Genetic Control of Cuticle Formation During Embryonic Development of *Drosophila melanogaster*

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ABSTRACT

The embryonic cuticle of *Drosophila melanogaster* is deposited by the epidermal epithelium during stage 16 of development. This tough, waterproof layer is essential for maintaining the structural integrity of the larval body. We have characterized mutations in a set of genes required for proper deposition and/or morphogenesis of the cuticle. Zygotic disruption of any one of these genes results in embryonic lethality. Mutant embryos are hyperactive within the eggshell, resulting in a high proportion reversed within the eggshell (the "retroactive" phenotype), and all show poor cuticle integrity when embryos are mechanically devitellinized. This last property results in embryonic cuticle preparations that appear grossly inflated compared to wild-type cuticles (the "blimp" phenotype). We find that one of these genes, *krotzkopf verkehrt* (*kkv*), encodes the Drosophila chitin synthase enzyme and that a closely linked gene, *knickkopf* (*knk*), encodes a novel protein that shows genetic interaction with the Drosophila E-cadherin, *shotgun*. We also demonstrate that two other known mutants, *grainy head* (*grh*) and *retroactive* (*rtv*), show the blimp phenotype when devitellinized, and we describe a new mutation, called *zeppelin* (*zep*), that shows the blimp phenotype but does not produce defects in the head cuticle as the other mutations do.

PATTERNING of the embryonic epidermis in Drosophila has been studied extensively by examining the cuticular pattern elements deposited by epidermal cells late in development. These structures serve as indelible markers of cell fates within the epidermis and have proven invaluable in genetic screens designed to identify mutations that disrupt cell fate decisions (Nüsslein-Volhard and Wieschaus 1980). However, little is known about the deposition of the cuticular layers during late stages of embryogenesis. Here we report the characterization of a set of Drosophila genes required for proper formation of the embryonic cuticle. We have isolated mutations in the Drosophila chitin synthase enzyme and demonstrate that these mutations are allelic with *krotzkopf verkehrt* (*kkv*), a gene identified in the Heidelberg screens for mutations affecting cuticle pattern (Jürgens *et al.* 1984).

kkv mutants show severe disruption of the head cuticle, which was the basis of their isolation in the earlier screen. The genetic screen in which our alleles were isolated involved devitellinizing embryos as well as dechorionating them, and this procedure reveals an additional phenotype associated with mutations in *kkv*. Embryos develop fully and move within the eggshell, but fail to hatch; when the vitelline membrane of these mutant eggs is broken by mechanical pressure, the embryos stretch to several times the size of a wild-type embryo, suggesting that the cuticle is much more elastic. We refer to this very distinctive embryonic phenotype as the "blimp" phenotype. Subsequently we found mutations in four other loci that yield similar blimp phenotypes. These are *knickkopf* (*knk*; Jürgens *et al.* 1984), *grainy head* (*grh*; Nüsslein-Volhard *et al.* 1984), and *retroactive* (*rtv*; Wieschaus *et al.* 1984) from the Heidelberg screens, and *zeppelin* (*zep*), a new mutation identified in our genetic screen.

We believe that further analysis of this collection of genes will provide insight into the process of cuticle formation and the orchestration of interactions between discrete epidermal cells during the formation of the continuous cuticular layers. Morphogenesis of the cuticle defines the structure of the larval body, forming its exoskeleton. This process is interesting not only from a developmental standpoint, but also from an economic one. Disruption of chitin synthesis provides a means of targeting insects without harming humans and other animals. For example, the active ingredient in a popular flea control medication for pets is lufenuron, a potent inhibitor of the chitin synthase enzyme (Dryden *et al.* 1999).

Insect cuticle is comprised of fibrils of chitin, a β1-4 linked polymer of *N*-acetyl-D-glucosamine, embedded in a matrix of proteins and lipids (Cohen 1987). Synthe-

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sis of the chitin chains, catalyzed by chitin synthase, appears to occur intracellularly. Analysis of the sheep blowfly (*Lucila caprina*) chitin synthase suggests an integral membrane protein with the catalytic domain located on the cytosolic face of the plasma membrane (Tellam *et al.* 2000). Thus the polymers must be extruded across the membrane, perhaps through a pore formed by the transmembrane domains of chitin synthase. The transport and deposition of chitin polymers may be the process disrupted by lufenuron and other pesticides in this class of benzoylphenyl ureas. These compounds are ineffective in blocking chitin synthesis in cell-free systems, suggesting that they do not directly inhibit the polymerization step of chitin synthase (Cohen 1985).

Lufenuron has been shown to disrupt molting of Drosophila larvae and to block development of eggs laid by mothers that were fed the pesticide (Wilson and Cryan 1997). We find that when such eggs are dechorionated and mechanically devitellinized, they show a blimp phenotype similar to our mutant embryos. This observation, along with the discovery that *kkv* encodes a chitin synthase, supports the idea that the stretchy cuticle aspect of our blimp phenotypic class results from disruption of the chitin biosynthetic pathway. Further analysis of the set of genes defined by these mutations may give insight into the biochemical and genetic cascade required for proper chitin deposition and cuticle formation during Drosophila embryogenesis.

MATERIALS AND METHODS

Drosophila stocks and culture: Previously described alleles of *kkv*, *knk*, *rtv*, *grh*, and *shg* were obtained from the Bloomington (*kkv*^{1}, *knk*^{1}, *rtv*^{11}, *grh*^{IM}, and *shg*^{2}) and Umea (*kkv*^{2}, *kkv*^{3}, *knk*^{2}, and *knk*^{4}) Stock Centers. All deficiency stocks used in this work were obtained from the Bloomington Stock Center. *l(3)82Fh* alleles were obtained from Adelaide Carpenter (University of Cambridge). *P*-element line *EP(3)3092* was obtained from Exelexis, *EP(3)0974* from Todd Laverty, and the other *P*-element lines described in this work from the Bloomington Stock Center. Data regarding the different alleles of the genes described in this article are summarized in Table 1.

Flies were reared on standard cornmeal-agar-molasses medium unless otherwise noted. For analysis of embryonic stages, eggs were collected on apple juice-agar plates. To examine embryonic cuticles, eggs were allowed to develop fully (24 hr at 25°), dechorionated in bleach, and then transferred to a microscope slide bearing a drop of Hoyer's medium mixed 1:1 with lactic acid (Wieschaus and Nüsslein-Volhard 1986). Mechanical devitellinization was performed by exerting gentle pressure on the coverslip to burst the vitelline membrane. Cuticle preparations were heated at 65° overnight before viewing.

