). Conversely, if Hh signaling is activated inappropriately and ectopic dpp is produced, overgrowth and duplications of the anterior compartment occur (Capdevila et al. 1994).

Anterior cells must efficiently activate a transcriptional program in response to the Hh signal and must also ensure that Hh target genes are not activated inappropriately in the absence of ligand. Loss of any of several negative regulators in the Hh signal transduction pathway gives rise to Hh target gene activation in the absence of Hh. These include the 12-pass transmembrane protein Patched (Ptc; Hooper and Scott 1989; Nakano et al. 1989), the kinesin-related protein Costal2 (Cos2; Robbins et al. 1997; Sisson et al. 1997), the C1 catalytic subunit of the serine-threonine kinase protein kinase A (PKA-C1; Jiang and Struhl 1995; Johnson et al. 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995), and the Cdc4-like protein Supernumerary limbs (Slimb; Jiang and Struhl 1998; Theodosiou et al. 1998). Recent experiments have uncovered additional potential positive roles for pka-C1 and cos2 in regulating Hh target gene transcription (Wang and Holmgren 2000).

Like cos2, Costal1 (Cos1) mutants result in phenotypes similar to hh gain of function (Figure 1; Whittle 1973, 1974; Grau and Simpson 1987), so mutant alleles of...
CosI are likely to allow inappropriate transcription of Hh target genes (Capdevila et al. 1994). The role of the zinc-finger transcription factor Cubitus interruptus (Ci) in Hh signal transduction is complex as it functions as both a negative and a positive regulator of transcription (Aza-Blanc et al. 1997).

The genetic data show that ptc, cos2, and pka-C1 are negative regulators of Hh target gene transcription. Unlike ptc and cos2, which appear to function exclusively in Hh signaling, pka-C1 plays many roles in addition to controlling transduction of the Hh signal. For example, pka-C1 is necessary for Drosophila oogenesis and neural function, as indicated by studies of pka-C1 hypomorphic alleles (Lane and Kalderon 1993; Skoulakis et al. 1993). Later analysis of pka-C1 null alleles in clones of cells revealed wing duplications and other patterning defects that linked pka-C1 to Hh signal transduction (Jiang and Struhl 1995; Johnson et al. 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995).

Pioneering studies of PKA showed how it is regulated by cAMP. In the absence of cAMP, PKA exists as a heterotrimer consisting of two catalytic (C) subunits and a homodimer of two regulatory (R) subunits, and the enzyme is inactive. Upon binding of two cAMP molecules to each R subunit, the C subunits are released as catalytically active monomers. Drosophila has two R subunits, RI and RII (Kalderon and Rubin 1988; Park et al. 2000). The ability of R subunits to influence PKA-C1 activity in the context of Hh signaling has been explored only by overexpression of a mutant RI subunit that cannot bind cAMP, which mimics pka-C1 loss-of-function phenotypes (Li et al. 1995; Kiger et al. 1999). The role of wild-type R subunits in transducing the Hh signal is unknown.

Hh signal transduction culminates in the control of the activity of the transcription factor Ci, which can act as either a repressor or an activator of Hh target genes. In the wing imaginal disc, Hh signaling acts through Ci to activate the transcription of dpp, ptc, and en. In the absence of Hh, Ci is proteolytically processed into a 75-kD repressor form that represses dpp, but not ptc or en (Methot and Basler 1999). PKA affects Hh signal transduction by phosphorylating Ci and thus promoting processing of Ci into the repressor form (Chen et al. 1998, 1999; Price and Kalderon 1999; Wang et al. 1999). It is unknown how the Hh signal either prevents PKA from phosphorylating Ci or activates an opposing phosphatase. Whether PKA also affects Hh signaling in other ways is unknown.

In previous work the CosI gene has been mapped to at least two locations, suggesting that similar genetic effects derive from more than one locus (Grau and Simpson 1987; Lasko and Pardue 1988). Here we have found that certain CosI alleles map to two loci, both of them encoding subunits of PKA. Our genetic studies show that different CosI alleles affect Hh signal transduction in distinct ways.

**MATERIALS AND METHODS**

**Fly stocks:** P-element lines were obtained from Bloomington Stock Center, Ziegled Stock Center, and Exelixis. F1 Gal4 was a gift of J. Kiger. For CosI+/+ heterozygous discs, BS3.0 dpp LacZ (Blackman et al. 1991) was used. For CosI+/+ clonal analysis, a line carrying dpp LacZ on the third chromosome was used (Glise et al. 2002).

**Mounting wings:** Wings were dissected in ethanol and mounted in a 1:1 solution of Permount and Xylene.

**Recombination mapping:** w; b pr cos2+/en Cos1+/P; w; b pr cos2+/en Cos1+/P; w b pr cos2+/en Cos1+/P; w; b pr cos2+/en Cos1+/P; and w; b pr cos2+/en Cos1+/P virgin females were mated to w; Sco/CyORoi males. Eye-color and wing phenotypes of progeny were scored. Due to variable penetrance of the wing duplication for cos2+/+ Cos1 chromosomes, only red-eyed progeny were counted to determine map distance. All P elements used for mapping were marked with miniwhite+.

For CosI+/+ mapping experiments, stocks were established from recombinant progeny by crossing to w; Sco/CyORoi males. DNA was made from the recombinant stocks using a Berkeley Drosophila Genome Project (BDGP) protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html) and the presence or absence of cos2+/+ on the recombinant chromosomes was determined by PCR.

**cos2+/+ genotyping PCR:** PCR was performed using HFtaq (BD Biosciences) with an annealing/extension temperature of 62°C. A forward primer that recognizes both cos2+ and cos2− genomic sequences (complete sequence 5′-TGCTGACGAA TAGCTGAGATCGT-3′) along with a cos2+–specific reverse primer (5′-TGACACGGTGATATTGAGG-3′) and a cos2+/+–specific reverse primer (5′-TGACTCCGCTATATCC GTAC-3′) were used. cos2+ and cos2− products could be

![Figure 1](image-url)
recognized due to a size difference (~650 bp for cos2+ vs. 400 bp for cos2–).

