

The *Drosophila* *dCREB-A* gene is required for dorsal/ventral patterning of the larval cuticle

Deborah J. Andrew^{1,*}, Anjum Baig¹, Purnima Bhanot^{1,†}, Sarah M. Smolik² and Katya D. Henderson¹

¹Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, Maryland 21205-2196, USA

²Vollum Institute for Advanced Biomedical Research, Oregon Health Science University L-474, 3183 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3098, USA

*Author for correspondence (e-mail: debbie_andrew@mail.bs.jhu.edu)

†Present address: Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, Maryland 21205-2196, USA

SUMMARY

We report on the characterization of the first loss-of-function mutation in a *Drosophila* CREB gene, *dCREB-A*. In the epidermis, *dCREB-A* is required for patterning cuticular structures on both dorsal and ventral surfaces since *dCREB-A* mutant larvae have only lateral structures around the entire circumference of each segment. Based on results from epistasis tests with known dorsal/ventral patterning genes, we propose that *dCREB-A* encodes a transcription factor that functions near the end of both the

DPP- and SPI-signaling cascades to translate the corresponding extracellular signals into changes in gene expression. The lateralizing phenotype of *dCREB-A* mutants reveals a much broader function for CREB proteins than previously thought.

Key words: cuticle differentiation, *d-CREBA*, *Drosophila melanogaster*, dorsal/ventral patterning, epithelium, salivary gland

INTRODUCTION

The CREB family of proteins are DNA-binding transcription factors that respond to extracellular signals (reviewed by Lee and Masson, 1993; Meyer and Habener, 1993). The family is named for the first member to be identified, Cyclic AMP Response Element Binding protein (CREB), which binds to DNA sequences required for the transcriptional response to increased intracellular cAMP levels. All CREB-related proteins contain a 'basic leucine zipper' domain (Dwarki et al., 1990) which mediates DNA binding and the formation of both homo- and heterodimers. CREB proteins bind as dimers to the palindromic consensus sequence TGACGTCA (Lin and Green, 1988). The ability of many CREB family members to activate transcription is regulated by phosphorylation events controlled by intracellular second messengers (reviewed by Meyer and Habener, 1993; Quinn, 1993). The phosphorylation state of various CREB proteins is regulated by several different kinases including the cAMP-dependent protein kinase A (PKA) (Gonzalez et al., 1989), calmodulin-dependent kinases (CaM kinases I and II) (Sheng et al., 1991), a Ras-dependent protein kinase, RSK2 (Ginty et al., 1994; Xing et al., 1996), and the p70 S6 kinase (de Groot et al., 1994), as well as the PP-1 phosphatase (Hagiwara et al., 1992; reviewed by Hunter, 1995). A protein known as CREB Binding Protein (CBP) binds only to the phosphorylated form of CREB (Chrivia et al., 1993; Kwok et al., 1994; Lundblat et al., 1995). Therefore, CBP is molecularly positioned to trigger transcriptional activation

after CREB binds DNA and is phosphorylated. Indeed, CBP also binds TFIIB, potentially linking CREB to the RNA polymerase II preinitiation complex (Chrivia et al., 1993; Kwok et al., 1994).

Different CREB family members have been implicated in a wide variety of activities including receptor-mediated induction of *fos* transcription (Sassone-Corsi et al., 1988; Boutillier et al., 1992; Peunova and Enikolopov, 1993; Ginty et al., 1994), virally induced transcription of the interferon β gene (Du and Maniatis, 1992), transcriptional regulation of various hormones and transcription factors (Montminy et al., 1986; Comb et al., 1986; Delegeane et al., 1987; McCormick et al., 1990), thymocyte activation (Barton et al., 1996), transcriptional response to circadian rhythms (Ginty et al., 1993; Stehle et al., 1993), consolidation of long-term memory in *Aplysia* (Dash et al., 1990) and *Drosophila* (Yin et al., 1994), both consolidation and repression of long-term memory in mice (Bourtchuladze et al., 1994; Goda, 1995; Bartsch et al., 1995), and male fertility in mice (Nantel et al., 1996; Blendy et al., 1996a). Many of these CREB activities had been based on experiments in cell culture and only in the past few years have studies of CREB function in animals been initiated.

The first experiments looking at CREB function in vivo were based on expressing dominant negative forms of CREB. The most-studied family member, CREB itself, is regulated by cAMP-dependent phosphorylation of Ser-133 by PKA. A growth hormone promoter was used to overexpress mutant

non-phosphorylatable CREB (Ser-133-Ala) in the pituitary gland (Struthers et al., 1991). Although this study did not control for the effects of overexpressing wild-type CREB, mice transformed with this construct were on average smaller than control animals. The dwarfed mice also had fewer somatotrophic cells in their pituitary glands, suggesting that wild-type CREB is required to activate genes needed for somatotrophs to proliferate. Early studies in cultured *Aplysia* neurons implicate CREB as a mediator of long-term potentiation (LTP) (Dash et al., 1990). Consistent with a role in LTP, the overexpression of a nonphosphorylatable CREB protein in *Drosophila* causes the loss of long-term memory (Yin et al., 1994) and overexpression of an activatable form enhances long-term memory (Yin et al., 1995).

In both mice and *Drosophila*, different members of the CREB family are expressed dynamically in various tissues (Lee and Masson, 1993; Smolik et al., 1992; Usui et al., 1993; Andrew et al., 1994). Studies that rely on overexpressing dominant negative proteins are therefore inherently flawed since each CREB family member can bind to and potentially regulate many different genes containing CRE elements. Thus, the overexpression of the dominant negative form of any given CREB protein may inhibit the function of other CREB family members by blocking their binding sites or titrating their regulators. To understand the role of specific CREB proteins, it is essential to study simple loss-of-function mutations in individual CREB genes. Then, starting with the loss-of-function phenotypes for the single genes, one can genetically identify other proteins that interact with specific CREBs to determine their *in vivo* pathways. For example, a loss-of-function CREB mutant in mice supports a role for this particular CREB protein in LTP (Bourtchuladze et al., 1994) but does not yet indicate a requirement for this CREB protein in controlling somatotroph cell proliferation (Hummler et al., 1994). Recent results suggest that this particular CREB mutant is not a complete loss-of-function mutation and that other isoforms, as well as other CREB-related proteins, may be upregulated to compensate for this mutation (Hummler et al., 1994; Blendy et al., 1996b). Knockout mutations in a mouse CREB family member, known as CREM (CAMP-Responsive Element Modulator), have specific effects on male fertility with both hetero- and homozygotes affected (Nantel et al., 1996; Blendy et al., 1996a). Heterozygous males have reduced fertility and homozygous males are completely sterile, due to a block at a specific stage of spermatogenesis.

In *Drosophila*, the genes encoding two CREB family members have been cloned, *dCREB-A* and *dCREB-B*. The *dCREB-B* gene is the more similar of the two *Drosophila* genes than to the mammalian *CREB* and *CREM* genes. *dCREB-B* contains the consensus sites for cAMP-dependent PKA phosphorylation and shows PKA-responsive transcriptional activation (Usui et al., 1993; Yin et al., 1995). *dCREB-A* is less homologous to the mammalian CREBs; it does not contain a PKA consensus phosphorylation site and its transcriptional activity is only mildly enhanced by cAMP (Abel et al., 1992; Smolik et al., 1992). Both proteins bind the CRE consensus site. Here, we report the characterization of loss-of-function mutations in the *Drosophila dCREB-A* gene. *dCREB-A* is expressed in several embryonic tissues including the salivary gland and epidermis, structures affected by loss-of-function mutations in the gene. Our analysis places *dCREB-A* near the

end of two signaling cascades controlling dorsal/ventral patterning in the epidermis, an unexpected finding that may have implications for similar pathways in vertebrates.

MATERIALS AND METHODS

Fly stocks

Lines *B204* and *l(3)3576* are from the enhancer-trap P element stock collections of the G.Rubin/C. Goodman and A. Spradling laboratories, respectively. Both enhancer-trap stocks were identified in a screen for embryonic salivary gland-expressing lines (Andrew et al., 1994). The inserts in both lines were mapped to the *dCREB-A* locus by *in situ* hybridization to polytene chromosomes (D. J. A., unpublished) and Southern mapping (Rose et al., submitted). *wR11*, *wR23*, *wR83* and *wR84* are lethal excision *w⁻* lines derived from line *B204*, which is a *w⁺* homozygous viable stock. *rR5* and *rR7* are lethal excision *ry⁻* lines derived from *l(3)3576*. *l(3)3576* is a *ry⁺* lethal insert and a *dCREB-A⁻* loss-of-function allele. Each of the *dCREB-A* mutant chromosomes is balanced over either *TM3-Ubx-lacZ* or *TM6B-Ubx-lacZ*. The balancer chromosome stocks were kindly provided by Ken Irvine (Irvine et al., 1993). Segment polarity and dorsal/ventral mutant stocks are described by Lindsley and Zimm (1992) and elsewhere as cited in the text. The HS-DPP stock, which contains four copies of the transgene, was provided by R. Padgett and R. Blackman. The genotype of each stock built and used in this work was independently verified by antibody staining and/or larval cuticle preparations.