Isolation of mutant alleles: New alleles of *kkv* (*kkv*^{DP14}, *kkv*^{DZ8}, *kkv*^{JH9}, and *kkv*^{LX5}) and the defining allele of *zeppelin* (*zep*^{LP13}) were generated in the course of an EMS mutagenesis designed to recover suppressors of *wingless* (*wg*) mutant phenotypes. This was a standard F_3 lethal screen performed in a *wg* mutant background and involved examining cuticle preparations from individual isogenized lines. Thus recessive mutations

TABLE 1

Summary of blimp class mutations

Gene	Map position	Deficiency	Identity	Homology	Alleles	Allelic strength	Codon change	Protein change
krotzkopf verkehrt	82F3	Df(3R)3-4	Chitin synthase/*CG2666*	*chitin synthase 2* (*CG7464*)	$\frac{kkv^{DZ8}}{kkv^{JH9}}$	Amorph	tgg → tag	W947@
					$\frac{kkv^{LX5}}{kkv^{DP14}}$	Amorph	ggc → gac	G1063D
					$\frac{kkv^{1}}{kkv^{2},kkv^{3}}$	Amorph	ND	ND
					l(3)82Fh	Amorph	ND	ND
						Amorph	ND	ND
knickkopf	85D11	Df(3R)by62	*CG6217*	*Skeletor* (*CG14681*), *CG12492*	*knk*^{1}	Amorph	tgg → tag	W299@
					knk^{2}	Amorph	aag → tag	K656@
					knk^{4}	Amorph	ttg → tag	L631@
zeppelin	81Fab	Df(3R)4-75	Unknown	NA	$\frac{zep^{LP13}}{rtv^{11}}$	Amorph	ND	ND
retroactive	10A7-9		Unknown	NA		Amorph	ND	ND
grainy head	54F1-5		*grainy head*	GATA factor	*grh*^{IM}	Amorph	ND	ND
Unknown	87B12-87E8	Df(3R)ry615	Unknown	NA	NA	NA	NA	NA

Underlined alleles were isolated in the genetic screen described here. All other mutations were provided by the Bloomington or Umea Stock Centers, with the exception of *l(3)82Fh*, which was a gift from Adelaide Carpenter, University of Cambridge.

ND, not determined; NA, not applicable; @, stop codon.

either linked to *wg* on the second chromosome or segregating independently could be assessed for their ability to alter the *wg* mutant phenotype. The unhatched eggs from 5304 independently derived mutagenized lines were examined and 42 lines showing altered cuticle pattern were retained for further analysis. Several mutations suppressed the *wg* phenotype and their characterization revealed new Wingless pathway components (van de Wetering *et al.* 1997; Cavallo *et al.* 1998; McCartney *et al.* 1999; A. Bejsovec, unpublished data). Other unlinked mutations were found that do not modify the *wg* mutant phenotype but that affect the cuticle pattern. Among this class were the five mutations, representing two complementation groups, that generate a blimp phenotype when the embryos are mechanically devitellinized.

Mapping of mutations: The blimp mutations, DZ8 and LP13, were mapped by meiotic recombination against *rucuca*, a multiply marked third chromosome. Both mutations were found to map between *st* and *cu* at an approximate genetic position of 47.0. DZ8 and the other mutations from the screen with which it had been found to be allelic, DP14, JH9, and LX5, disrupt the embryonic head cuticle, as do *kkv* and *knk*, two previously identified embryonic lethal mutations that map close to this position. Therefore, we obtained alleles of these genes from the Bloomington Stock Center to perform complementation tests and found that our *blimp* mutations fail to complement kkv^1, kkv^2, and kkv^3. We also discovered that *knk* shows a blimp phenotype when it is mechanically devitellinized. This aspect of the *kkv* and *knk* phenotypes had been overlooked previously because prior work had not included mechanical devitellinization in the preparation of cuticles.

kkv and *knk* had been erroneously mapped to *Df(3R)Tpl10* and *Df(3R)by10*, respectively (Jürgens *et al.* 1984). We determined that *kkv* maps instead to *Df(3R)3-4*, which removes 82F3-4;82F10-11, and also that it is allelic to *l(3)82Fh* (Carpenter 1999). *knk* maps to *Df(3R)by62*, which removes 85D11-13;85F6. We mapped both loci and the zep^{LP13} mutation more precisely by using *P*-element-mediated male site-specific recombination (Chen *et al.* 1998). *P* elements used for mapping *kkv* were the following: *P{lacW}l(3)L3051*, *P{PZ}l(3)02255*, *P{PZ}l(3)09904*, *EP(3)0974*, *P{PZ}l(3)03644*, and *P{PZ}Itp-r83A*. *P* elements used for mapping *knk* were the following: *P{PZ}l(3)10477*, *P{lacW}l(3)s2681*, *EP(3)3092*, *P{PZ}tws01436*, and *P{PZ}tws02414*. *P* elements used for mapping *zep* were *P{PZ}shk1*, *P{lacW}l(3)L7251*, and *P{lacW}l(3)j4D1*.

kkv and *knk* mutant chromosomes were marked with *st* and *ca*, crossed into a Δ*2-3* background to provide transposase, and placed *in trans* to the various *P*-bearing chromosomes. Males of the proper genotype were crossed back to a strain carrying an independently isolated allele of *kkv* or *knk*, respectively, on a chromosome also marked with *st* and *ca*. Thus only one class of recombinant, either *st* or *ca*, will be recovered in a given cross since *kkv* and *knk* homozygous progeny die as embryos. If *st* recombinants are viable, then the mutation must lie distal to the *P* element, and if *ca* recombinants are viable, the mutation is proximal to the *P* element. We scored ~2000 flies from each cross. For each *P* element tested, we observed at least five recombinants of one class with one or no recombination events of the other class. Rearrangements can occur during the recombination event (Chen *et al.* 1998), with duplications of the wild-type counterpart of the mutant locus producing a recombinant that would otherwise be inviable. Thus, some crosses produced both *st* and *ca* recombinant offspring. However, such rearrangements are rare events; by scoring enough recombinants we were able to determine and exclude the recombinant class that represented the exception.

Plasmid rescue: Genomic sequence flanking the *P* elements used in the male recombination experiments was obtained from the Berkeley *Drosophila* Genome Project, with the exception of *P{PZ}l(3)09904*. We performed plasmid rescue from this strain as described (Wilson *et al.* 1989). Genomic DNA from ~10 flies was digested with *Xba*I and added to a 200-μl ligation reaction. The reaction was phenol/chloroform extracted, ethanol precipitated, and resuspended in 10 μl Tris-EDTA. DH5-α cells (GIBCO-BRL, Gaithersburg, MD) were transformed and plated on Luria broth-kanamycin. DNA was extracted from six resulting colonies and subjected to restriction analysis to verify that all were identical. Two were subsequently chosen for sequencing.

Sequence analysis of mutations: Candidate genes within the intervals defined by the male site-specific recombination experiments were examined for mutational changes between wild-type (Oregon-R) and the mutant stocks. PCR primers were designed using GCG software and tested using the Amplify program (W. R. Engels, University of Wisconsin-Madison). PCRs were performed on genomic DNA, products were purified using Wizard DNA preps (Promega, Madison, WI), and sequencing was performed on an automated sequencer (IBI, New Haven, CT). To enhance the quality of certain sequences, dITP was incorporated into the PCR reaction (Dierick *et al.* 1993). For *kkv*, the following PCR primers were used to amplify a 2-kb fragment that encodes the most highly conserved region of the protein: 5′-tatccatttagagcgcccac-3′ and 5′-tgaacgaggttggagaactg-3′. For *knk*, two sets of primers were used to amplify two overlapping fragments spanning the entire *knk* open reading frame: 5′-agcgaacccgcgaaaaaacc-3′ and 5′-gacccaaaacctgaacgcac-3′ were used to amplify a 1.8-kb fragment containing the proximal part of the *knk* coding region, while 5′-cttggactcaaagaccattc-3′ and 5′-agcatacattctacacacgc-3′ were used to amplify a 1.8-kb fragment containing the distal portion of the *knk* sequence.