5'-rapid amplification of cDNA ends for Cost1A sequences: RNA was isolated from dechorionated 0- to 7-hr collections of b pr cos2+/ Ex Cos1/+/CyO or b pr en cos2+/ bw sp embryos using RNAZOL (Teltest). 5'-rapid amplification of cDNA ends (RACE) was performed according to manufacturer’s protocol (Roche, Indianapolis). A primer directed at base pairs 562–607 of the RII mRNA (5'-TTGTCATCTGCTTCTG-3') was used for 5'-tagged first-strand synthesis. One round of HF Tag PCR (BD Biosciences) was performed using a primer directed at base pairs 555–553 (5'-TGTCATCTGCTTCTTGG GGAAACC-3') of the RII mRNA and a 5'-tag-specific primer. No detectable product was obtained, so a 1:10 dilution of the reaction was made and used in a second round of PCR using a primer at base pairs 482–464 (5'-CGAAAACGTAGTTGCGACG-3') of the RII mRNA and a 5'-tag-specific primer. The resulting products were TA cloned (Invitrogen, San Diego) and sequenced.

Allele sequencing: Genomic DNA was isolated using a BDGP protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html) or DNEasy kit (QIAGEN, Chatsworth, CA). PCR was performed using the HF Tag kit (BD Biosciences). PCR products were purified from agarose gels (QIAGEN) and subjected to automated sequencing.

To generate homozygous mutant DNA for sequencing, a b pr cos2+/ Ex Cos1/+/CG19 (CyO-Kc-Gal4, UAS-GFP) stock was used. Adults 0–2 days old were placed in collection bottles in the dark and provided with apple/agarose collection caps with yeast paste. Caps were changed twice a day for 2 days. On the third and subsequent days, two 1-hr prelays were discarded. A 2-hr collection was then aged an additional 4.5 hr at room temperature. The resulting 4.5- to 6.5-hr embryos were dechorionated and hand sorted. Non-green fluorescent protein (GFP)-containing embryos were used to make genomic DNA. Purity of the sort was determined by PCR for cos2+ and cos2–. Only sorted pools that had no detectable PCR product for cos2– were used for further analysis.

Preparation of protein extract from homozygous Cost1A embryos for Western analysis and kinase assays: Homozygous b pr cos2+/ Ex Cost1A embryos were obtained as previously described in Allele sequencing except that collections were sorted using automated methods (Furlong et al. 2001). Purity of the sort was determined by examining sorted embryos for GFP. Sorted embryos were incubated for 10 min on ice in hypotonic lysis buffer [10 mm Tris, pH 7.4, 0.2 mm MgCl2, and complete mini protease inhibitor (Roche)] and then dounce homogenized. Sucrose and EDTA were added to final concentrations of 0.25 m and 1 mm, respectively. Extracts were spun at 100,000 × g for 1 hr at 4°C. The protein concentration of the resulting S100 was determined in triplicate using a Bradford Assay (Bio-Rad, Richmond, CA). For Western analysis, rabbit anti-RII sera were used at 1:2000. Kinase assays were performed using the Amphi-ECT PKA assay system (Promega, Madison, WI). CAMP concentrations were varied. Reactions were performed for 10 min at 30°C. Specific activity was determined by subtracting from each experimental condition the counts incorporated in the presence of 1 μM of the PKA inhibitor-(6-22)-amide (see supplementary Figure 3 at http://www.genetics.org/supplemental/). Each assay was performed in triplicate. Wild type vs. mutant was compared at each concentration of cAMP using Student’s t-test.

Clonal analysis: A G13 FRT 42B Cost1A chromosome was generated, recombined with wild-type chromosomes for seven generations, and then balanced over CyO. A cross of y,w, hsFLP; G13 FRT 42B Cost1A/CyO virgin to y,w, hsFLP; G13 FRT Ubi-GFPods males was allowed to lay for 2 days. Larvae were heat-shocked at 37°C for 1 hr on the third and fourth days or on the fourth and fifth days.

Antibody staining of imaginal discs: Third instar larvae were dissected in PBS and fixed in Brower’s fix for 1 hr on ice or in 4% paraformaldehyde in PBS for 13 min at room temperature. Larvae were washed in PBS, 0.1% Tween, and 0.2% BSA. Primary antibodies were hybridized in wash buffer overnight at 4°C. Larvae were washed and secondary antibodies were hybridized at room temperature for 1 hr. After additional washes, discs were mounted in Vectashield and imaged using confocal microscopy. Primary antibody concentrations were as follows: rabbit polyclonal anti-RII, 1:2000; rat monoclonal anti-α-Ci Ctermin (2A1), 1:10; rabbit polyclonal and mouse anti-β-gal (Promega), 1:5000.

Overexpression studies: JW1 Gal4 was crossed to EP(2)2162 or EP(2)2277 as a control. All crosses were carried out at 29°C.

RESULTS

Cost1A, Cost1B, Cost1C, and Cost1D encode dominant-negative alleles of pka-CI: Two conflicting map locations have been published for Cost1A, Cost1B, and Cost1C. They were mapped by meiotic recombination to a position distal to cn and proximal to vg on the right arm of the second chromosome (2R). Two other alleles, Cost and Cost, were not mapped but were considered to be allelic to Cost because of their dominant wing phenotype and lethality when tested in trans to other Cost alleles (Grau and Simpson 1987). Cost was later placed at a different map location on 2R distal to vg at 50A1–50A2. This map location was based on the discovery of a deficiency in the region that produced duplications when placed in trans to a recessive allele of cost (Lasko and Pardue 1988).

To resolve this discrepancy, meiotic map locations were determined for all dominant alleles of Cost. This was accomplished by measuring map distances between Cost alleles and individual white+ (w+) P-element insertions. Cost results in dominant wing duplications and the P-element results in dominant eye pigmentation in a w background, so recombinant progeny can be scored in the first generation by scoring wing and eye phenotypes. Because of variable penetrance of Cost (which can range from 3 to 95%, depending on the allele and positive or negative selection for the phenotype; Grau and Simpson 1987), some recombinant progeny will not be scored as such and thus recombination percentages are underestimations of map distance. In addition, only w+ progeny were scored for calculating map distances.