Antibodies and embryo staining

Preparation and isolation of the rat and rabbit polyclonal antisera to DSC73 (D. J. A. and B. S. Baker, unpublished data) will be described in detail elsewhere. The EN mouse monoclonal antibody (mAb4D9) was provided by the Kornberg laboratory (Patel et al., 1989) and the WG rabbit polyclonal antibody was provided by the Nüsse laboratory (van den Heuvel et al., 1989). α -TWI and α -SNAIL antibodies were prepared by S. Roth (Roth et al., 1989). mAb2A12, which stains trachea at high levels from embryonic stages 14 through 17, was provided by N. Patel. mAb68G5D3 (Giniger et al., 1993) and α -CRB mAb (Tepass and Knüst, 1990a) antibodies stain the luminal surface or apical membranes, respectively, of the salivary gland and trachea (and other invaginating ectoderm) from the initiation of invagination (embryonic stage 11) throughout embryogenesis. The mouse monoclonal antibody to β -gal was purchased from Promega Biotec (Madison, WI). Two different batches of *dCREB-A* antisera were used in this work. Both give identical staining patterns in embryos that match the accumulation pattern of *dCREB-A* transcripts (K. D. H. and D. J. A., unpublished). One batch of antiserum was prepared as described by R. Rose and S. M. S. (unpublished). The second batch was prepared by first obtaining a full-length *dCREB-A* cDNA from the Nick Brown 4-8 and 12-24 hour libraries by double stranded PCR using a forward primer (5'-GGCATATGGAATTCTACGATGGCG-3') that engineered a 5' *NdeI* site at the amino-terminal methionine of the coding sequence and a reverse primer (5'-GGCATATGCCCTAGGATATCGA-3') that engineered a *NdeI* site 3' of the carboxy-terminal stop. The 1594 *NdeI* fragment isolated from the PCR reaction was ligated into the pET15b (Novagen) expression vector, fusing six histidines to the amino terminus of *dCREB-A*. This vector was then introduced into competent BL21(DE3) cells (Novagen) for overexpression. *dCREB-A* protein was purified in inclusion bodies (Rio et al., 1986) 3 hours after induction with 0.1 mM IPTG. Rat polyclonal antibodies were raised (HRP Inc) against 1 mg of the renatured inclusion body protein. Antisera from the bleeds were used at dilutions of 1:5,000 through 1:20,000.

Embryo fixation and staining were performed as described elsewhere (Reuter et al., 1990). Homozygous mutant embryos were identified either by the absence of staining with anti- β -gal which

stains embryos carrying the balancer chromosome *Ubx-lacZ* insert or by the absence of *dCREB-A* staining, or both criteria. Antibody-stained embryos were visualized and photographed using Nomarski optics on a Zeiss Axiophot microscope. Ektar 25 print film (Kodak) was used for photography.

Cuticle preparations

Cuticle preparations were done as described by Andrew et al. (1994). HS-DPP embryos were collected for 3–4 hours, aged 1 hour, and incubated in a 37°C water bath for 40 minutes. Cuticle preparations were done 24 hours later. Preparations were examined using either phase or dark-field optics and photographed with Kodak TMAX 100 print film.

RESULTS

The *Drosophila dCREB-A* gene is expressed in several tissues during embryogenesis

Two novel *Drosophila* genes, *dCREB-A*, and *dCREB-B*, were identified by screening a cDNA expression library for proteins that bind the canonical CRE consensus, TGACGTCA (Smolik et al., 1992; Usui et al., 1993). *dCREB-B* was also later cloned and characterized by another group as one of several splicing isoforms of the *dCREB2* gene, specifically *dCREB2-c* (Yin et al., 1995). *dCREB-A* RNA and protein are expressed dynamically during embryogenesis (Fig. 1A–F) in a pattern that does not overlap with that of *dCREB-B* (Usui et al., 1993). The highest level of *dCREB-A* is detected in the salivary gland primordia, initially in both the presecretory cells and a subset of the duct cells (Fig. 1A,D; embryonic stages 9 through early 11; stages according to Campos-Ortega and Hartenstein, 1985), and later in only the secretory cells (Fig. 1B–F; late embryonic stage 11 and throughout larval life). *dCREB-A* expression in the salivary gland depends on the homeotic gene *Sex combs reduced* (*Scr*), since *Scr*[−] embryos do not express *dCREB-A* in the region of the salivary gland and embryos expressing SCR in new places also express *dCREB-A* in new places (Andrew et al., 1994). *dCREB-A* is also expressed at lower levels in other tissues, including the trachea, a subset of neuroblasts, the proventriculus, the amnioserosa, the epidermis, and the foregut and its derivatives. *dCREB-A* protein first appears in the foregut primordia by embryonic stage 6 and persists in the foregut derivatives until the end of embryogenesis. *dCREB-A* protein accumulation in the amnioserosa begins during embryonic stage 8 and disappears during stage 13 (Fig. 1A). Transient expression of *dCREB-A* protein is observed in a subset of neuroblasts from stage 9

through stage 11. *dCREB-A* is detected in the proventriculus from embryonic stage 13 to 17 (Fig. 1E,F). Tracheal expression of *dCREB-A* is first detected at the time of tracheal pit formation (stage 11) and persists in the dorsal trunk tracheal cells throughout embryogenesis (Fig. 1C,E). The epidermal cells, which secrete the larval cuticle just prior to hatching, begin to express *dCREB-A* during stage 11 in a subset of cells in each segment, with accumulation of *dCREB-A* in all epidermal cells by embryonic stage 13 (Fig. 1E). This level of *dCREB-A* protein in the epidermis persists through the remainder of embryogenesis.

dCREB-A was also cloned in another laboratory and the corresponding protein, called BBF-2 by this group, was shown to bind fat body- and liver-specific regulatory elements (Abel et al., 1992). However, *dCREB-A* is not expressed in the fat body during any developmental stage based on both RNA in situ and immunostaining of whole embryos, immunostaining of larvae (K. D. H. and D. J. A., data not shown) and RNA in situ to sectioned adults (Smolik et al., 1992).

Loss-of-function mutations in *dCREB-A* lateralize and weaken the larval cuticle

The *dCREB-A* gene maps to cytological position 71C/D by in situ hybridization to polytene chromosomes (Smolik et al., 1992). At the time of the cloning of the *dCREB-A* gene, no mutations were known to correspond to this cytological location. However, in an ‘enhancer-trap’ screen for genes expressed in the embryonic salivary gland, we obtained two independent inserts in *dCREB-A* (Andrew et al., 1994). Line *B204* from the Goodman and Rubin enhancer-trap collection contains a single P element insert in 71D and expresses β-galactosidase (β-gal) in a subset of the cells normally expressing *dCREB-A*, in both the salivary gland and amnioserosa (Fig. 1G). Line *l(3)3576* from the Spradling collection also contains a single P element insert in 71D, but expresses β-gal in all cells that normally express *dCREB-A* protein (Fig. 1H,I). The P element insert in line *B204* maps approximately 3.5 kb upstream of the *dCREB-A* transcription start site (Rose et al., submitted) and does not disrupt *dCREB-A* function. The P element in line *l(3)3576* maps within the first intron (R. Rose and S. M. S., unpublished) and inactivates the *dCREB-A* gene.

Using standard excision mutagenesis (Hamilton and Zinn, 1994), we obtained four homozygous lethal *white*[−] (*w*[−]) derivatives of line *B204*(*w*⁺), named *dCREB-A*^{wR11}, *dCREB-A*^{wR23}, *dCREB-A*^{wR83} and *dCREB-A*^{wR84}, and two homozygous lethal *rosy*[−] (*ry*[−]) derivatives of line *l(3)3576* (*ry*⁺), named *dCREB-A*^{rR5} and *dCREB-A*^{rR7}. Each of the six lethal excision deriva-

Table 1. Complementation tests among *dCREBA* mutant alleles

X	R11	R23	R83	R84	Df(3L)BK10	l(3)3576	R5	R7
R11	–	–	–	–	–	–	–	–
R23	130:0	–	–	–	–	–	–	–
R83	93:0	78:0	–	–	–	–	–	–
R84	135:0	89:0	196:0	–	–	–	–	–
Df(3L)BK10	160:0	114:0	113:0	142:0	–	–	–	–
l(3)3576	344:0	324:0	477:0	590:0	358:0	–	–	–
R5	305:0	228:0	404:0	360:0	171:0	261:0	–	–
R7	248:0	244:0	522:0	329:0	147:0	359:0	299:0	–
B204	265:112	32:39	177:64	101:40	–	191:97	141:80	38:26

Numbers reflect the ratio of third chromosome balancer-carrying heterozygous adult flies to adult flies not carrying balancer third chromosomes. Data were pooled from pair matings which were done in both directions with respect to the gender of the parents.