Sequence similarity searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.* 1990). Sequence alignments were performed using the ClustalW program (http://www.ebi.ac.uk/clustalw/; Thompson *et al.* 1994). Transmembrane predictions were generated using the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0; Sonnhammer *et al.* 1998) and the TMPRED program (http://www.ch.embnet.org/software/TMPRED_form.html).

Lufenuron treatment: Lufenuron (Sigma, St. Louis/Fluka) was dissolved in 95% ethanol at a stock concentration of 10 mg/ml. Working concentrations ranged from 0.1 to 10 μg/ml and were achieved by diluting into 95% ethanol and adding 100 μl to a standard fly vial containing 5 ml of solidified medium, which had been scored with a spatula to accelerate absorption of the supplement. Control vials were handled the same way but with no lufenuron added to the 95% ethanol supplement.

RESULTS

Characterization of mutations that disrupt embryonic cuticle formation: Mutations in two genes, originally named *blimp* and *zeppelin*, were isolated coincidentally in an F_3 lethal genetic screen for mutations that modify the *wg* mutant phenotype. This screen identified important new components in the Wg/Wnt signaling pathway, such as *dTCF* (van de Wetering *et al.* 1997; Cavallo *et al.* 1998) and *dAPC2* (McCartney *et al.* 1999). However, the screen also yielded several mutations that did not suppress *wg* but that did alter the appearance of the embryonic cuticles. Homozygous mutant embryos fail to hatch but are initially hyperactive, often reversing

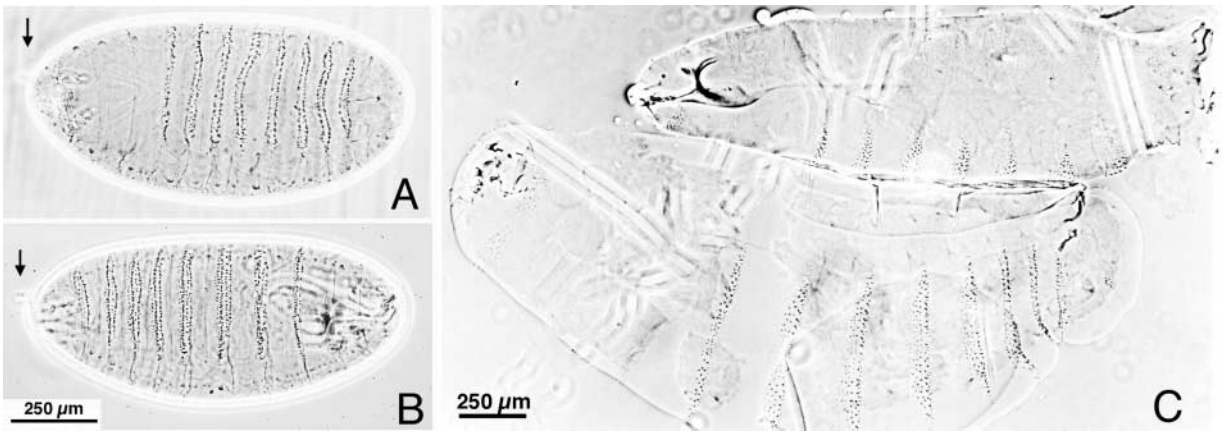

FIGURE 1.—The "retroactive" and blimp phenotypes. (A) *kkv* homozygous mutant embryo oriented correctly within the eggshell, with anterior to the left. Arrow points to the micropyle, a specialization of the eggshell that marks the anterior of the egg. (B) *zep* homozygous mutant embryo showing reversed orientation. The micropyle of the eggshell is to the left (arrow), but anterior head structures in the embryo are to the right. Note that the head structures of the *zep* embryo appear normal, whereas the *kkv* mutant head skeleton is degenerated. (C) A devitellinized cuticle preparation of embryos from a kkv^{DZ8} stock shows the blimp phenotype of a *kkv* homozygote (bottom) compared with a wild-type *kkv*/+ heterozygous sibling (top).

themselves within the eggshell (Figure 1, A and B). When the mutant embryos are mechanically devitellinized, the resulting cuticle preparations stretch to a much greater extent than do wild-type cuticles (Figure 1C and Figure 2). We refer to the inflated appearance of these embryonic cuticles as the blimp phenotype, and this is the source of the gene names. Both genes were mapped meiotically against the *rucuca* multiply marked chromosome and found to lie between *st* and *cu*, roughly at genetic map position 47.0.

In addition to the blimp mutant phenotype, all four independently isolated mutant alleles of *blimp* show a defect in the embryonic head skeleton and slight denticle belt abnormalities (Figure 1C and Figure 2, A and C). Two previously identified genes, *kkv* and *knk*, had been mapped to genetic map positions 47.5 and 49.1,

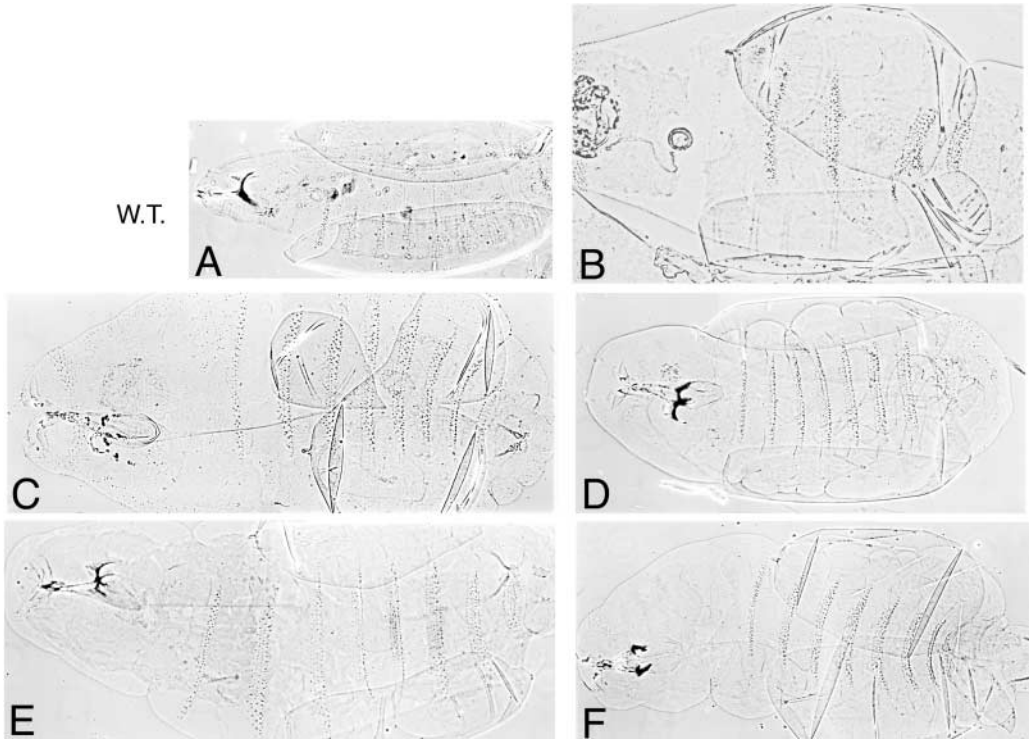

FIGURE 2.—The blimp class of mutations. (A) A wild-type mechanically devitellinized cuticle preparation shows the normal extent to which the embryonic cuticle is flattened by a coverslip. (B) An embryo derived from a wild-type female that has been fed on lufenuron-treated food shows much greater stretching of the cuticle after devitellinization, comparable to the kkv^{DZ8} mutant phenotype (C). Defects in the head skeleton of the lufenuron-treated specimen also resemble those observed in the *kkv* mutant embryo. (D) knk^{1} mutant embryos also show cuticle stretching although the head skeleton defects are milder. (E) zep^{LP13} mutant embryos show stretching to the same extent as *kkv* mutant embryos, but the head skeleton is much more wild type in appearance. rtv^{11} mutant embryos (F) and grh^{IM} embryos (not shown) also show excessive stretching when flattened under a coverslip. The degree of cuticle stretching is somewhat variable between preparations and so is not as accurate a measure of allelic severity as is degree of head cuticle disruption. A–F are at the same scale.