The previously unmapped allele Cost was mapped using a panel of P elements on the second chromosome. Surprisingly, Cost recombined readily with all P-transposable elements that were located on 2R, indicating that Cost does not map to either previously published map location for Cost (supplementary Figure 1 at http://www.genetics.org/supplemental/). For P elements on chromosome arm 2L, recombination percentages with Cost decreased as P elements farther and farther from the centromere were used, until polytene band 30C, indicating that Cost maps close to polytene band 30C. This was confirmed by the lack of recombination be-
between Cos1\textsuperscript{a} and the k07104 P element insertion at 30C. With P elements distal to 30C, recombination percentages increased with P elements closer and closer to the telomere of chromosome arm 2L (supplementary Figure 1 at http://www.genetics.org/supplemental/). Cos1\textsuperscript{f} also mapped to this location (data not shown). Cos1\textsuperscript{f} and Cos1\textsuperscript{a} were difficult to map due to low penetrance, yet both failed to recombine with the k07104 P element, indicating a map location near 30C (data not shown).

Inspection of the 30C region to which Cos1\textsuperscript{f}, Cos1\textsuperscript{a}, Cos1\textsuperscript{b}, and Cos1\textsuperscript{c} mapped revealed the presence of the gene encoding the protein kinase A C1 subunit (\textit{pka-C1}). Because loss of \textit{pka-C1} function activates Hh target gene transcription (Jiang and Struhl 1995; Li et al. 1995; Strutt et al. 1995), there was reason to suspect that \textit{pka-C1} could be the Cos1 gene located near 30C. The \textit{pka-C1} protein-coding sequence was sequenced using DNA obtained from Cos1\textsuperscript{f}, Cos1\textsuperscript{a}, Cos1\textsuperscript{b}, and Cos1\textsuperscript{c} heterozygous flies and point mutations were found in the gene in each of the four stocks. Cos1\textsuperscript{f} and Cos1\textsuperscript{a} each contained the same base-pair substitution that translated into a change in protein sequence of E to K at amino acid (aa) 130. Cos1\textsuperscript{b} contained a base-pair substitution that translates to an E-to-K change at aa 173 and Cos1\textsuperscript{c} contained a base-pair change that translates to a G-to-D substitution at aa 189 (Figure 2). These changes are not present in wild-type Canton-S flies or on the CyO balancer chromosome. All three residues are conserved from yeast to human.

On the basis of these data, Cos1\textsuperscript{f}, Cos1\textsuperscript{a}, Cos1\textsuperscript{b}, and Cos1\textsuperscript{c} should be renamed \textit{pka-C1}\textsuperscript{f}\textsuperscript{col-2}, \textit{pka-C1}\textsuperscript{a}\textsuperscript{col-3}, \textit{pka-C1}\textsuperscript{b}\textsuperscript{col-4}, and \textit{pka-C1}\textsuperscript{c}\textsuperscript{col-9}.

\textbf{Cos1\textsuperscript{f} encodes a gain-of-function allele of \textit{pka-RII}:} Unlike the other Cos1 alleles, Cos1\textsuperscript{f} did not recombine readily with P elements in the \textit{cn} to \textit{vg} region of chromosome 2R (supplementary Figure 2 at http://www.genetics.org/supplemental/). Specifically, Cos1\textsuperscript{f} recombination percentages with P element insertion k13906 at 46D1 and with P element insertion EP(2)2170 at 46E1 were 0.05%, indicating that Cos1\textsuperscript{f} maps near these two P elements. Because the Cos1\textsuperscript{f} chromosome used for mapping is also marked with an allele of cos2, cos2\textsuperscript{f}, the linear order of the centromere, Cos1\textsuperscript{f}, and the P element could be determined for each recombinant chromosome. cos2\textsuperscript{f} and Cos1\textsuperscript{f} are linked (they are separated by \textsim 500 kb) and the gene order is centromere, cos2\textsuperscript{f}, Cos1\textsuperscript{f}, telomere (Grau and Simpson 1987). Recombination events between Cos1\textsuperscript{f} and the P element would result in recombinant chromosomes that either include or exclude cos2\textsuperscript{f}, depending on the proximal/distal relationship of Cos1\textsuperscript{f} and the P element. The presence of cos2\textsuperscript{f} on a Cos1\textsuperscript{f} P element recombinant chromosome would indicate that Cos1\textsuperscript{f} is proximal to the P insertion. Conversely, the absence of cos2\textsuperscript{f} would indicate that Cos1\textsuperscript{f} is distal to the P insertion.

Cos2\textsuperscript{f} contains a transposon insertion in the 3′-untranslated region (UTR) of cos2 (Sisson et al. 1997; data not shown) so the presence of cos2\textsuperscript{f} can be easily elucidated by PCR using a cos2 and a primer specific to the insert. Using this method, Cos1\textsuperscript{f} was found to be distal to P element insertion k13906 at polytene band 46D1 and proximal to P element insertion EP(2)2170 at polytene 46E4 (data not shown). No recombinants were detected between Cos1\textsuperscript{f} and P element insertion EP(2)2162 at polytene band 46D1 (\textit{n} = 6640), P element insertion EP(2)2324 at polytene band 46D7 (\textit{n} = 5935), and P element insertion EP(2)2277 at polytene band 46D7 (\textit{n} = 9776; supplementary Figure 2 at http://www.genetics.org/supplemental/). The mapping of Cos1\textsuperscript{f} to polytene chromosome location 46D–E is in agreement with the previously identified meiotic map location (61) published by Grau and Simpson (1987).

