Development 117, 135-148 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Homeostatic balance between dorsal and cactus proteins in the *Drosophila* embryo

Shubha Govind†, Lynn Brennan* and Ruth Steward

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

*Present address: Insecticide Discovery, American Cyanamid, PO Box 400 Princeton, NJ 08543-0400, USA
†Author for correspondence

SUMMARY

The maternal-effect gene *dorsal* encodes the ventral morphogen that is essential for elaboration of ventral and ventrolateral fates in the *Drosophila* embryo. Dorsal belongs to the rel family of transcription factors and controls asymmetric expression of zygotic genes along the dorsoventral axis. The dorsal protein is cytoplasmic in early embryos, possibly because of a direct interaction with cactus. In response to a ventral signal, dorsal protein becomes partitioned into nuclei of cleavage-stage syncytial blastoderms such that the ventral nuclei have the maximum amount of dorsal protein, and the lateral and dorsal nuclei have progressively less protein.

Here we show that transgenic flies containing the *dorsal* cDNA, which is driven by the constitutively active *hsp83* promoter, exhibits rescue of the *dorsal*$^{-}$ phenotype. Transformed lines were used to increase the level of dorsal protein. Females with dorsal levels roughly twice that of wild-type produced normal embryos, while a higher level of dorsal protein resulted in phenotypes similar to those observed for loss-of-function *cactus* mutations. By manipulating the *cactus* gene dose, we found that in contrast to a dorsal/cactus ratio of 2.5 which resulted in fully penetrant weak ventralization, a cactus/dorsal ratio of 3.0 was acceptable by the system. By manipulating dorsal levels in different *cactus* and dorsal group mutant backgrounds, we found that the relative amounts of ventral signal to that of the dorsal-cactus complex is important for the elaboration of the normal dorsoventral pattern. We propose that in a wild-type embryo, the activities of dorsal and cactus are not independently regulated; excess cactus activity is deployed only if a higher level of dorsal protein is available. Based on these results we discuss how the ventral signal interacts with the dorsal-cactus complex, thus forming a gradient of nuclear dorsal protein.

Key words: *Drosophila*, *dorsal*, *cactus*, dorsoventral, polarity, morphogen, signal transduction

INTRODUCTION

Positional information along the dorsoventral axis in the *Drosophila* embryo is initially specified by the activities of several maternally expressed genes that function to create a gradient of nuclear dorsal (dl) protein (Steward et al., 1988; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; for recent reviews see Govind and Steward, 1991; Ferguson and Anderson, 1991). The activity of dl is controlled by its relocalization from the cytoplasm to the nucleus. Dorsal belongs to the rel family of transcription factors; its function as a transcriptional activator and repressor of zygotic genes is critical in the establishment of embryonic dorsoventral polarity. Nuclear dl protein spatially restricts the expression of zygotic genes along the dorsoventral axis in a concentration-dependent manner, which ultimately results in blastoderm cells assuming their appropriate identities.

The 300 amino acid 'rel domain' is shared by members of the rel family of proteins which include dl, c-rel and the p50 and p65 subunits of NF-κB (Chen et al., 1983; Wilhelmsen et al., 1984; Steward, 1987; Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991; Ruben et al., 1991). The nuclear import of the rel proteins is regulated by the structurally related IκB-α, IκB-β/pp40 and cactus (cact) proteins (reviewed by Lienhard Schmitz et al., 1991; St. Johnston and Nüsslein-Volhard, 1992). dl is a sequence-specific DNA-binding protein that recognizes a motif akin to the one recognized by the mammalian transcription factor NF-κB (Ip et al., 1991; Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991). dl protein self-associates, and most probably functions as a homodimer (Govind et al., 1992).

Genes such as *snail* and *twist* are activated by high levels of nuclear dl protein ventrally and are required for the formation of mesoderm and mesectoderm (Thisse et al., 1987; Leptin and Grunewald, 1990; Alberga et al., 1991). Other genes like *zerknüllt*, *decapentaplegic* and *tolloid* are repressed by the same high dl concentrations ventrally and by lower dl concentration laterally (Rushlow et al., 1987; St. Johnston and Gelbart, 1987; Shimell et al., 1991). Therefore, they are expressed only dorsally and result in the production of the amnioserosa and dorsal epidermis.