TABLE 2

Frequency of the retroactive phenotype in blimp class mutant stocks compared to wild type (Oregon-R)

Genotype	Total no. of mutant embryos	No. of reversed mutant embryos	% of mutant embryos showing reversal
rtv^{11}	95	9	8.9
kkv^{DZ8}	89	7	7.8
knk^{1}	85	2	2.4
zep^{LP13}	75	20	26.6
OreR	625	0	0

respectively, and both produce defects in the embryonic head skeleton when mutated. Consequently we tested both mutations for failure to complement our *blimp* and *zeppelin* mutations. All four of our *blimp* alleles fail to complement *kkv*, indicating that they are new mutations in this known gene. However, our *zeppelin* mutation complements both *knk* and *kkv*, and therefore *zep* represents a new gene.

In the course of our complementation tests, we discovered that the original *kkv* and *knk* mutations produce a blimp phenotype when the embryos are mechanically devitellinized (Figure 2D). The genetic screens in which these alleles were isolated (Jürgens *et al.* 1984) did not involve mechanical devitellinization of the cuticle preparations, and so the inflated-cuticle aspect of their phenotypes had been missed. For this reason, we reexamined other mutations from these screens, which had been described as having defects in the embryonic head skeleton. We found that in addition to *kkv* and *knk*, *rtv* and *grh* produce blimp phenotypes (Figure 2F and Table 1). Thus we have defined five different genes that are required for normal cuticle integrity.

The *retroactive* gene, as the name suggests, was also noted to show reversal within the eggshell. We find that *kkv* embryos show roughly the same frequency of reversal as do *retroactive* embryos: ~8–9% are reversed relative to wild type (Table 2). *knk* mutants show a lower frequency of reversal, ~2%, whereas *zeppelin* mutants show a far greater frequency of reversal, approaching 27% (Table 3). This phenotype probably reflects the increased elasticity of the cuticle, allowing the mutant embryos to move more freely within the confines of the eggshell than do wild-type embryos.

The cuticle defects, particularly the disruption of the head skeleton, are most severe in *kkv* and *grh* mutants. All alleles of *kkv*, both those isolated previously and those identified in this screen, produce similar phenotypes. When removed from the vitelline membrane, *kkv* and *grh* mutant embryos are very flaccid and are not motile although they are able to contract their body wall muscles. All three alleles of *knk* and the one available allele of *rtv* produce milder defects in the head skeleton and denticle belts. When removed from the vitelline membrane they are more robust than the *kkv* and *grh* mutants, and they are motile but die within hours after removal from the eggshell. The head skeleton and denticle belts of *zep* mutants are almost wild type (Figure 2, A and E) and these embryos are sometimes able to hatch on their own, although they die at roughly the same stage as the *knk* and *rtv* mutants. The degree of cuticle expansion can vary among cuticle preparations due to uncontrollable differences in the mechanical devitellinization process. However, the severity of head defects, flaccidity, and motility are consistent within the alleles of each complementation group. Thus the phenotypic effects of the blimp class mutations can be ranked from most to least severe: *kkv*, *grh* > *rtv*, *knk* > *zep*.

We suspect that at least one more gene may be associated with a blimp phenotype. Embryos homozygous for the small deficiency *Df(3R)ry615*, which deletes cytological positions 87B12–87E8, show a phenotype similar to that of *zep*. Thus a sixth gene involved in cuticle formation may reside within this interval.

Mapping mutations that produce the blimp phenotype: We chose to focus our further analysis on *kkv*, *knk*, and *zep*, since these three genes lie close together on the third chromosome. First, we refined our mapping by analyzing deficiencies from the Bloomington Stock Center. We find that *kkv* fails to complement *Df(3R)3-4* (82F3-4;82F10-11) but does complement the overlapping deficiency *Df(3R)ME15* (81F3-6;82F5-7). This narrowed down the interval where *kkv* maps to between 82F5 and 82F11 (Figure 3), contradicting earlier work that had placed it between 83C1 and 84B2 (Jürgens *et*

TABLE 3

Genetic interaction between *shotgun* and blimp class mutations

Cross	Total no. of mutant embryos	No. of mutant embryos showing fragmented cuticle	% of mutant embryos showing fragmented cuticle
shg^{1}/+;*rtv*^{11}/+ males × *rtv*^{11}/*TM3* females	102	7	7
shg^{1}/+;*kkv*^{DZ8}/+ males × *kkv*^{DZ8}/*TM3* females	99	4	4
shg^{1}/+;*knk*^{1}/+ males × *knk*^{1}/*TM3* females	114	42	37
shg^{1}/+;*zep*^{LP13}/+ males × *zep*^{LP13}/*TM3* females	123	39	31