Examination of the sequence within the maximal chromosome region that must contain at least part of Cos1\textsuperscript{f} (between \textit{P} element k13906 and \textit{P} element EP(2)2170, an interval of 156 kb) revealed 26 genes predicted by the Drosophila genome project. One of these 26 genes encodes a regulatory subunit of protein kinase A, \textit{pka-RII}. \textit{pka-RII} was already known to influence PKA catalytic activity in adult Drosophila (Park et al. 2000), but the role of \textit{pka-RII} in regulating PKA-C1 activity in Hh signaling has not been elucidated. Because \textit{pka-RII} can regulate PKA catalytic activity in certain circumstances, it was a candidate for the Cos1\textsuperscript{f} mutation.

Genomic DNA from homozygous mutant Cos1\textsuperscript{f} embryos was isolated and used as a template for PCR reactions to amplify the \textit{pka-RII} genomic region. When primers directed against a portion of the second intron of \textit{pka-RII} were used, no product was obtained for Cos1\textsuperscript{f} mutants, but the expected product was obtained from wild-type Drosophila DNA (data not shown). 5′ RACE was employed to determine the sequence of the \textit{pka-RII} mRNA in Cos1\textsuperscript{f} mutants. The mutant transcript contained the third exon of \textit{pka-RII} (which contains the ATG) but the first and second exons were missing. In their place was sequence from the \textit{14-3-3} and JRA genes, which are located \textsim 100 kb distal to \textit{pka-RII}. The se-
Drosophila Costal1 Mutants and PKA
sequence of the mutant mRNA is consistent with the underlying aberration being an inversion. No other changes were detected in the coding sequence for pka-RII in Cos1\^I mutants.

To investigate the effect of the inversion on pka-RII expression, marked homozygous mutant clones of Cos1\^I were generated in the wing imaginal disc using the flipase-flipase recombinase target system (HARRISON and PERRIMON 1993) and were stained with an antibody directed against PKA-RII (RII). In cells containing one mutant copy of Cos1\^I, a moderate level of staining for RII was observed. In cells wild-type for Cos1\^I, a low or background level of staining was observed. In homozygous Cos1\^I cells, a high level of RII protein was detected (Figure 3). This result suggests that the pka-RII gene is the relevant gene for this Cos1 allele and that the underlying mutation causes overproduction of the regulatory subunit of the PKA enzyme.

Cos1 mutant embryos have mutant phenotypes indicative of misregulation of Hh signaling during embryogenesis (GRAU and SIMPSON 1987). RII protein levels were assayed in extracts prepared from cos2\^v1 Cos1\^I homozygous mutant embryos to determine whether RII is also overproduced in Cos1\^I mutants at early stages of development. For this purpose, homozygous cos2\^v1 Cos1\^I mutant embryos were obtained by automated sorting from a GFP-balanced stock (FURLONG et al. 2001). Compared to wild type, Cos1\^I mutants contain very high levels of RII protein (Figure 3). Kinesin heavy chain, detected with a specific antibody, was used to demonstrate that equal amounts of total protein were present for each genotype. As previously reported, Drosophila RII migrates as a doublet with an apparent molecular weight of ~50 kDa (PARK et al. 2000; Figure 3).

One explanation for the high levels of RII protein found in Cos1\^I mutants is that pka-RII is a transcriptional target of Hh signaling. If this were the case, high levels of RII should be found in cells mutant for other negative regulators of Hh target gene transcription. To investigate this possibility, clones of homozygous cos2 mutant

Figure 3.—RII protein levels are elevated in Cos1\^I mutants. (A–F) Cos1\^I clones were induced in wing imaginal discs. (A) A merged image of B and C. (B) Two copies of GFP mark homozygous wild-type cells (bright green) and lack of GFP marks Cos1\^I homozygous mutant cells. (C) RII protein stain. (D–F) Detail of clone marked by arrow in A; arrowheads in E and F mark edges of Cos1\^I homozygous mutant clones. RII levels are highest in Cos1\^I homozygous mutant cells and intermediate levels of RII are detected in cells heterozygous for Cos1\^I. (G) Western blot for RII. Protein extract was made from wild-type (Canton-S) embryos or homozygous b pr cos2 V1 cn Cos1 A1 mutant embryos. RII antibody recognizes a doublet at ~50 kDa. RII levels are elevated in mutant compared to wild type. An antibody against kinesin heavy chain was used to demonstrate that equal amounts of protein were loaded in each lane.
cells were generated and stained for RII. No increased staining for RII was detected in cos2 clones (data not shown), indicating that pka-RII is not a transcriptional target of Hh.

To determine whether pka-RII overexpression is sufficient to phenocopy Cos1 mutants, the GAL4-upstream activating sequence (UAS) system was used to overproduce RII in the developing wing. The transposable element EP(2)2162 is inserted 5' to the transcriptional start of pka-RII (Park et al. 2000). The enhanced promoter (EP) type of P elements contains UAS sites that allow GAL4-mediated overexpression of downstream genes (Rorth et al. 1998). EP(2)2162 is in the correct orientation to drive overexpression of RII (Park et al. 2000). JW1 Gal4 expresses Gal4 in the wing imaginal disc (Kiger et al. 1999; Kiger and O'Shea 2001) and was used to activate pka-RII transcription by taking advantage of the UAS element in EP(2)2162. Activation of pka-RII transcription resulted in outgrowths and duplications of the costa region of the wing, a Cos1 phenotype. When JW1 Gal4 was used to express EP(2)2277, a control EP element, no duplications were observed, indicating that the phenotype is not a result of GAL4 expression alone (Figure 4). Duplications also occurred when RII was overproduced with the E132 Gal4 driver (data not shown).

In summary, a small interval that contains pka-RII and must also contain part of Cos1 was identified by recombination mapping. In total, three lines of evidence support that pka-RII is the gene affected in Cos1 mutant: (1) an inversion with a breakpoint in pka-RII was found in Cos1 mutant, (2) RII is overproduced in Cos1 mutant, and (3) overexpression of wild-type pka-RII is sufficient to mimic a Cos1 phenotype. On the basis of these data, Cos1 should be renamed pka-RIICos1A1.