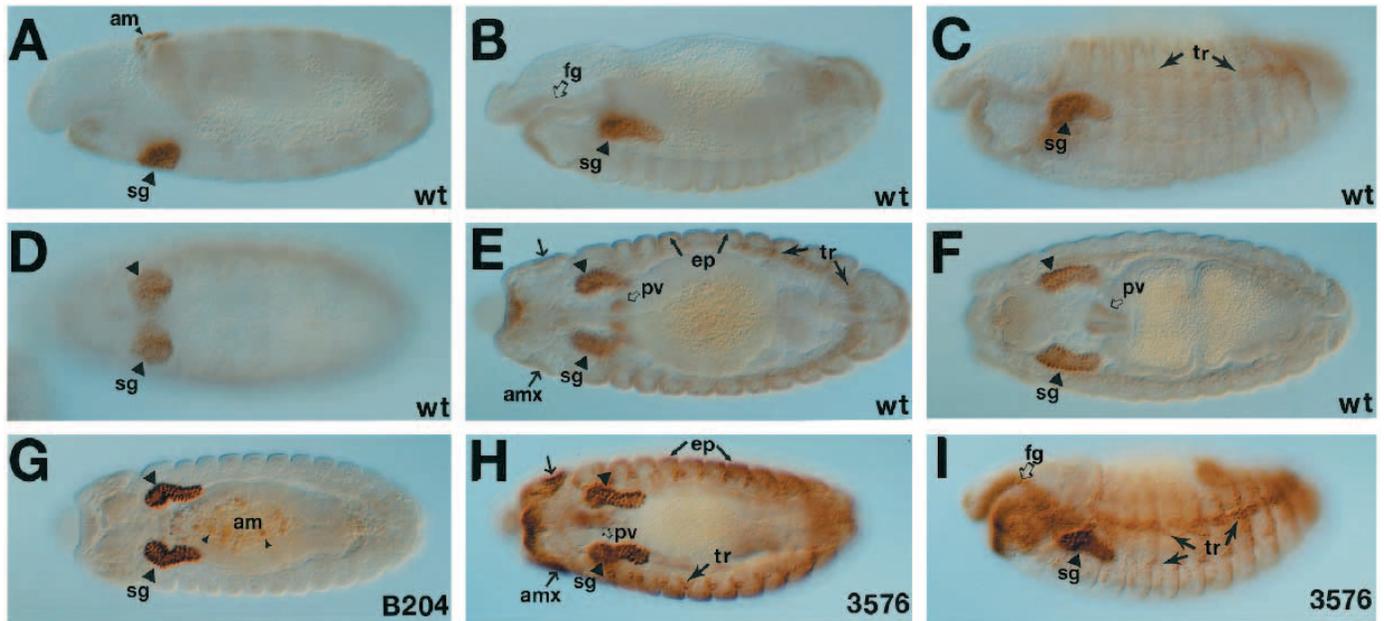


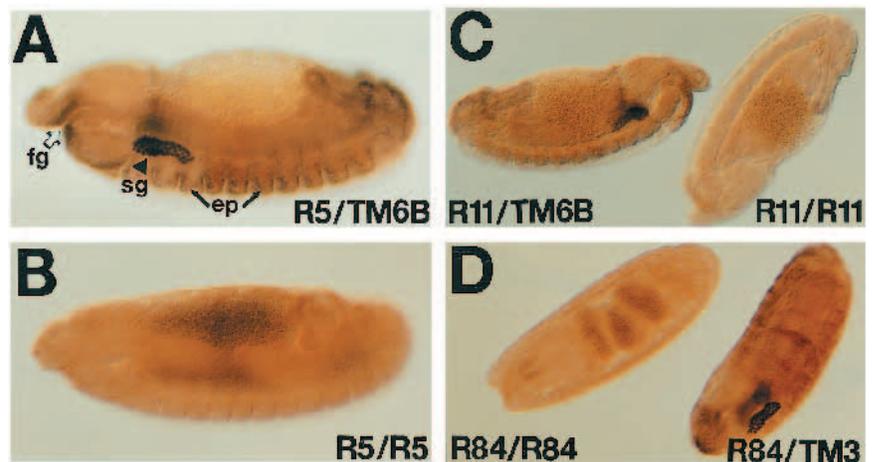
Fig. 1. Expression of wild-type dCREB-A protein is compared with expression of β -gal in embryos carrying enhancer-trap inserts, *B204* and *l(3)3576*. A-F are wild-type embryos stained with rat polyclonal antisera directed against the dCREB-A protein (R. Rose and S. M. S., unpublished). G-I are embryos stained with β -gal antiserum. G is an embryo from enhancer-trap line *B204* and H and I are embryos from the enhancer-trap line *l(3)3576*. dCREB-A protein can be detected in the salivary gland (sg), proventriculus (pv), trachea (tr), amnioserosa (am), anterior maxillary complex (amx), foregut (fg) and epidermis (ep). β -gal expression in enhancer-trap line *B204* reflects a subset of the normal dCREB-A pattern (sg and am) and β -gal expression in enhancer trap line *l(3)3576* shows all aspects of the dCREB-A pattern although detectable expression of β -gal in these tissues persists longer than detectable expression of dCREB-A.

tives fails to complement each of the others as well as the original insert line, *l(3)3576*, and a deficiency for the region, Df(3L)BK10 (Table 1). The following results suggest that all seven *dCREB-A* alleles (the six excision alleles and the *l(3)3576* insert) are single loss-of-function mutations in only the *dCREB-A* gene. (1) None of the seven alleles produces detectable levels of *dCREB-A* protein during embryogenesis (Fig. 2B-D). (2) All six excision alleles lack only a few hundred bp of DNA, either from the transcribed region (rR5 and rR7) or from the region immediately upstream of the 5' end of the *dCREB-A* gene (wR11, wR23, wR83 and wR84)

(R. Rose and S. M. S., unpublished). (3) The *l(3)3576* allele was created by a single P element insertion in the gene since precise excisions of the *l(3)3576* insert complement Df(3L)BK10, a deficiency spanning 71C1,2 to 71F4,5 (Lindsley and Zimm, 1992). (4) As discussed in detail below, the phenotypes of all seven alleles are indistinguishable from each other and, for some aspects of the phenotype, indistinguishable from homozygous Df(3L)BK10 embryos.

dCREB-A mutants exhibit severe defects in the larval cuticle. The most obvious defect, visible at even low magnifications, is a weakening of the cuticle and a decrease in the overall

Fig. 2. *dCREB-A* mutants produce no detectable *dCREB-A* protein. A shows a heterozygous *dCREB-A*⁻ embryo with α -dCREB-A staining (black) in the foregut (fg), salivary gland (sg) and epidermis (ep). The light brown non-nuclear β -gal staining, due to the presence of the TM6B-*Ubx-lacZ* balancer chromosome, is visible in the epidermis of the abdominal segments. B is a homozygous *dCREB-A*^{R5} embryo, which stains with neither α -dCREB-A nor α - β -gal. C and D show staining of homozygous and heterozygous *dCREB-A* mutant siblings (*dCREB-A*^{R11} and *dCREB-A*^{R84}). The embryos in C are approximately embryonic stage 15, a stage when β -gal staining due to the presence of the TM6B-*Ubx-lacZ* balancer chromosome is most visible in the nerve cord. The embryos in D are embryonic stage 16 when β -gal staining due to the presence of the balancer chromosome, TM3-*Ubx-lacZ*, is visible in the epidermis and second midgut constriction (stages according to Campos-Ortega and Hartenstein, 1985).