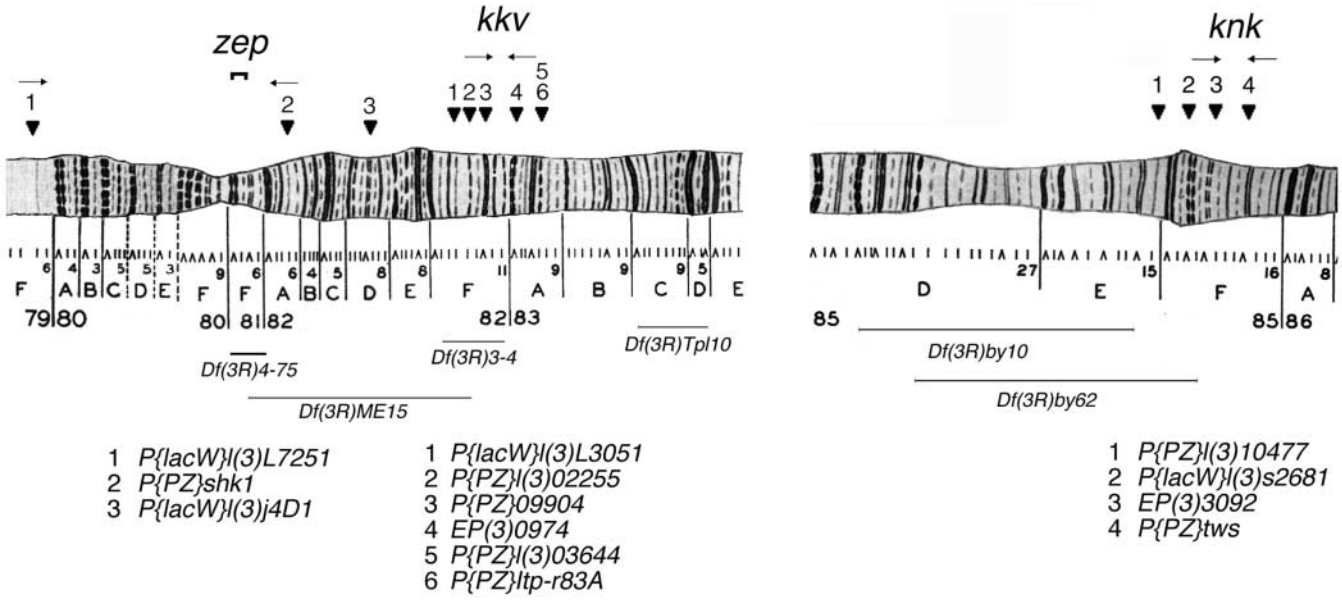

FIGURE 3.—Genetic mapping of *kkv*, *knk*, and *zep*. All three genes map to the third chromosome near the centromere. *zep* (left) appears to be located within the centromeric heterochromatin, as it maps within the interval deleted by *Df(3R)4-75* (81Fab) and is not included within the interval deleted by *Df(3R)ME15* (81F3-6;82F5-7). *kkv* (middle) lies within the interval deleted by *Df(3R)3-4* (82F3-4;82F10-11) and has been positioned between *P{PZ}09904* (82F8-9) and *EP(3)0974* (83A1-2). *knk* (right) maps to the interval deleted in *Df(3R)by62* (85D11-13;85F6) but is not included in the interval deleted in *Df(3R)by10* (85D8;85E10-13). It has been more precisely positioned between *EP(3)3092* (which is erroneously assigned to 87C8-9 by the Berkeley *Drosophila* Genome Project) and two *P* insertions at 85F12-13, *P{PZ}tws*^{01436} and *P{PZ}tws*^{02414}.

al. 1984). Likewise, we have determined a new cytological position for *knk*: *knk* fails to complement *Df(3R)by62* (85D11-13;85F6) but it complements *Df(3R)by10* (85D8; 85E10-13), the deficiency to which it had been previously assigned (JÜRGENS *et al.* 1984). Thus its new interval is 85E10–85F6 (Figure 3). *zep* fails to complement *Df(3R)4-75*, a small deficiency within the centromeric heterochromatin (81Fab). In all cases, the phenotypes generated by the mutation *in trans* to the noncomplementing deficiency are indistinguishable from the homozygous mutant phenotype. Therefore our new alleles of *kkv*, *kkv*^{DP14}, *kkv*^{DZ8}, *kkv*^{JH9}, and *kkv*^{LX5}, and of *zep*, *zep*^{LP13}, as well as the previously isolated allele of *knk*, *knk*^{1}, are likely to represent null mutations at these loci.

To correlate these cytological positions with the molecular map, we used male site-specific recombination to place our genes with respect to *P*-element insertions within each interval. Although meiotic recombination does not occur in Drosophila males, crossing over can be induced at the position of *P*-element insertions when transposase is present in the male germline (PRESTON and ENGELS 1996). This observation has been adapted for mapping mutations rapidly at molecular resolution, since *P*-element insertions can be positioned on the genomic scaffold by performing plasmid rescue techniques and analyzing the recovered flanking DNA (CHEN *et al.* 1998).

Unfortunately, the heterochromatic region where *zep* is located has not yet been sequenced and so we have not yet identified the gene responsible for this mutant phenotype. However, both *kkv* and *knk* lie within characterized regions of the genome and we were able to identify the transcription units corresponding to these genetic loci.

Molecular analysis of *kkv*, a chitin synthase gene: We determined that *kkv* lies distal to *P{PZ}l(3)09904* (82F8-9) and proximal to *EP(3)0974* (83A1-2; Figure 3). We identified the genomic sequence flanking *P{PZ}l(3)09904* by sequencing a plasmid rescue fragment, and we obtained genomic sequences flanking *EP(3)0974* from the Berkeley *Drosophila* Genome Project. By comparing these sequences with the Drosophila genome sequence (ADAMS *et al.* 2000), we refined the location of *kkv* to a 40-kb interval containing nine predicted genes. Within this region we identified a candidate gene that could explain the elastic cuticle phenotype behind the blimp phenotype: a predicted transcript, *CG2666*, encoding a putative chitin synthase.

Chitin synthase genes have been identified not only in sheep blowfly (TELLAM *et al.* 2000) and mosquito (IBRAHIM *et al.* 2000), but also in *Saccharomyces cerevisiae* as chitin is a major component of the fungal cell wall (CABIB *et al.* 2001). All of these enzymes share two conserved domains (Figure 4 and Figure 5, A and B), referred to as con1 and con2, each of which contains several invariant residues essential for the catalytic activity of the yeast chitin synthase (NAGAHASHI *et al.* 1995; YABE *et al.* 1998). We performed sequence analysis on our *kkv* mutants, concentrating on a 2-kb exon of

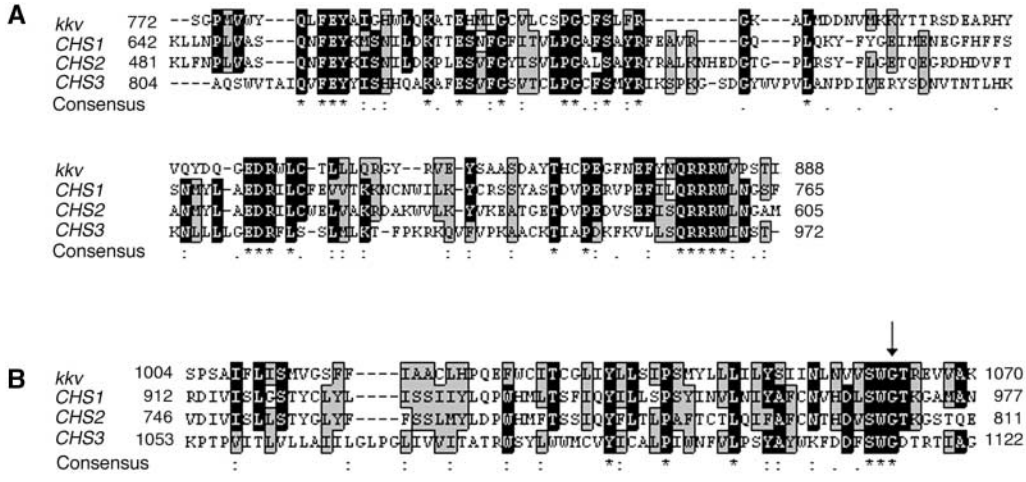

FIGURE 4.—Comparison of conserved domains from *kkv* and yeast chitin synthases. (A) Alignment of the con1 domain from *kkv* and from the three *S. cerevisiae* chitin synthases, *CHS1*, *CHS2*, and *CHS3*. This domain is highly conserved among all chitin synthases and contains several clusters of invariant residues. (B) Alignment of the con2 domain from *kkv* and the yeast *CHS1*, *CHS2*, and *CHS3*. Although this domain is less highly conserved than con1 overall, there are several invariant residues, including the C-terminal SWG motif that is required for catalytic activity. kkv^{DZ8} introduces a stop codon at Trp 947, so that the protein is truncated before the con2 domain, and kkv^{JH9} changes an invariant glycine within the SWG motif (arrow) to an aspartic acid.