PKA activity is affected in pka-RIICos1A1 mutants: If pka-RIICos1A1, and thus RII overproduction, activates Hh signaling by regulating PKA-C1, PKA catalytic activity should be altered in pka-RIICos1A1 mutants. To address this question, PKA kinase assays were performed using extract from wild-type [Canton-S (CS)] or cos2 pka-RIICos1A1 homezygous mutant embryos (Figure 5 and supplementary Figure 3 at http://www.genetics.org/supplemental/). Compared to wild type, the basal level of activity (no added cAMP) of PKA in pka-RIICos1A1 mutants is decreased. At the lowest level of CAMP tested, pka-RIICos1A1 mutants, PKA activity is actually lower than in the presence of no added cAMP. This is in contrast to the wild-type situation where, even at the lowest level of cAMP, PKA activity is increased compared to the basal level of activity. At CAMP concentrations of 0, 0.005, and 0.05 μM, PKA activity in pka-RIICos1A1 mutants is decreased compared to wild type. At higher concentrations of CAMP (0.5 and 5 μM), PKA activity in pka-RIICos1A1 mutants was actually higher than that in wild type. At all concentrations tested, the activities of PKA in CS and pka-RIICos1A1 extracts were statistically different from each other on the basis of Student’s t-test (P < 0.05; Figure 5).

In conclusion, pka-RIICos1A1 results in decreased basal PKA catalytic activity but increased cAMP-stimulated PKA activity. The increase in cAMP-stimulated PKA activity is apparent only when high levels of cAMP are added, indicating that RII overproduction is likely to repress PKA catalytic activity in vivo. This supports the hypothesis that RII exerts its effects on Hh target gene transcription by inhibiting PKA-C1 activity.

Only certain cells are sensitive to pka-RII overexpression: Cos1 was named due to the mutant phenotype of duplications limited to the costa region of the wing (Whittle 1973, 1974). Are only costa cells sensitive to the action of pka-RIICos1A1? Levels of full-length Ci (the activator form) and of dpp transcription were examined in cos2 pka-RIICos1A1/ + mutant discs. In the absence of Hh, Ci is proteolytically cleaved to a 75-kD repressor form. Near the source of Hh, this proteolysis is inhibited and full-length Ci accumulates. This accumulation of full-length Ci can be monitored using 2A1, a monoclonal antibody that specifically recognizes full-length Ci.
Figure 5.—PKA activity is affected in Cos1\textsuperscript{AI} mutants. Kinase assays were performed using extract from wild type (CS) and b pr cos2\textsuperscript{V1} cn Cos1\textsuperscript{AI} homozygous mutant embryos. Activity is measured as picomoles of ATP per minute per microgram of protein. cAMP concentration varied from 0 to 5 μM. Values for CS are shaded and for b pr cos2\textsuperscript{V1} cn Cos1\textsuperscript{AI}, solid. All reactions were performed in triplicate and standard deviations are indicated by error bars. Activity is the average of separate reactions performed in triplicate. The standard deviation is also given. For each concentration of cAMP, PKA activities in CS and b pr cos2\textsuperscript{V1} cn Cos1\textsuperscript{AI} extract were significantly different from each other using Student's t-test (P values range from <0.0007 to <0.0345).

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<td>5.984</td>
<td>0.236</td>
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Ci (Aza-Blanc et al. 1997). cos2\textsuperscript{V1} pka-RII\textsuperscript{Gal4AI}/+; dpp lacZ wing discs were stained with 2A1 to monitor Ci. They were also labeled with anti-LacZ to monitor dpp transcription (Figure 6A). In cos2\textsuperscript{V1} pka-RII\textsuperscript{Gal4AI}/+ discs, dpp lacZ is expressed in the costa region in addition to its normal expression just anterior to the A/P boundary. The costa region also has levels of full-length Ci comparable to, or even higher than, those seen at the A/P boundary where Hh signaling is active. No ectopic hh is detected in pka-RII\textsuperscript{Gal4AI} mutants (data not shown). Therefore, RII overexpression can mimic the response of cells to Hh in a region of the disc where Hh is not present. In cos2\textsuperscript{V1} Cos1\textsuperscript{AI}/+ discs, RII protein is expressed at equal levels throughout the entire disc (Figure 6A' and data not shown), but ectopic high levels of full-length Ci and ectopic dpp transcription occur only in the region of the disc fated to form the costa of the adult wing (Figure 6, A'' and A'').

To further investigate if the effects of RII are limited to the costa region or if all cells in the anterior compartment are competent to respond to RII, pka-RII\textsuperscript{Gal4AI} clones and JWI-Gal4-EP(2)2162 RII overproducing cells were generated throughout the wing imaginal disc (Figure 6, B–F). dpp transcription was assayed in these discs.
Figure 6.—Ci protein and dpp transcription in RII overexpressing cells. (A–A’’) A third instar b pr cos2 cn pha-RII(CalI)/+; dpp-LacZ wing imaginal disc. (B–B’’) A third instar wing imaginal disc with a pha-RII(CalI) homozygous mutant clone just anterior to the A/P boundary (arrow). (C–C’’) Close up of clone from B, revealing the detail of full-length Ci accumulation and dpp-LacZ expression. (D–D’’) A third instar wing imaginal disc in which RII is overproduced using JW1 Gal4 and EP(2)2162. (A’’) Anti-RII staining reveals that RII levels are uniform throughout the disc. (A’’) 2A1 antibody visualizes full-length Ci. Only in the presumptive duplication is Ci stabilized at levels comparable to or higher than those found at the A/P boundary. The high levels of dpp LacZ observed in the duplication (A’’) indicate that dpp is fully transcriptionally activated by Ci activator and not merely derepressed due to low levels of Ci75. (B’’) 2A1 staining reveals full-length Ci. Full-length Ci accumulates throughout the pha-RIII(CalA) homozygous mutant clone. (D’’) Anti-RII staining reveals that JW1 Gal4 drives pha-RII expression in patches of cells throughout the disc. (D’’) Anti-LacZ staining reveals the normal expression of dpp-LacZ at the A/P boundary and additional areas of ectopic expression. (D’’’) 2A1 staining reveals full-length Ci. Full-length Ci accumulates in anterior cells that overproduce RII. (E–E’’) A close-up view of the A/P boundary of the wing pouch of the disc shown in D. Arrows indicate groups of cells that overproduce RII, have increased amounts of full-length Ci, and do express dpp-LacZ. (F–F’’) A close-up view of the costa region of the disc shown in D. Many cells in the costa region ectopically express dpp-LacZ. A, B, C, D, E, and F are merged images. A’, D’, E’, and F’, anti-RII; B’ and C’, GFP; A’’, B’’, C’’, D’’, E’’, and F’’, anti-LacZ to visualize dpp-LacZ; A’’’, B’’’, C’’’, D’’’, E’’’, and F’’’, 2A1 antibody to visualize full-length Ci.