Genetic studies have identified twelve maternal genes

that code for essential components of the pathway that establishes dorsoventral polarity (Nüsslein-Volhard, 1979; Anderson and Nüsslein-Volhard, 1984, 1986; Schüpbach and Wieschaus, 1989). Of these, eleven genes constitute the dorsal group of genes. Females that are homozygous for null mutations in any of these eleven genes produce dorsalized embryos that lack the ventral and lateral pattern elements and consequently all cells in the embryo produce dorsal epidermis and amnioserosa (Anderson and Nüsslein-Volhard, 1986). In contrast, loss-of-function mutations in *cact* cause ventralization of the embryo: homozygous mutant females produce embryos which show a decrease or loss of dorsal epidermis and expansion of the ventrolateral neurogenic epidermal anlagen (Schüpbach and Wieschaus, 1989; Roth et al., 1991). In addition to the loss-of-function alleles, some gain-of-function alleles of the dorsal group genes (for example *Toll* and *easter*) have been isolated (Anderson et al., 1985a; Schneider et al., 1991; Chasan and Anderson, 1989). These exhibit lateralizing or ventralizing phenotypes. However, gain-of-function mutations in *cact* result in dorsalization of the embryo (Roth et al., 1991).

The selective relocalization of dl is positively regulated by the dorsal group gene products but negatively controlled by cact protein. In dorsalized embryos, dl protein remains in the cytoplasm. However, in ventralized embryos the dl nuclear gradient is extended dorsally (Steward, 1989; Roth et al., 1989, 1991). Thus, mutations in any of the dorsal group genes or *cact* result in an altered slope of the nuclear dl gradient, leading to altered cell fates. Based on these genetic and antibody staining experiments, a direct interaction between the dl and cact proteins was proposed (Roth et al., 1991; Govind and Steward, 1991). This complex would be disrupted by a ventral signal, encoded by the dorsal group genes acting upstream of *dl* and *cact* (Hashimoto et al., 1988, 1991; Stein et al., 1991). Here we scrutinize this model by exploring the quantitative nature of the activities by varying the relative levels of dl, cact and the ventral signal. We have used a construct consisting of the *dl* cDNA driven by the maternally active *hsp83* promoter which rescues the dl^- phenotype to modulate the endogenous levels of dl protein.

An initial increase in dl protein levels above wild type did not have any phenotypic effect. However, a roughly two-fold excess led to saturation of the cytoplasmic inhibitory activity of cact, resulting in partial ventralization of embryos. Manipulation of dl protein levels in *cact* mutant backgrounds showed that there is a homeostatic balance between dl and cact proteins and that the concentration of the dl-cact complex is important for elaboration of the wild-type phenotype. We propose that the ventral signal affects both dl and cact proteins to result in graded nuclear localization of dl protein. In this model free or bound dl may be recognized by the ventral signal, while only bound cact is a target. In addition, the level of the ventral signal relative to that of the dl-cact complex is also important for obtaining the wild-type dorsoventral pattern.

MATERIALS AND METHODS

Plasmids and constructs

hsp83dl: A *Bam*HI-*Rsa*I fragment of the *hsp83* promoter was cloned into the *Bam*HI-*Eco*RV site of BS+. This approximately 900 bp region contains the heat shock and ovary-specific enhancer elements (Zimmerman et al., 1983). A *Sma*I-*Sal*I fragment of *dlB6* (Steward, 1987), containing the entire *dl* coding region, 270 bp of the 5′-non-coding region, 135 bp of the 3′-non-coding region including the putative poly(A) signal, was introduced into the *Sal*I-*Hin*dIII (blunt) fragment of the *hsp83*BS+ plasmid. This insert was then transferred to the pCaSpeR transformation vector (Pirrotta, 1988) as a *Not*I-*Kpn*I fragment which contains the w^+ gene for selection of transformants (Klemenz et al., 1987).