CG2666 that contains the con1 and con2 domains of this fly chitin synthase. In our kkv^{DZ8} allele, we detected a mutation that changes tryptophan 947 of the predicted amino acid sequence to a stop codon (tgg → tag). This would result in a truncated protein that lacks the con2 domain. The kkv^{JH9} allele contains a mutation that converts glycine 1063 to aspartate (ggc → gcc). This substitution disrupts an invariant SWG motif in the con2 domain (Figure 4). Thus both of these mutations would be expected to abolish catalytic activity of the chitin synthase enzyme. We also examined the sequence spanning the con1 and con2 domains from the existing *kkv* alleles kkv^{1}, kkv^{2}, and kkv^{3}, but were unable to find any nucleotide changes within this region. Presumably the genetic lesions in these alleles lie elsewhere in the gene, either 5′ or 3′ to the highly conserved region examined (Figure 5A).

The gene annotation for *CG2666* released by the Berkeley *Drosophila* Genome Project predicts a chitin synthase enzyme, but was not accurate with regard to the predicted transcript. To determine the correct *kkv* gene structure, we performed a sequence similarity search against the protein database (ALTSCHUL *et al.* 1990). Using regions of high homology with other chitin synthases, we deduced the proper coding regions for *kkv* from the genomic sequence available from the Berkeley *Drosophila* Genome Project. As expected, *kkv* shows sequence similarity to a large number of chitin synthases from various species, with highest similarity to *Caenorhabditis elegans*, mosquito, sheep blowfly, and a second Drosophila gene, *CG7464* (Figure 5C). We used two different computer programs, TMHMM and TMPRED, to determine the topology of the predicted multiple transmembrane domains. As with the sheep blowfly chitin synthase, the *kkv* sequence appears to have 17–18 transmembrane domains oriented such that the catalytic domains of the enzyme face the cytosol.

Molecular characterization of *knk*, a novel protein gene: We determined that *knk* lies distal to the *P*-element insertion *EP(3)3092* and proximal to *P{PZ} tws01436* and *P{PZ} tws02414* (Figure 3). Genomic sequences from these insertions were obtained from the Berkeley *Drosophila* Genome Project, and they were found to flank a 20-kb interval containing three predicted transcription units. We performed sequence analysis on the three existing *knk* mutations for all three candidate genes: *CG6217*, *CG6208*, and *CG3940*. All three *knk* alleles carry nonsense mutations within the predicted reading frame of *CG6217* (Figure 6A). knk^{1} converts tryptophan 299 to a stop codon (tgg → tag), knk^{2} changes lysine 656 to a stop codon (aag → tag), and knk^{4} mutates leucine 631 to a stop codon (ttg → tag). None of the mutant stocks showed significant nucleotide changes in the other two genes. Therefore we propose that *CG6217* represents the *knk* gene.

The transcription start site has not been defined for *CG6217/knk*, and so we do not know if the entire 5′ untranslated region is included within the first exon predicted for the gene. The gene spans 2695 bp and consists of six exons, with conceptual translation yielding a protein of 689 amino acids. The protein sequence shows similarity to two other Drosophila genes: *Skeletor* and *CG12492* (Figure 6B). *Skeletor* encodes a protein thought to be a component of the nuclear spindle matrix, but its true function is not known as no mutant alleles have yet been isolated (WALKER *et al.* 2000). *CG12492* is a predicted gene of unknown function. Thus it is not obvious from molecular analyses what function *knk* may play in the process of embryonic cuticle formation.

We believe that other loci in the fly genome are also involved in cuticle integrity. Our genetic screen was not designed specifically to recover blimp class phenotypes, and so our isolation of new *kkv* alleles was purely fortu-

itous. Since we did not recover *knk* alleles, it is clear that our small-scale screen did not approach saturation for this type of mutant phenotype. Furthermore, in the course of our deficiency analysis of the blimp mutations, we found a deficiency, *Df(3R)ry516*, which complements *kkv*, *knk*, and *zep*, but that produces a blimp phenotype when homozygous. This suggests that a blimp class gene resides within the interval defined by the breakpoints of this deficiency, cytological positions 87B12–87E8. The Drosophila genome annotations place the *knk* homologs, *Skeletor* at 86C2 and *CG12492* at 95C5-7, as well as the second fly chitin synthase *CG7464* at 79B2-3, outside of this cytological region. However, it is worth noting that determining the exact breakpoints of deficiencies by cytology is difficult and that *Skeletor* in particular might be the gene responsible for the phenotype associated with this deficiency. In the absence of mutations in *Skeletor*, we cannot eliminate this possibility.

Finally, *knk* function does not appear to be additive with *kkv* function; *knk kkv* double-mutant embryos show a phenotype indistinguishable from the *kkv* single-mutant embryos (data not shown). Since *kkv* is epistatic, *knk* appears to function upstream of chitin deposition in the cuticle formation process. Alternatively, *knk* may act parallel to the *kkv* chitin synthase in a pathway that independently influences chitin deposition. Although *kkv* mRNA can be detected in the epidermis of late-stage embryos (not shown), *knk* mRNA was not detected at significant levels at any stage of embryogenesis, using standard digoxygenin whole mount *in situ* hybridization protocols. This suggests that very low levels of gene product are sufficient to mediate wild-type *knk* gene activity.

Lufenuron treatment produces a *kkv* phenocopy: Disruption of chitin synthesis by treatment with lufenuron, a potent inhibitor, kills Drosophila larvae during ecdysis to the next larval instar (Wilson and Cryan 1997). Feeding lufenuron to mature adult Drosophila does not affect the health of the individuals but does disrupt the development of eggs laid by treated females. The embryos were reported to show hyperactivity within the eggshell but fail to hatch from the egg perhaps because of defects in the head skeleton. We repeated these experiments to determine if the embryos would also show a blimp phenotype when mechanically devitellinized. We find that cuticle preparations of embryos from lufenuron-treated mothers are very similar in appearance to those of *kkv* mutant embryos (Figure 2, A–C). Thus inhibition of chitin synthesis by artificial means produces a phenotype like that of loss-of-function mutations in a chitin synthase gene. We presume that lufenuron fed to the mothers is incorporated into the eggs for inhibition of chitin synthase to occur in the embryo.

Genetic interactions with *shotgun*, the Drosophila E-cadherin: The identification of *kkv* as a chitin synthase and the ability of a chitin synthesis inhibitor to phenocopy *kkv* shows that disrupting synthesis or deposition

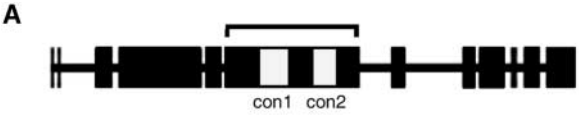

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FIGURE 5.—Predicted gene structure and amino acid sequence of *kkv*. (A) Schematic diagram of predicted intron-exon structure for the *kkv* genomic region and the relative positions of con1 and con2, the two protein domains that are highly conserved among all chitin synthases. The 2-kb region that was examined for mutations is indicated by a bracket above the diagram. (B) Predicted amino acid sequence of the complete *kkv* protein. This differs from the sequence available from the Berkeley *Drosophila* Genome Project. However, similar conclusions about the *chitin synthase-1* gene structure were reached by R. Tellam and are cited in Tellam *et al.* (2000) as well as in a Flybase error report for *CG2666*, dated July 2000, at http://flybase.bio.indiana.edu/.bin/fbidq.html?FBrf0129248. The con1 domain is underscored and the con2 domain is doubly underscored. (C) ClustalW alignment of *kkv* sequence with other insect chitin synthases: the sheep blowfly, *L. caprina*, and the mosquito, *A. aegypti*, as well as a second chitin synthase found in the Drosophila genome, *CG7464*. *kkv* shows greater similarity to the enzyme from *L. caprina* than to the second Drosophila chitin synthase.