by staining for dpp LacZ. In pha-RII(CalA1) homozygous mutant clones near the boundary, dpp-LacZ is not transcribed even though high levels of full-length Ci are present throughout the clone (Figure 6B and close-up view in 6C). However, cells in the costa region that are heterozygous for pha-RII(CalA1) contain high levels of full-
length Ci and also transcribe dpp (Figure 6B). Absence of dpp-LacZ expression in pka-RII<sup>cos1</sup> homozygous mutant clones was observed in clones located in several regions of the anterior compartment (data not shown). When RII is overproduced using JW1-Gal4 and EP(2) 2162, cells that contain high levels of RII contain high levels of full-length Ci. JW1-Gal4 mediates RII overproduction in small groups of cells throughout the wing imaginal disc (Figure 4A and Figure 6, D’, E’, and F’). However, in the pouch region, only a few of the sporadic cells that overproduce RII and have high levels of full-length Ci also transcribe dpp (Figure 6E’‘). In the costa region, many cells that overproduce RII and have high levels of full-length Ci transcribe dpp (Figure 6D’’).

In all situations in which RII is overproduced, only certain cells transcribe the Hh target gene dpp-LacZ. These responsive cells are found in more anterior regions of the disc. This argues for the existence of additional components of the Hh transduction machinery that are expressed or function only in limited regions of the wing imaginal disc.

**DISCUSSION**

Cos1 mutations strongly influence Hh signaling, but the identity of the gene has been mysterious for some time. Here we provide evidence in favor of PKA genes as the sites of Cos1 mutations. Four alleles of cos1 were mapped to polytene chromosome location 30C while a fifth allele, Cos1<sup>12</sup>, was mapped to a different chromosome arm at polytene band 46E. We conclude that the two genes had previously been characterized as one because Cos1<sup>12</sup> was seminiable only in trans to the other Cos1 alleles, and all alleles have the same genetic and phenotypic characteristics in combination with cos2 alleles. We have identified the two genes as encoding subunits of PKA.

Two different map locations have previously been published for Cos1. Neither polytene band 30C nor 46E (this work) is consistent with the 50A1–50A2 map location for Cos1. Cos1 was placed at 50A1–50A2 due to the discovery of a deficiency that deleted the region and resulted in wing duplications when in trans to a recessive allele of cos1 (Lasko and Pardue 1988). It seems quite possible that a third gene was mapped that has genetic and phenotypic characteristics similar to the two Cos1 genes that we have mapped. It was assumed in those studies that the Cos1 phenotype results from haplo-insufficiency and that the Cos1 phenotype is due to the deficiency and not to a second site mutation. For example, along with the deficiency, the chromosome may carry an allele of cos2 that caused the observed phenotype.

The location of Cos1<sup>19</sup> is consistent with the Grau and Simpson (1987) map location, indicating that the Cos1<sup>19</sup> stock used in these experiments is the same stock used in their research. The map locations for our copies of the Cos1<sup>2</sup> and Cos1<sup>1</sup> mutations are not in agreement with their previously published map locations. Details of the genetic methods used were not presented in the original mapping article, so it is difficult to explain the discrepancy. One possibility is that in the many years that have passed since the original article there were mistakes in stock labeling. This is a distinct possibility since both Cos<sup>1</sup> and Cos<sup>1</sup> contain the same base-pair change and thus may have originally been the same stock. Both stocks are likely to carry the original Cos<sup>1</sup> allele as it was not previously mapped and the original Cos<sup>1</sup> allele was mapped to the opposite arm of chromosome 2.

**Cos1<sup>2</sup>, Cos1<sup>1</sup>, Cos1<sup>8</sup>, and Cos1<sup>9</sup> are dominant-negative alleles of pka-CI:** We identified pka-CI as a candidate for the gene mutated in Cos<sup>1</sup>, Cos1<sup>8</sup>, Cos1<sup>9</sup>, and Cos1<sup>1</sup>. We detected sequence changes at the DNA level at the pka-CI locus, and these changes translated to substitutions in the coding sequence. These point mutations could influence PKA activity in several possible ways. First, they could render PKA-C1 catalytically inactive and give dominant phenotypes due to haplo-insufficiency. Alternatively, the point mutations could destabilize the encoded protein to such a degree that the mutant protein would be degraded and thus function as a protein null. This scenario would also result in dominant phenotypes due to haplo-insufficiency. These haplo-insufficiency explanations are unlikely to be correct. pka-CI is a recessive negative regulator of Hh signaling and heterozygosity for pka-CI null alleles or for deficiencies that delete pka-CI does not result in any obvious phenotype. Second, the mutations could render PKA-C1 constitutively active. The unregulated activity could be responsible for the dominant wing duplication phenotypes. However, expression in the wing imaginal disc of a mutant PKA-C1 that cannot be regulated by cAMP interferes with the normal expression pattern of the Hh target gene pte, indicating that constitutive PKA-C1 activity antagonizes Hh signal transduction (Jiang and Struhl 1995; Li et al. 1995).