hsp83dlSpe: This is the *Spe*I fragment of the *hsp83dl* in BS+ construct described above (Fig. 1A). The *hsp83* promoter contains a *Spe*I site at the −488 position (Xiao and Lis, 1989). This construct therefore lacks 392 bp when compared to the parent *hsp83dl* plasmid. The *Spe*I site in the 3′ trailer excludes the putative *dl* poly(A) signal (it is 57 bp 5′ to the putative poly(A) signal). Therefore, the poly(A) signal in the 3′ repeat of the P-element in pCaSpeR was most likely used in vivo. Both the *hsp83dl* and *hsp83dlSpe* constructs were made simultaneously to see if one worked better than the other.

Drosophila strains

The following genotypes were used for crosses described.

dl stocks: *al dp b Df(2L)TW119 cn bw/b Cy, b pr cn sca In(2L)dlH/b Cy, b pr cn sca In(2L)dlT/b Cy* (Steward and Nüsslein-Volhard, 1986), *al dp b* dl^1*pr cn sca sp/Cy* (protein null allele of *dl*, Roth et al., 1989).

cact stocks: *b* $cact^{A2}$*/CyO,* $cact^{PD}$*cn bw/CyO,* $cact^{E10}$*/CyO, b* $cact^{E10}$ *px sp/CyO, Df(2L)E10RN2/CyO,* (this is a revertant of $cact^{E10}$ and causes complete loss of *cact* function), *Df(2L)TE116GW21/CyO, b* $cact^{BQ}$ *pr cn/CyODTS100* (see Roth 1990; Roth et al., 1991 for description of *cact* alleles).

dl group stocks: spz^{rm7} *ru th st ri roe* p^p *e/TM3* and pll^{122} *ru e wo ro ca/TM3Sb* were obtained from K. V. Anderson, Tl^{rm9} *e/TM3,* Tl^{rm10} *e/TM3,* Tl^{10b} *mwh e /TM3 Sb Ser/T(1;3)OR60, Df(3R)* ro^{80b} *st e/TM3,* Tl^{r444} *st e/TM3 Ser* (Schnieder et al., 1991), $ea^{5.13}$ *th st c p in n* p^p*/TM3/T(1;3)OR60* (Chasan and Anderson, 1989).

Other stocks: w^1/w^1*;* Δ*2-3/*Δ*2-3* (Robertson et al., 1988), double balancer stocks *CyO/Sp;* ry^{506} ftz^{9H34} e^s*/TM3 Sb* and *CyO/Sp;* svp^{e22}*/TM6 Ubx* were obtained from Y. Hiromi, $Dp(2;3)osp^3$ *Ki* (Mohler and Wieschaus, 1986), tud^1*/CyO* (Boswell and Mahowald, 1985).

P-element transformations and genetic crosses

To obtain flies carrying DNA inserts in their germline (Spradling and Rubin, 1982), w^1/w^1*;* Δ*2-3/*Δ*2-3* virgins were mated with w^1/Y males. Eggs collected half an hour after laying were dechorionated and injected with different DNA constructs. All injected adults were mated with w^1 flies and transformants were screened by eye color.

The chromosome carrying the *hsp83dl* cDNA insert was crossed into dl^- background according to the scheme shown in Fig. 1B. Twenty-one independent stably transformed lines were obtained for the *hsp83dlSpe* construct, whereas 15 lines were isolated for the *hsp83dl* construct. Individuals from 8 lines for the *hsp83dlSpe* and 12 lines for the *hsp83dl* construct were crossed into *dl* null background ($In(2L)dl^T/In(2L)dl^H$ or $In(2L)dl^T/Df(2L)TW119$) where no dl-specific poly(A) RNA is detected (Steward et al., 1984). An embryo was considered completely rescued if it hatched into a viable larva. Six of the 8 lines carrying the *hsp83dlSpe* insert and 6 of the 12 lines with the *hsp83dl* insert showed some rescue. In addition to the variability among different lines, the expressivity was variable among individual females of a single line. Standard crosses were performed to introduce the w^1 mutation into mutant *dl*, dorsal group or *cact* backgrounds. Subse-