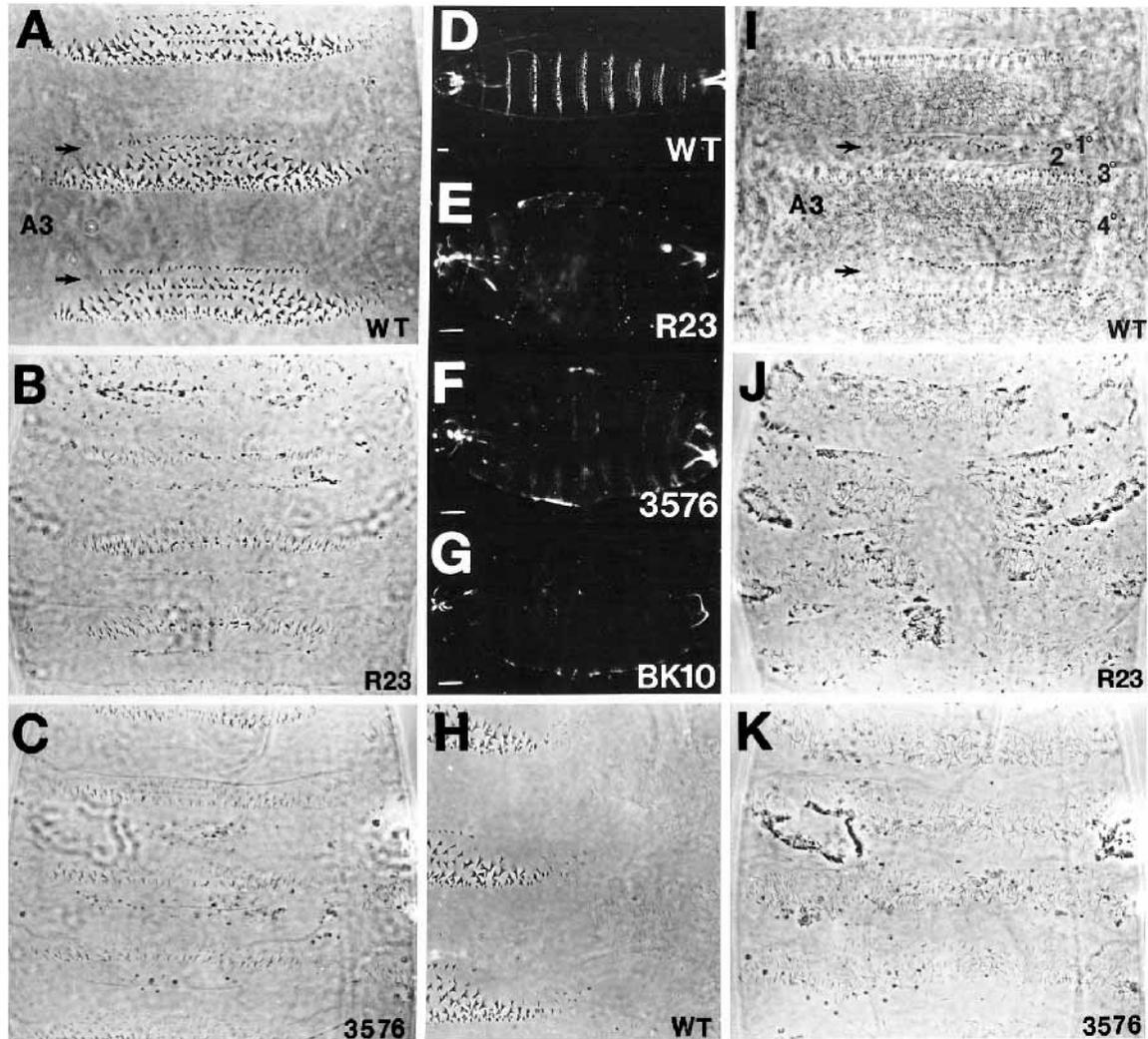


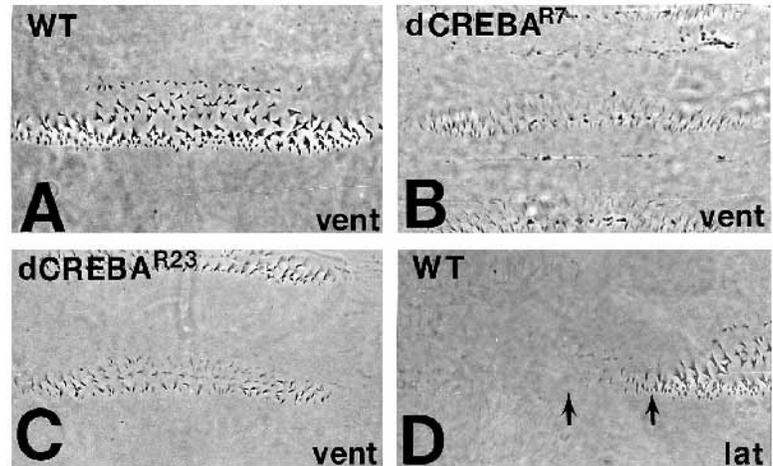
Fig. 3. *dCREB-A* mutant larvae are shorter than wild-type and have patterning defects. A-C are phase contrast views of the ventral cuticle of wild-type, WT, (A) and *dCREB-A* mutant (B,C) larvae. D-G are dark-field micrographs of wild-type (D) and *dCREB-A* mutant (E-G) larvae. *dCREB-A* mutant larvae are about 40% the length and the same width as wild-type larvae; the micrograph of the wild-type larva (D) is taken at half the magnification of the *dCREB-A* mutants (E-G) as indicated by the scale bars in the lower left of each panel. (H) lateral and (I) dorsal phase contrast views of wild-type larvae. The arrows in A and I indicate the limits on the third abdominal segment, A3. The assignment of 1°, 2°, 3° and 4°, hairs (I) is according to Heemskerk and DiNardo, 1994. J and K are dorsal, phase contrast views of *dCREB-A* mutant larvae. Note the holes in the dorsal cuticles of the *dCREB-A* mutant larvae (J,K). R23 is homozygous for the *dCREB-A*^{wR23} allele and 3576 is homozygous for the *dCREB-A*³⁵⁷⁶ allele.

length of the homozygous mutant animals; *dCREB-A* homozygotes are on average 40% the length of their heterozygous siblings (Fig. 3D-G; note that the wild-type larva in D was photographed at half the magnification of the mutant larvae in E-G). Cuticular weakening is evident by the frequent 'blow-outs' or 'holes' which occur at random positions in the exoskeleton of homozygous animals (Fig. 3J,K). The cuticular weakening and the decrease in larval length could be related. At all earlier embryonic stages, homozygous *dCREB-A* mutants are the same length as their heterozygous siblings, suggesting that the length differences arise during the formation of the larva (Fig. 2C,D). Correspondingly, synthesis of cuticle proteins does not begin until the final stages of embryogenesis; both weaker and smaller larval cuticles might be expected if cuticle protein synthesis were impaired. Alternatively, *dCREB-A* mutant larvae could be shorter because they have patterning defects

(see below). Larvae mutant for either segment polarity or dorsal/ventral genes are typically much shorter than wild-type larvae. The cuticle phenotypes of animals homozygous for *Df(3L)BK10* are indistinguishable from those of any of the *dCREB-A* alleles, suggesting that with regard to the cuticle defects, each of the seven alleles is null.

A more surprising effect of *dCREB-A* mutations was found in the patterning of the epidermis. The ventral surface of *dCREB-A* mutants have denticle belts comprising between 5 and 6 rows of small, irregularly pigmented denticles separated by bands of naked cuticle (Fig. 3B,C). The dorsal surface of the *dCREB-A* mutants is covered by bands of uniformly sized randomly aligned hairs also separated by bands of naked cuticle (Fig. 3J,K). The dorsal hairs in the *dCREB-A* mutants are most similar in size and morphology to both a subset of dorsal hairs, the so-called 4° hairs (Fig. 3I; Heemskerk and

Fig. 4. The ventral larval cuticle of *dCREB-A* mutants is composed of pattern elements normally found in a ventrolateral position in wild-type larval cuticle. (A) ventral (vent) and (D) lateral (lat) phase contrast micrographs of wild-type larval cuticles. B and C are ventral (vent) phase contrast views of *dCREB-A* mutant larvae homozygous for two different *dCREB-A* alleles, *dCREB-A^{R7}* (B) and *dCREB-A^{wR23}* (C). The arrows in D indicate the position of structures in wild-type larvae that are most similar to the structures on the entire ventral surface of *dCREB-A* mutant larvae (B) and (C). Note that in wild-type larvae the smaller gray-pigmented denticles are more laterally positioned than the denticles with light bases and dark tips (D). In the *dCREB-A* mutants (B) and (C), the smaller gray pigmented denticles are found in the most lateral position, but are also concentrated in what in wild-type larvae is the most ventral position.

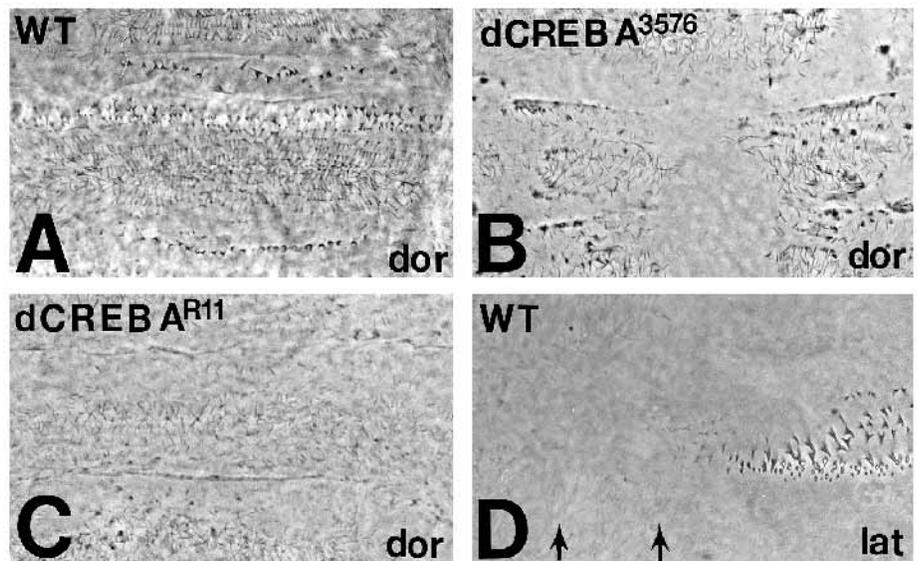


DiNardo, 1994), and the dorsolateral hairs of wild-type larvae; however, the dorsal hairs of *dCREB-A* mutants are neither as orderly nor as densely packed as the dorsal 4^o hairs of wild-type embryos. Rather, the loose irregular spacing of these hairs is more reminiscent of those seen in a dorsolateral position in wild-type embryos. The altered cuticle pattern in the *dCREB-A* mutants can be explained in at least three ways. *dCREB-A* may be required for the differentiation of specific cell types and its loss may result in the production of only a subset of denticle and hair types normally found. This phenotype could be related to the weakening of the cuticle. Alternatively, *dCREB-A* may be required for normal anterior-posterior patterning, and is therefore part of the segment polarity hierarchy. Finally, *dCREB-A* may function in the dorsal-ventral system to specify both dorsal and ventral fates, and the observed patterns are due to lateralization of the mutant larval cuticle. As described next, our evidence supports the last possibility.