We therefore favor a third possibility: that pka-CI<sup>Cos1-2</sup>, pka-CI<sup>Cos1-3</sup>, pka-CI<sup>Cos1-8</sup>, and pka-CI<sup>Cos1-9</sup> encode dominant-negative (dn) versions of PKA-C1 that produce stable, full-length protein with reduced catalytic activity. There are precedents for the formation of dn pka-C1 mutants in Drosophila. A previously described dominant mutation that causes wing duplications maps near pka-C1. Although the molecular nature of this mutation was not ascertained, it was assumed that the mutation was a pka-C1 allele (Pan and Rubin 1995). Overproduction of a catalytically inactive mutant form of PKA-C1 in an otherwise wild-type background results in wing duplications, indicative of inappropriate activation of Hh signaling (Kiger and O’Shea 2001).

PKA-C1, like all other protein kinases, contains a catalytic core. Within the catalytic core of PKA-C1 and all kinases are defined subdomains that contain conserved amino acids and that play known roles in catalyzing the
phosphate transfer reaction. The amino acids affected in \textit{pka-C1}^{Col-2}, \textit{pka-C1}^{Col-3}, \textit{pka-C1}^{Col-4}, and \textit{pka-C1}^{Col-8} mutants are part of the catalytic core and are likely to be required for PKA-C1 catalytic activity.

\textit{pka-C1}^{Col-8} contains a substitution of D for G at aa 189 (all numbering of amino acids is taken from the protein sequence of Drosophila PKA-C1). This amino acid is the third amino acid in the DFG triad located in subdomain VII. This triad is conserved in essentially all serine/threonine and tyrosine kinases. In PKA, the triad has been implicated in binding the metal ion that coordinates the \(\beta\)- and \(\gamma\)-phosphoryl oxygens of the ATP used as a phosphate donor. Mutation of the D residue in the triad renders PKA inactive (Zoller \textit{et al.} 1991). Therefore, it is likely that the substitution in \textit{pka-C1}^{Col-8} results in a catalytically inactive kinase.

\textit{pka-C1}^{Col-1} contains a K substitution for E173. This amino acid lies in subdomain VI between D169 and N174, two residues implicated in ATP binding (Hanks \textit{et al.} 1988). This region is implicated in substrate binding specificity as most serine/threonine kinases share the to regulation of cells. On the basis of \textit{pka-C1}^{Col-1} could still incorporate into complexes and render the entire complex ineffective.

\textit{Cos1^II} is a gain-of-function allele of RII: \textit{pka-RII}^{Gal4} was recovered in a screen using \(\gamma\)-rays as a mutagen. Unlike most chemical mutagens, which tend to cause point mutations, \(\gamma\)-rays tend to produce larger aberrations such as chromosome deficiencies or rearrangements. A change consistent with a 100-kb inversion was found in the 5' UTR of \textit{pka-RII} in \textit{pka-RII}^{Gal4} mutants. No other changes in RII coding sequence were detected but higher levels of RII were found in \textit{pka-RII}^{Gal4} mutant cells. On the basis of cos2 clone results, this is not due to regulation of \textit{pka-RII} by Hh signaling. In addition, \textit{pka-RII}^{Gal4} clones located in the posterior compartment of the wing disc have high levels of RII but do not express C, the only transcription factor known to function in Hh signaling (data not shown). Taken together, the data indicate that the high levels of RII observed in the wing disc are unique to \textit{pka-RII}^{Gal4} mutants. \textit{pka-RII} overexpression is likely to cause the inappropriate activation of Hh target gene transcription observed in \textit{pka-RII}^{Gal4} mutants. Previous work (Li \textit{et al.} 1995) has shown that overexpression of a mutant RI that cannot bind cAMP can activate Hh target gene transcription. The data presented here are the first to show that the other regulatory subunit found in Drosophila, \textit{pka-RII}, can influence Hh signaling and that overproduction of a wild-type R subunit of PKA is sufficient to activate Hh target gene transcription.

Antibody staining for RII in wing imaginal discs allowed us to observe that RII levels are increased in \textit{pka-RII}^{Gal4} mutants but did not allow us to examine absolute levels or possible post-translational modifications. We therefore used Western analysis to examine RII protein levels in \textit{pka-RII}^{Gal4} mutant embryos. RII protein exists in two forms due to differential phosphorylation of a consensus PKA phosphorylation site in RII (Foster \textit{et al.} 1984). In Drosophila whole-fly extract, RII can be labeled by radiophosphate at low, but not high, levels of cAMP (Park \textit{et al.} 2000). Increasing the cAMP concentration leads to the dissociation of R and C subunits, so the phosphorylation probably occurs when the RII subunit is part of a heterotetrameric holoenzyme (Rangel-Aldao and Rosen 1976). In \textit{pka-RII}^{Gal4} mutants, the majority of the extra RII is the faster-migrating, non-catalytically inactive subunit essentially “poisons” each dimer of a larger multiprotein complex. In this case, a catalytically inactive PKA-C1 could still incorporate into complexes and render the entire complex ineffective.
zymes. In this situation, essentially all PKA-C1 is predicted to be in complex with RII.

Unlike the four alleles pka-C1<sup>gal-1</sup>, pka-C1<sup>gal-3</sup>, pka-C1<sup>gal-4</sup>, and pka-C1<sup>gal-5</sup> that are likely to be amorphic and function as pka-C1-dominant negatives, pka-RII<sup>gal-141</sup> is likely to be a hypermorphic gain-of-function allele. Genetically, increased pka-RII function is predicted to mimic loss of pka-C1 function. The wing duplications caused by Gal4-driven overexpression of pka-RII were, exactly as predicted, similar to the effects of pka-RII<sup>gal-141</sup>. Overproduction of RII could influence PKA-C1 activity in two ways. First, RII could inhibit the catalytic activity of PKA by binding to the catalytic subunit. Second, R subunits can associate with A-kinase anchoring proteins (AKAPs) that localize PKA holoenzymes to specific subcellular locations (Scott and McCartney 1994). RII overproduction could mislocalize PKA-C1 within the cell via association with various AKAPs.