of chitin alone can account for the blimp phenotype. However, we believe that two of the blimp class genes, *knk* and *zep*, may function in the epidermis prior to cuticle deposition because both interact genetically with mutations in the Drosophila E-cadherin, encoded by *shotgun* (*shg*).

shg is provided both maternally and zygotically and is required for oogenesis as well as for subsequent embryonic development (Tepass *et al.* 1996; Godt and Tepass 1998). Zygotic loss of *shg* function produces defects only in those tissues that undergo dramatic morphogenesis, indicating that maternal *shg* product pro-

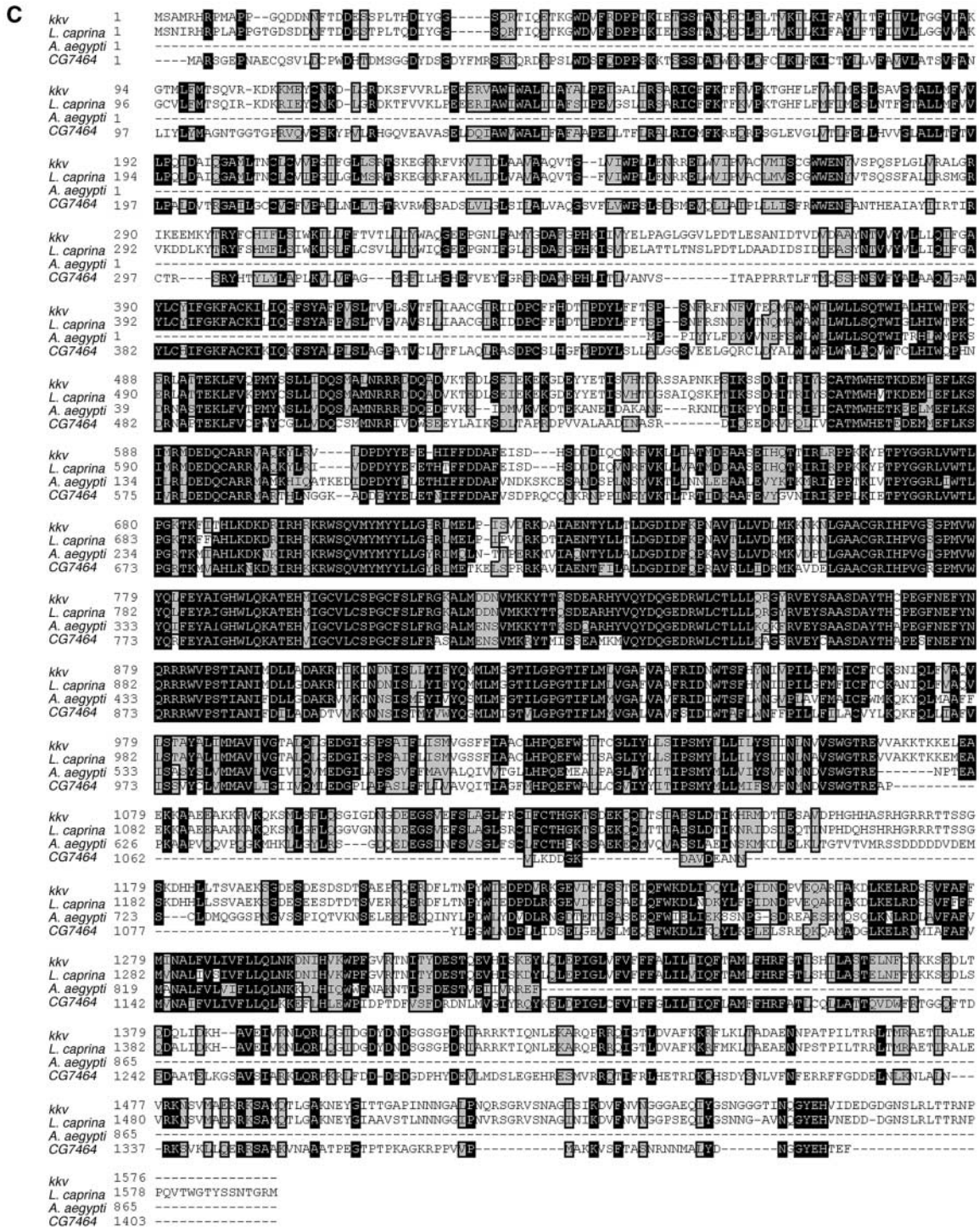

FIGURE 5.—*Continued.*

vides sufficient cadherin-mediated adhesion for most embryonic epithelia but not for those subject to mechanical stress. The embryonic ventral epidermis undergoes substantial cell rearrangements and degenerates in *shg* mutant embryos, resulting in a fragmented cuticle (NÜSSLEIN-VOLHARD *et al.* 1984). *shg* loss-of-function mutations are completely recessive, but in embryos homozygous for either *knk* or *zep*, heterozygosity for *shg* leads to a fragmented cuticle phenotype similar to that of a *shg* homozygote (Table 2). This effect is not observed for *kkv*, *rtv*, or *grh*, the other blimp class genes. Thus *knk* and *zep* seem likely to encode proteins that are more broadly involved in epidermal development. In the absence of these gene functions, a single gene dose of *shg* becomes inadequate for epithelial cell adhesion in the ventral epidermis.