The denticles present in the *dCREB-A* mutants have neither the morphology nor the orientation of the ventral denticles in wild-type larvae (Fig. 4A-C). The *dCREB-A*⁻ ventral denticles are, however, indistinguishable from those found at a ventrolateral position in wild-type larvae (Fig. 4D): small gray-pigmented denticles found at the most lateral position, and

somewhat larger denticles, which are gray at the base and black at the tip, located slightly more ventrally (Fig. 4D). Likewise, the hairs on the dorsal surface of *dCREB-A* mutants (Fig. 5B) are most similar in arrangement and morphology to those found in a dorsolateral position in wild-type larvae (Fig. 5D). Because the morphology and arrangement of the ventral denticles and dorsal hairs in *dCREB-A* mutants accurately represent structures normally found on the lateral surface of wild-type larvae, we believe the altered cuticle pattern is not due to a failure to differentiate denticles or hairs, but rather arises because wild-type *dCREB-A* is required for correct patterning of the epidermis along the dorsal-ventral axis. The absence of a correlation between the size of the denticles in wild-type and the size of denticles found in the same position in *dCREB-A* mutants also argues against a failure to differentiate denticles in the *dCREB-A* mutants. The positioning of denticle types suggests that the ventral surface in *dCREB-A* mutants is a mirror image duplication of normal ventrolateral pattern elements, with the most lateral denticle type (small gray-pigmented denticles) now positioned most ventrally (Fig. 4). The dorsal surface of *dCREB-A* mutants may be a mirror reflection of normal dorsolateral pattern elements, but the similarity of hair structure on the dorsolateral surface of wild-type

Fig. 5. The dorsal larval cuticle of *dCREB-A* mutants is composed of pattern elements that are normally found in a dorsolateral position in wild-type larval cuticles. (A) dorsal (dor) and (D) lateral (lat) phase contrast micrographs of wild-type larval cuticles. (B,C) Phase contrast views of the dorsal (dor) surface of *dCREB-A* mutant larvae homozygous for two different *dCREB-A* alleles, *dCREB-A³⁵⁷⁶* (B) and *dCREB-A^{R11}* (C). The arrows in D indicate the position of structures in wild-type larvae that are most similar to the structures on the entire dorsal surface of *dCREB-A* mutant larvae (B,C). dor, dorsal views; lat, lateral views.



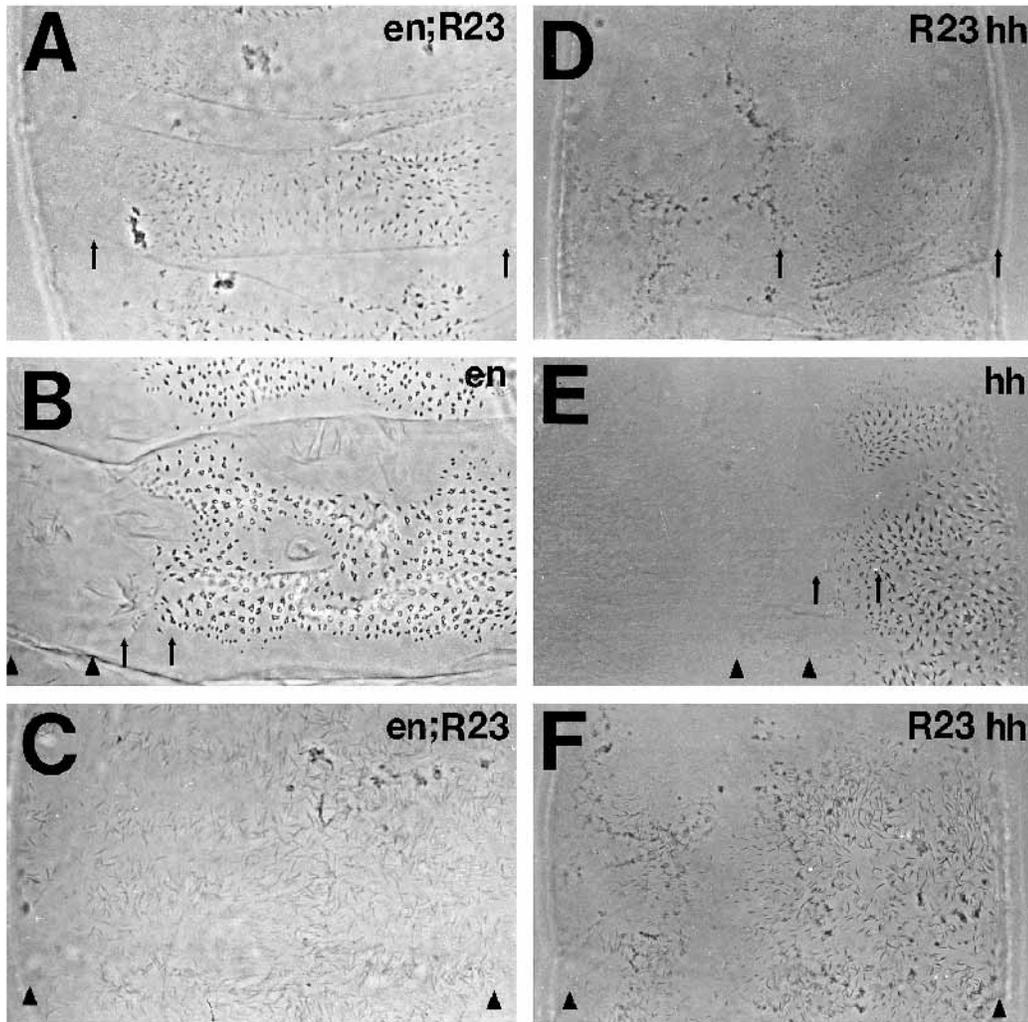


Fig. 6. Cuticle defects in larvae mutant for both *dCREB-A* and the segment polarity genes suggest simple additive phenotypes. Phase contrast micrographs of cuticles from larvae homozygous for loss-of-function mutations in both *en* (*en^{IM99}*) and *dCREB-A* (*dCREB-A^{wR23}*) (A,C), an *en* (*en^{IM99}*) homozygous mutant larva (B), larvae homozygous for loss-of-function mutations in both *hh* (*hh^{AC}*) and *dCREB-A* (*dCREB-A^{wR23}*) (D,F), and a *hh* (*hh^{AC}*) mutant larva (E). The arrows in panels A, B, D and E indicate denticles of either known (B,E) or apparent (A,D) ventrolateral identity. The arrowheads in B, C, E, and F indicate hairs of either known (B,E) or apparent (C,F) dorsolateral identity.

and dorsal surface of mutant animals did not allow us to examine this possibility.

Double mutants of *dCREB-A* and segment polarity genes give simple additive phenotypes

To test the idea that *dCREB-A* is involved in dorsal-ventral and not anterior-posterior patterning, we constructed animals homozygous for both segment polarity mutations and *dCREB-A* mutations. For each segment polarity mutation tested, including loss-of-function alleles of *engrailed* (*en^{IM99}* and *en^{CX1}*; Cadigan et al., 1994; Schuske et al., 1994), *wingless* (*wg^{CX4}*; van den Heuvel et al., 1993), *gooseberry-2* (*gsb^{DIIx62}*; Zhang et al., 1994), *Cubitus interruptus* (*Ci^D*; Pankratz and Hoch, 1995) and *hedgehog* (*hh^{AC}*; Basler and Struhl, 1994), the double mutant phenotype with *dCREB-A* mutations is simply additive. In Fig. 6 we compare the phenotypes of an *en*;*dCREB-A* larva (Fig. 6A,C) to an *en* larva (Fig. 6B) and a *hh* *dCREB-A* larva (Fig. 6D,F) to a *hh* larva (Fig. 6E). The denticles on the ventral surface of the double-mutant embryos have the same morphology and arrangement as those in the ventrolateral position in larvae mutant for each segment polarity gene alone (compare Fig. 6A and 6B and Fig. 6D and 6E). Correspondingly, the dorsal surface of each double mutant has the same arrangement of hairs and naked cuticle as the dor-

solateral position of each segment polarity mutant alone (compare Fig. 6C with 6B and Fig. 6F with 6E). The simple additivity of *dCREB-A* and segment polarity phenotypes supports the hypothesis that *dCREB-A* is not involved in segment polarity. Further support for this argument is provided by our finding that the expression patterns of the proteins encoded by two segment polarity genes, *WG* and *EN*, are unchanged in *dCREB-A* mutant embryos (data not shown).