**RII overproduction modulates PKA activity:** The most likely way for overproduced RII to influence Hh signaling is by reducing PKA catalytic activity. The results of our PKA kinase assays are consistent with this hypothesis. In the heterotetrameric PKA holoenzyme, R subunits have two roles: inhibiting the catalytic activity of C subunits and protecting C subunits from degradation (Amieux et al. 1997; Brandon et al. 1998). Loss of R subunits results in increased basal activity of PKA due to the presence of free catalytically active C subunits, but in a decrease in cAMP-induced PKA activity because the C subunits are not protected from degradation. In pka-RII<sup>EpP2162</sup> mutants, RII levels are reduced by 95% and cAMP-induced PKA catalytic activity is reduced by almost 60%, indicating that C subunits have been degraded (Park et al. 2000). In pka-RII<sup>col-141</sup> mutants, high levels of RII should protect C subunits from degradation. At basal levels of cAMP, the stabilized pool of C subunits should remain associated with RIIs and the overall effect would be a decrease in basal PKA activity compared to wild type, which is precisely what we observed.

With the addition of cAMP, RII subunits should release C subunits and PKA activity should increase. At high levels of cAMP, essentially all C subunits should be released from RII-mediated inhibition. In this situation, mutants should contain higher levels of PKA activity than wild type because the high levels of RII have protected C subunits from degradation, resulting in an overall increase in the amount of C subunits. For the most part, our measurements of PKA activity in pka-RII<sup>gal-141</sup> compared to wild type were exactly as we predicted: basal PKA activity was decreased in pka-RII<sup>gal-141</sup> mutants compared to wild type while cAMP-induced PKA activity was vastly higher in pka-RII<sup>gal-141</sup> mutants than in wild type. However, at the lowest level of cAMP tested, a slight reduction in PKA activity from the basal level was observed for pka-RII<sup>gal-141</sup> mutants. This contrasts to the wild-type situation, where any added cAMP results in PKA activity higher than basal levels. One possible explanation for the slight decrease of PKA activity in the mutant could be that the initial release of a small amount of C subunits triggers the degradation reaction. At higher levels of cAMP, this effect is masked because more C subunits are released than can be immediately processed by the degradation machinery. Although RII overexpression results in higher levels of cAMP-induced PKA activity compared to wild type, the basal level of PKA activity is lower in the mutant than in wild type.

In the future, it will be interesting to examine the roles of wild-type R subunits in Hh signal transduction. If pka-RII normally plays a role in transducing the Hh signal, pka-RII mutants should possess Hh phenotypes. The only reported pka-RII mutant, pka-RII<sup>Fy72162</sup>, reduces adult RII levels by >95% and is homozygous viable with no phenotypes indicative of aberrant Hh signaling (Park et al. 2000). RII and RII may function redundantly. Another possibility is that residual levels of RII may be sufficient for normal regulation of Hh target gene transcription. The roles of R subunits in transducing the Hh signal may be revealed only by the creation of the double mutant carrying null alleles of RI and RII.

The wing duplication phenotype of pka-RII<sup>gal-141</sup> indicates that Hh target gene transcription has been inappropriately activated in response to pka-RII overexpression in the anterior compartment of the wing imaginal disc. Although RII protein levels are uniform throughout pka-RII<sup>gal-141</sup> heterozygous wing discs, Ci is stabilized and dpp is transcribed only in the presumptive duplication. Not every cell in the presumptive duplication that contains high levels of full-length Ci transcribes dpp. The high levels of full-length Ci should indicate that Ci repressor levels are low, so dpp, at the very least, should be derepressed. This sensitivity of only certain cells to high levels of full-length Ci resulting from RII overexpression was also observed when very high levels of RII were generated in pka-RII<sup>gal-141</sup> homozygous clones or by GAL4-UAS-mediated expression. In general, anterior-most cells are the most sensitive to pka-RII overexpression. One possible explanation is that more medial cells contain higher levels of cAMP and thus higher levels of RII are required to suppress PKA-C1 activity.

A similar situation is observed in clones mutant for a component of the SCF ubiquitin ligase complex, Roc1<sub>a</sub>. In Roc1<sub>a</sub> mutant clones, dpp is transcribed only in the most anterior clones even though high levels of full-length Ci are detected in all anterior clones. This is surprising because the appearance of full-length Ci in these cells must correspond to a decrease in Ci<sub>75</sub> repressor levels. In the absence of Ci<sub>75</sub>, dpp should be transcribed because it is derepressed. One possible interpretation is that in medial cells less full-length Ci is stabilized in response to pka-RII overexpression or loss of Roc1<sub>a</sub>. Because full-length Ci forms at the expense of Ci<sub>75</sub>, small reductions in full-length Ci levels translate
into small increases in Ci75 levels. This small increase in Ci75 could provide enough repressor activity to repress dpp. Immunoﬂuorescence may not be sensitive enough to detect subtle differences in full-length Ci levels between medial and lateral cells. Our studies of Costal-1, together with previous work by others, provide a powerful genetic link between the kinesin-related molecule Cos2 and PKA. The genetic interaction may be due to the sensitive balance between Ci, which is modiﬁed by PKA, and Cos2, or to additional activities that more directly link PKA to Cos2. In imaginal disk development precisely controlled Hh signal transducer activities are crucial for pattern formation, building the perfect forms of wings and legs.

We thank P. Simpson and I. Guerrero for providing Cos1 stocks, J. Kiger for providing JWI-Gal4, and D. L. Jones for the dpp LexA line on the third chromosome. We thank the Stanford Protein and Nucleic Acid facility and J. Kline for oligo synthesis and helping us. We thank E. Furlong for help with embryo sorting. The RII antibody was a gift from D. Kalderon. 2A1-producing hybridomas were a gift from R. Holmgren. We thank D. Kalderon, J. Hirsh, C. Cronmiller, J. Ferrell, and all the members of the Scott laboratory for helpful discussions. L.S.C. was a Howard Hughes Medical Institute predoctoral fellow. L.S.C. also wishes to thank the Baxter Foundation. M.P.S. is an Investigator of the Howard Hughes Medical Institute.

LITERATURE CITED