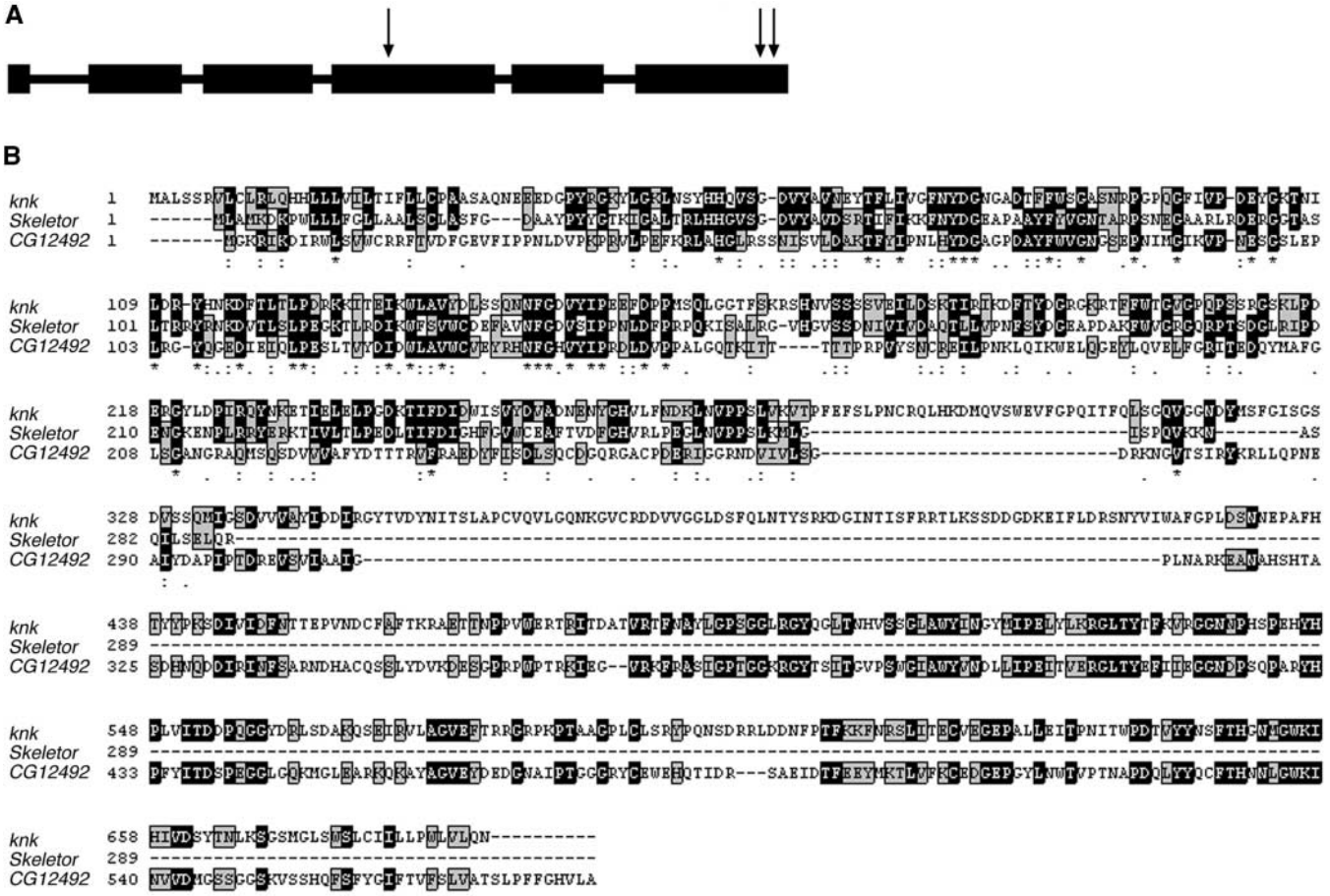

FIGURE 6.—Predicted gene structure and amino acid sequence of *knk*. (A) Schematic diagram of predicted intron-exon structure for the *knk* genomic region. Arrows mark the positions of nonsense mutations found in *knk* mutant strains. knk^{1} changes the Trp 299 codon from UGG to UAG. Interestingly, both knk^{2} and knk^{4} are base transversions rather than the more typical transitions generated by EMS: knk^{2} changes the Lys 655 codon from AAG to UAG, and knk^{4} changes the Leu 632 codon from UUG to UAG. (B) ClustalW alignment of the *knk* (*CG6217*) gene product (protein product accession no. AAF54497) and the two most closely related Drosophila proteins, encoded by *Skeletor* (*CG14681*) and *CG12492*.

The epidermal tissue of the blimp class mutants appears structurally indistinguishable from that of wild-type embryos. Epidermal cell membrane-associated proteins such as Coracle (FEHON *et al.* 1994), Armadillo (PEIFER *et al.* 1991), and dAPC2 (MCCARTNEY *et al.* 1999) show normal localization and reveal no discernible difference between mutant and wild-type embryos in cell size, shape, or number (data not shown). Thus it remains to be determined what aspect of epidermal cell function is disrupted in *knk* and *zep* mutant embryos.

DISCUSSION

We have defined a set of genes that are required for proper development of the Drosophila embryonic cuticle. Mutations in these genes are zygotic lethal and result in flaccid embryos with very elastic cuticles that stretch to a remarkable degree when flattened beneath a coverslip. Three of the genes associated with this blimp phenotype, *kkv*, *knk*, and *zep*, map to a small region near the centromere of the third chromosome, but since at least two appear to be structurally unrelated, they do not appear to represent a gene cluster. We have determined the molecular identities of *kkv* and *knk*, with *kkv* encoding a chitin synthase enzyme and *knk* encoding a novel protein of unknown function.

The blimp phenotype can be accounted for by a failure of the epidermal cells to deposit cuticle properly. Loss-of-function mutations in *kkv*, the chitin synthase, or inhibition of chitin synthesis with the benzoylphenyl urea, lufenuron, both produce this stretchy cuticle effect. A second Drosophila chitin synthase, encoded by *CG7464*, has not been characterized genetically and so we do not know if it participates in formation of the embryonic cuticle. The chitin synthases in yeast, *CHS1*, *-2*, and *-3*, each perform a distinct function in cell wall formation: *CHS1* repairs damaged chitin during cell separation, *CHS2* is required for primary septum formation, and *CHS3* is involved in all other chitin synthesis (CABIB *et al.* 2001). Specialization among the insect

chitin synthases is likely as well; the enzyme isolated from the mosquito *Aedes aegypti* appears to be specific for the midgut epithelium, where it contributes to the peritrophic matrix that surrounds the blood bolus after a meal (Ibrahim *et al.* 2000). The authors postulate that a second as-yet-unidentified chitin synthase in *A. aegypti* directs cuticular synthesis of chitin. In contrast, in the sheep blowfly, *L. caprina*, chitin synthase is expressed in larval epidermal cells and so is likely to be involved in formation of the exoskeleton (Tellam *et al.* 2000).

Another blimp class gene, *grainy head*, was previously known to affect head skeleton and embryonic cuticle (Bray and Kafatos 1991). *grh* encodes a GATA family transcription factor and activates the transcription of a number of genes during development, one of which is *Dopa-decarboxylase* (*Ddc*). This enzyme is synthesized in the cuticle-secreting layer of cells at the end of embryogenesis; the dopamine produced undergoes further metabolism and oxidation to produce quinones that crosslink cuticular proteins (Scholnick *et al.* 1983). Thus, loss of *grh* function would result in weakening of the cuticle indirectly through its failure to activate *Ddc* expression.

The contribution of the remaining blimp class genes to cuticle formation is not clear. *rtv* and *zep* remain uncharacterized at the molecular level; we have characterized *knk*, but its molecular identity does not provide insight into its possible role. We suspect that further analysis of *knk* and *zep* may reveal a function within the epidermal cell layer that involves cell-cell adhesion, because their loss of function sensitizes the embryo to reduction in zygotic DE-cadherin dose. This could indicate that *knk* and *zep* have a general role in maintaining epidermal integrity, which indirectly affects cuticle deposition. Since *kkv* is epistatic to *knk*, *knk* could function upstream of the chitin synthase, for example, by localizing the enzyme to the correct membrane domain. Alternatively, *knk* and *zep* may be directly involved in the extrusion or assembly of cuticle matrix components, and disruption of this process in mutant embryos places abnormal stress on the adherens junctions that link epidermal cells together. In any case, further study of these two genes may shed light on the interplay between the epidermal epithelium and the overlying cuticular layers that it must construct during late stages of embryogenesis.

These late-stage events appear to be coordinated by the steroid hormone, 20-hydroxyecdysone. Genes that regulate ecdysone levels in the embryo influence both epidermal integrity and cuticle deposition (Chavez *et al.* 2000). However, mutations in ecdysone regulatory genes produce a broader spectrum of defects than do the blimp class mutations, including severe disruption of morphogenetic movements throughout embryogenesis. In contrast, *knk*, *zep*, and the other blimp class mutations do not disrupt early stages of development and these mutant embryos have normal morphology. Thus, the blimp class genes may be a subset of the targets of ecdysone regulation during late stages of embryogenesis, where they orchestrate the final stages of epidermal development and cuticle secretion.

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