dCREB-A is epistatic to known dorsal/ventral patterning genes

Because the D/V patterning defects in *dCREB-A* mutants appear limited to structures produced by the epidermis, and because other known D/V patterning genes affect both internal and external structures, we hypothesized that *dCREB-A* acts late in the D/V pathway. To test this idea, epistasis tests were done with two D/V patterning genes, the *decapentaplegic* (*dpp*) gene, which encodes a TGF β -like secreted signaling protein required to pattern the dorsal half of the embryo and larva (Irish and Gelbart, 1987; Padgett et al., 1987), and the *spitz* (*spi*) gene, which encodes a TGF α -like secreted signaling protein required to pattern the most ventral structures in the embryo and larva (Mayer and Nüsslein-Volhard, 1988; Rutledge et al., 1992). In *dpp* null larvae, the entire cuticle is

ventralized (Fig. 7A). In *dpp;dCREB-A* larvae, the entire cuticle is lateralized (Fig. 7B); we see only the small gray-pigmented denticles and denticles with dark tips and light bases characteristic of ventrolateral structures. This result suggests that *dCREB-A* acts either downstream of, or parallel to, *dpp*. We also compared the phenotypes of *HS-DPP* larvae to *HS-DPP; dCREB-A* double mutant larvae. In induced *HS-DPP* larvae, ventral pattern elements are severely reduced and are replaced by the hairs normally found on the dorsal surface of wild-type larvae (i.e., the effect is opposite to that of losing *dpp* function) (Fig. 7C). The only ventral pattern elements that remain in *HS-DPP* embryos are found at the most ventral position and appear to have lateral identities, being primarily denticles with light bases and dark tips (arrowheads in Fig. 7C). In induced *HS-DPP; dCREB-A* mutant larvae, nearly the entire larval surface is covered by structures normally found in a dorsolateral position, similar to the dorsal surfaces of *dCREB-A* single mutants. The remaining 'ventral structures' appear to be even more lateralized than those in the *HS-DPP* larvae, consisting almost entirely of the small gray-pigmented denticles normally found at the most lateral position on the ventral surface (arrowheads in Fig. 7D). We also compared *spi* and *dCREB-A* single mutants to the *spi;dCREB-A* double mutants. In *spi* mutants, the most ventral denticles are absent, and denticle rows are fused between segments (Mayer and Nüsslein-Volhard, 1988; Fig. 7E). In *spi; dCREB-A* mutants, we see narrower denticle bands with some fusion of denticles between segments; however, the denticles have the same morphology found in *dCREB-A* mutants alone (Fig. 7F) suggesting that *dCREB-A* also either functions downstream of, or in parallel to, the *spi* signal in patterning the ventral epidermis.

To determine where in the D/V pathway *dCREB-A* functions and to learn what other structures might also be affected by *dCREB-A* mutations, we examined the distribution of several tissue-specific markers whose expression is controlled by DPP- and/or SPI-signaling. The distribution of TWIST and SNAIL, two proteins expressed in ventrally localized presumptive mesoderm, is unaltered in *dCREB-A* mutants. This result is not surprising since neither the mesoderm nor its derivatives detectably express *dCREB-A*, and the visceral and somatic musculature are apparently normal at all embryonic stages. The *snail* gene is also expressed later in the wing, haltere and genital imaginal disc primordia (Alberga et al., 1991) and thus indicates if the early development of these structures is normal in *dCREB-A* mutant embryos. SNAIL accumulation in the wing, haltere and genital imaginal disc primordia is unaltered in *dCREB-A* mutant embryos compared to their wild-type siblings (data not shown). Similarly, two other proteins, ORTHODENTICLE, which is normally expressed and required in a stripe three to four cells wide along the ventral

midline in germ band extended embryos (Finkelstein et al., 1990; Wieschaus et al., 1992), and FASCICLIN III, which is expressed in the epidermis (Patel et al., 1987; Raz and Shilo, 1993), are not detectably altered in *dCREB-A* mutants. We also examined expression of KRUPPEL (KR) protein in *dCREB-A* mutants. KR, which is expressed at later stages in the nuclei of the amnioserosa, the most dorsal tissue in the developing embryo (Gaul et al., 1987; Raftery et al., 1995), is also apparently unaffected in *dCREB-A* mutants, despite high *dCREB-A* expression in the amnioserosa (Fig. 1). The amnioserosa, however, does not contribute to the larval epidermis. The only antibody we tested that had altered expression patterns in *dCREB-A* mutants was directed against DSC73, a secreted protein expressed at late embryonic stages in the epidermal cells that produce denticles and hairs (D. J. A. and B. Baker, unpublished). The altered DSC73 accumulation patterns in *dCREB-A* embryos are subtle when compared to wild-type embryos but do correspond to the changes in denticle and hair patterns in the *dCREB-A* mutant larvae. We observe a decrease in the levels of DSC73 on the dorsal and ventral surfaces compared to levels of DSC73 in lateral positions, which appear unchanged. We also stained *dCREB-A* mutants with markers

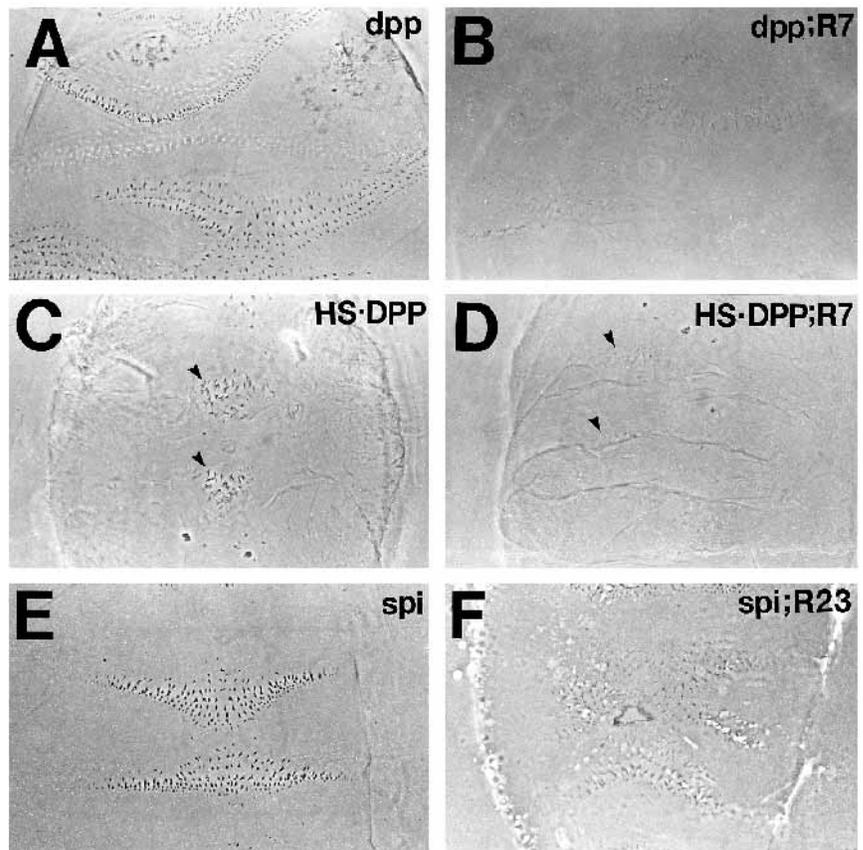


Fig. 7. *dCREB-A* is epistatic to mutations in *dpp* and *spi*. (A) *dpp*⁴⁸ homozygous first instar larval cuticle. (B) *dpp*⁴⁸;*dCREB-A*^{R7} homozygous first instar larval cuticle. (C) Induced *HS-DPP* first instar larval cuticle. (D) Induced *HS-DPP; dCREB-A*^{R7} first instar larval cuticle. (E) *spi* homozygous first instar larval cuticle. (F) *spi*^{IIA14};*dCREB-A*^{R23} first instar larval cuticle. Arrowheads in C and D indicate remaining ventral structures in the induced *HS-DPP* embryos. For C and D, 3- to 4-hour-embryo collections were heated to 37°C for 40 minutes, aged 24 hours, fixed and prepared as described by Andrew et al. (1994).

for the trachea, foregut and proventriculus. No obvious defects were observed in these tissues at any stage of embryogenesis. The staining results strongly support the hypothesis that *dCREB-A* functions very late in D/V patterning and may affect patterning in only the epidermis.

dCREB-A is required for the structural integrity of the salivary gland

Our interest in *dCREB-A* was initially based on its high level expression in the salivary gland beginning at the time salivary glands are first determined and extending throughout larval life (Fig. 1; Smolik et al., 1992; Andrew et al., 1994). Expression of *dCREB-A* in the salivary gland is activated by the homeotic gene, *Scr*, and that activation is blocked by the trunk gene, *teashirt* (*tsh*), and the posterior homeotic gene, *Abdominal-B* (*Abd-B*) (Andrew et al., 1994). As with two other salivary gland genes, *fork head* (*fh*) (Panzer et al., 1992) and *tracheal-less* (*trh*) (Isaac and Andrew, 1996), activation of *dCREB-A* in the salivary gland by *Scr* is also blocked by *dpp* (data not shown).

We stained salivary glands in *dCREB-A* mutants with antibodies to two different epitopes, one an unknown antigen expressed in the salivary gland lumen and other invaginating ectoderm, mAb68G5D3 (D3) (Giniger et al., 1993), and one expressed on the apical surface of the salivary gland and other epithelia, α -CRUMBS (α -CRB) (Tepass et al., 1990a,b;

Wodarz et al., 1995). Based on these markers, the only observed defect is that the salivary glands are 'crooked' (Fig. 8B). To quantitate this phenotype, we stained embryos from the *dCREB-A*, Df(3L)BK10, and wild-type stocks with antibodies to both *dCREB-A* and CRB. We then scored embryos as having smooth, slightly crooked or crooked salivary glands (Fig. 8C). The *dCREB-A* mutant embryos show an increase in the severity and frequency of crooked salivary glands compared to their *dCREB-A*⁺ siblings. Homozygous Df(3L)BK10 embryos showed the highest degree of crookedness suggesting that either the excision alleles of *dCREB-A* have some residual *dCREB-A* function or the deficiency also removes other genes that affect the integrity of the salivary gland.

DISCUSSION

dCREB-A and dorsal-ventral patterning

dCREB-A mutant larvae have lateralized cuticle. The most ventral denticles are replaced by denticles normally found in a ventrolateral position, and the most dorsal hairs are replaced by hairs normally found in a dorsolateral position (Fig 3-5). This phenotype is reminiscent of that of larvae lacking both *dpp* function and function of any of three maternal effect genes *Toll*, *pelle*, or *dorsal*, genes which are normally required for

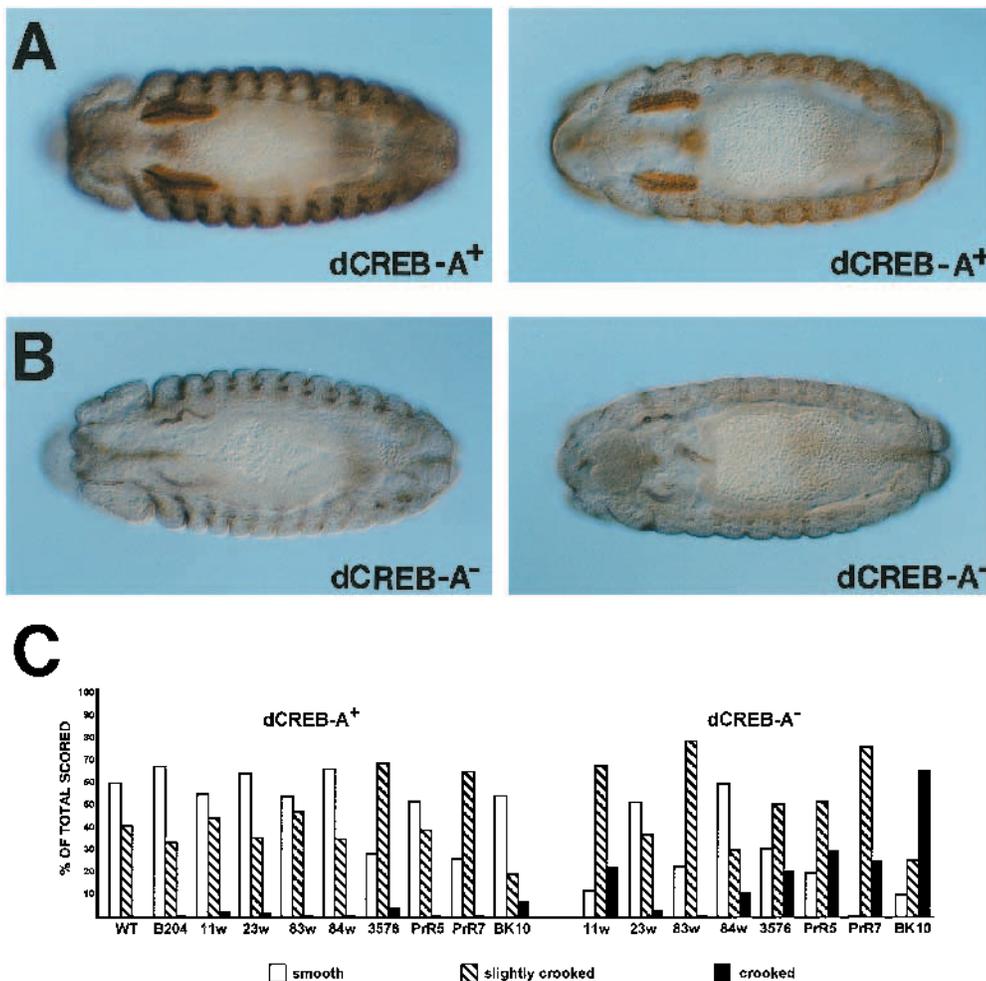
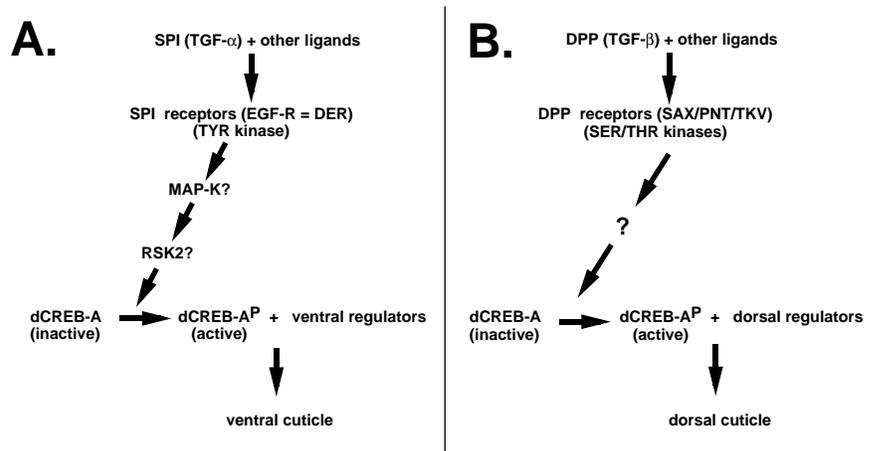


Fig. 8. *dCREB-A* mutants have crooked salivary glands. Overnight embryo collections of all the *dCREB-A* alleles, original P element stocks (B204 and 1(3)3576), Df(3L)BK10 and Oregon-R were fixed and stained serially with α -CRB (in black) and α -*dCREB-A* (brown). Representative wild-type (*dCREB-A*⁺) (A) and mutant (*dCREB-A*⁻) (B) embryos are shown. The histogram in C indicates the frequency of *dCREB-A*⁺ and *dCREB-A*⁻ embryos with smooth (no obvious bends in the salivary gland), slightly crooked (bends but no change in direction) and crooked (significant bends involving part of the salivary gland pointing in a new direction) salivary glands. Scoring of each embryo was based on the most extreme phenotype seen in either of the two glands. Only embryos between embryonic stages 13 and 15 were scored.

Fig. 9. Models placing *dCREB-A* among the known components for dorsal/ventral patterning. (A) This model places *dCREB-A* downstream of SPI-signaling in patterning the ventral cuticle. SPI and other ligands activate a SPI receptor, one of which is the EGF-R or DER (Raz and Shilo, 1993). Once activated, EGF-R functions as a tyrosine kinase that can either directly or indirectly phosphorylate and activate *dCREB-A*. Candidate intermediate kinases include the RAS-dependent MAP-K which is known to phosphorylate RSK-II, which in turn phosphorylates CREB in PC12 cells in response to either EGF or NGF stimulation (Xing et al., 1996). Phosphorylated *dCREB-A* then collaborates with the ventral regulators to activate expression of ventral-specific epidermal target genes. (B) On the dorsal surface, *dCREB-A* could be downstream of the DPP-signaling pathway. DPP and other ligands bind to and activate the DPP receptors, SAX, TKV and PNT, which are known serine/threonine kinases (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Letsou et al., 1995). Ligand binding results, either directly or indirectly, in the phosphorylation and activation of *dCREB-A*. Phosphorylated *dCREB-A* then collaborates with other dorsally localized regulators to activate expression of dorsal epidermal genes.



ventral fates (Irish and Gelbart, 1987). The similarity in phenotypes suggests that *dCREB-A* is part of the pathway for dorsal/ventral patterning of the larval epidermis. Since *dCREB-A* affects only the epidermis, whereas all other known genes in this hierarchy affect multiple germ layers, we propose that *dCREB-A* functions near the end of embryogenesis. Consistent with this idea, the distribution of known dorsal/ventral proteins is unaltered in the *dCREB-A* mutant embryos, and mutations in *dCREB-A* are epistatic to *dpp* and *spi*, which act early. Because the *dCREB-A* mutant phenotypes prevail in larvae doubly mutant for *dCREB-A* and either *dpp* or *spi* (Fig. 7), it is likely that *dCREB-A* functions either downstream of the two signaling cascades or in parallel to both.

dCREB-A is a member of a family of proteins whose transcriptional activity is regulated by phosphorylation events triggered by extracellular signals (reviewed in Meyer and Habener, 1993). We propose that in the ventral epidermis (Fig. 9A), *dCREB-A* functions downstream of SPI and its activity is regulated, directly or indirectly, by a phosphorylation event triggered by the binding of SPI to its receptor, which is a *Drosophila* EGF receptor homolog (EGF-R/DER) and functional tyrosine kinase (Raz and Shilo, 1993; reviewed by Shilo and Raz, 1991). Activated *dCREB-A* would then collaborate with ventrally localized transcription factors to activate expression of ventral effector molecules. In the dorsal epidermis (Fig. 9B), *dCREB-A* functions downstream of DPP, and its activity is regulated, directly or indirectly, by a phosphorylation event triggered, in this case, by the binding of DPP to its receptors, which encode serine/threonine kinases (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994; Letsou et al., 1995). Activated *dCREB-A* would then act in concert with dorsally localized transcription factors to activate expression of dorsal effector molecules. An alternative to the proposed models is that *dCREB-A* acts in parallel with downstream effector molecules whose activities are regulated by either SPI- or DPP-signaling. In this alternative model, the phosphorylation state of *dCREB-A* would not be altered as a consequence of D/V signaling.

dCREB-A protein is found in the nuclei of all epidermal cells

by stage 13 and is thus poised to be posttranslationally regulated by either the SPI- or DPP-signaling pathways. In the models proposed above, *dCREB-A* activity is regulated by a phosphorylation cascade. Although *dCREB-A* has no consensus PKA phosphorylation site and its transcriptional activity is only mildly enhanced by cAMP, *dCREB-A* does have three CaM Kinase II/Ribosomal S6 Kinase II (RSK2) phosphorylation sites, one of which also fits the consensus phosphorylation site for GSK-3 (Smolik et al., 1992).

Evidence for parallel pathways in mammalian systems exists for the proposed role of *dCREB-A* in both ventral and dorsal patterning (Fig. 9A). Either NGF or EGF treatment of PC12 cells (pheochromocytoma cells from adrenal medulla) can stimulate RAS-dependent phosphorylation of CREB (Ginty et al., 1994). The active CREB kinase has been identified as the pp90^{rsk} family member RSK2, which is phosphorylated and activated by the Ras/Mitogen-Activated Protein Kinase (MAPK) pathway (Xing et al., 1996). RSK2 translocates to the nucleus upon growth factor stimulation (Chen et al., 1992) and phosphorylates serine residues contained in the consensus sequence R-X-X-S (Erikson and Maller, 1988; Rivera et al., 1993); three such consensus sequences exist in *dCREB-A*. Consistent with our proposed model for dorsal patterning, a DPP homolog, TGFβ1, induces cAMP-independent phosphorylation of a CREB protein in mink lung cells, supporting the notion of a common regulatory pathway for dorsal patterning in flies and TGFβ signaling in mammals (Kramer et al., 1991).

The identification of a phosphorylation-dependent transcription factor, such as *dCREB-A*, downstream of either SPI- or DPP-signaling is not an unexpected finding. The receptors for both ligands are protein kinases and both signaling events are expected to lead to differences in gene expression. *dCREB-A* function in D/V signaling appears limited to the epidermis, and thus, different phosphorylation-dependent transcription factors must respond to D/V signaling in other cell types. One such factor may be the MAD (Mothers Against DPP) protein (Raftery et al., 1995; Sekelsky et al., 1995; Hoodless et al., 1996; Liu et al., 1996). Mutations in MAD cause phenotypes similar to *dpp* mutations in midgut morphogenesis and larval

development (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996). A human MAD homologue, known as SMAD1 or MADR1, activates transcription when fused to a heterologous DNA binding protein (Liu et al., 1996), and is only translocated to the nucleus after being phosphorylated as a consequence of receptor activation by BMP2 or BMP4, which are DPP homologs (Hoodless et al., 1996; Liu et al., 1996). MAD does not have a recognizable DNA binding motif (Sekelsky et al., 1995) and has not yet been shown to physically contact DNA; therefore, MAD may act through other DNA-binding proteins.

Another potential example of a transcription factor downstream of DPP is the *schurri* (*shn*) gene, which is required to mediate DPP-signalling events in both the visceral mesoderm and underlying endoderm (Grieder et al., 1995). In the absence of *shn* zygotic function, DPP-dependent midgut expression of *Ubx*, *wg*, *lab*, and *dpp* itself, does not occur. In the absence of both zygotic and maternal *shn*, dorsal cuticular structures are missing, further linking the function of *shn* to *dpp*. The distribution of *shn* transcripts suggests that its expression is not regulated by DPP. *shn* encodes a protein similar in sequence to a family of mammalian zinc finger transcription factors; perhaps the activity of the SHN protein is regulated by DPP-signalling.

Another class of transcription factors that could function downstream of DPP- and SPI-signaling is the homeotic gene family. One excellent potential example is SCR. SCR activates expression of salivary gland genes in parasegment 2 only ventrally where *dpp* is not expressed (*fkh*, Panzer et al., 1992; *trh*, Isaac and Andrew, 1996; *dCREB-A*, data not shown). In the absence of *dpp* function SCR activates salivary gland genes around the entire circumference of parasegment 2, and when DPP is expressed everywhere, SCR does not induce salivary gland genes. Similarly, SPI activity blocks the activation by SCR of salivary gland genes whose expression is limited to the more dorsal secretory cell population (Panzer et al., 1992). We do not yet know if SCR is differentially phosphorylated in response to DPP and SPI. However, another homeotic protein, *UBX*, is differentially phosphorylated in a tissue-specific manner (Lopez and Hogness, 1991), consistent with the idea that D/V signaling may affect its phosphorylation state and subsequent activities.

To test our model for where *dCREB-A* fits in dorsal ventral patterning, we must first identify late-acting DPP- and SPI-dependent target genes in the epidermis and demonstrate that activation (or repression) of these targets by either DPP or SPI depends on *dCREB-A*. This result would unequivocally place *dCREB-A* in the same pathway as known D/V determinants but would not reveal if *dCREB-A* protein is directly affected by these signaling cascades. We must also ask whether or not *dCREB-A* protein is phosphorylated as a consequence of either DPP or SPI induction. If so, then it is likely that *dCREB-A* is directly downstream of the known D/V signaling cascades. Experiments addressing the above issues are in progress.

***dCREB-A* and salivary gland formation**

dCREB-A RNA is detectable in the salivary gland less than 1 hr after SCR protein appears in parasegment 2 (D. J. A., unpublished data), and continues to be expressed to very high levels in the salivary gland throughout larval life. We therefore expected that loss-of-function mutations in *dCREB-A* would

significantly affect the salivary gland; however, the only salivary gland defect seen in *dCREB-A* mutant embryos is crooked salivary glands, a relatively mild phenotype overlapping that of wild-type embryos (Fig. 8). The crooked phenotype could be related to the defects we observe in the larval cuticle. The salivary glands and epidermis both consist of polarized secretory epithelial cells. In both tissues, *dCREB-A* may be required for high level expression of secreted gene products, such as the proteins that comprise the exoskeleton and lining of the salivary gland. The increase in crookedness of the salivary gland and the weakening of the larval cuticle could be explained if in *dCREB-A* mutants these proteins are not produced at sufficient levels. Transmission electron micrographs of the salivary glands and epidermis of *dCREB-A* mutants may reveal similar defects in the two tissues. Alternatively, since there are D/V patterning defects in the cuticles of *dCREB-A* mutant larvae, salivary glands may require D/V patterning information to orient themselves. If D/V patterning information is lost in the *dCREB-A* mutants, we might predict crooked salivary glands. Finally, the defects in the epidermis and salivary glands in the *dCREB-A* mutants could be unrelated. The identification of downstream targets for *dCREB-A* should reveal more about *dCREB-A* function in the epidermis and salivary gland as well as in other tissues that express the gene but do not have obvious embryonic defects when *dCREB-A* function is missing.

Conclusions

We have characterized the first loss-of-function mutation in a *Drosophila* CREB family member, the *dCREB-A* gene. Past studies have implicated CREB proteins in LTP, growth regulation, and other transcriptional activation events linked to extracellular signaling. Our study suggests that *dCREB-A* participates in two critical signaling cascades potentiated by the secreted molecules, SPI and DPP, members of the TGF α and TGF β family, respectively. It will be exciting to test if CREB proteins function in related pathways controlled by the vertebrate homologues of TGF α and TGF β , as is suggested by studies in cell culture (Kramer et al., 1991; Ginty et al., 1994; Xing et al., 1996).

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