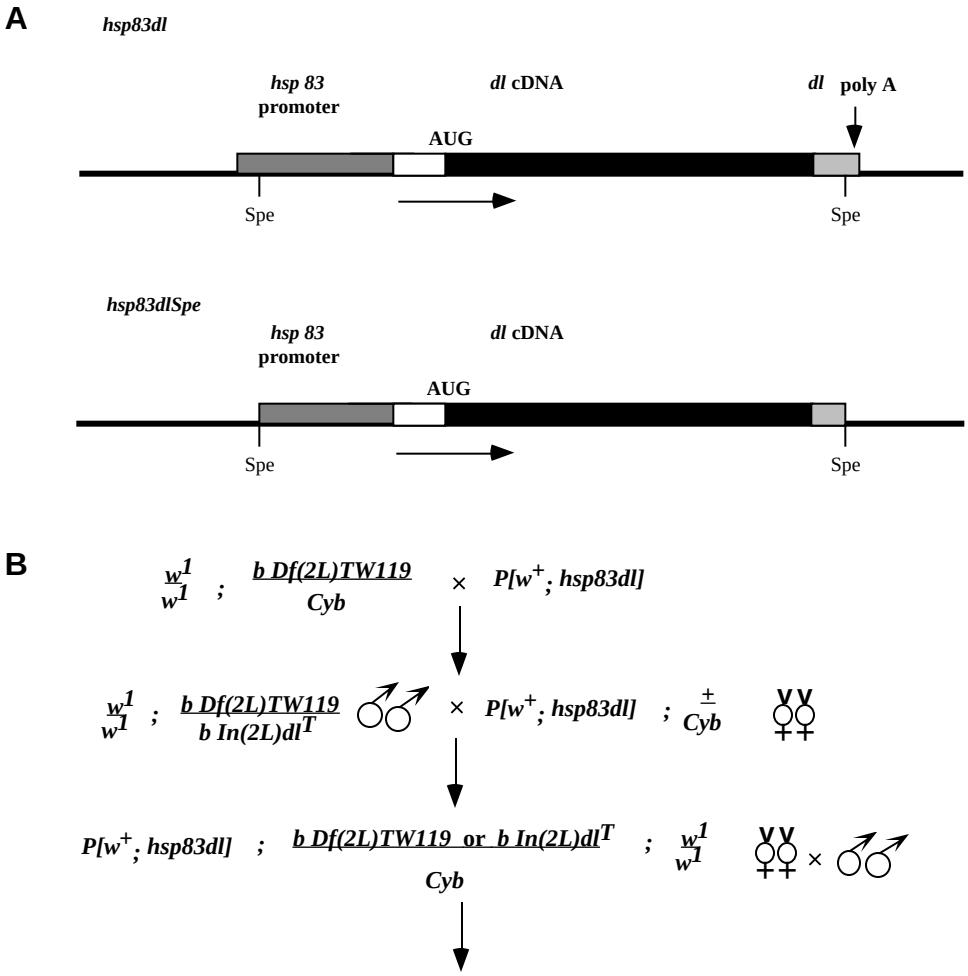

Fig. 1. (A) Structures of the *hsp83dl* cDNA constructs in pCaSpeR used for P-element germ-line transformation. *hsp83dl* and *hsp83dlSpe* contain the constitutively active and maternally expressed heat shock 83 promoter driving the *dl* cDNA. These constructs were made in the transformation vector pCaSpeR and inserted in the *Drosophila* germ-line as described in the Materials and methods and transformants were isolated on the basis of eye color. Both these constructs showed similar properties in their ability to rescue. (B) Crosses done to transfer the transgenic insert into dl^- background. For complete genotypes of chromosomes used, see Materials and methods. These crosses were performed when the transgenic insert was either on the 1st or the 3rd chromosomes. Inserts on the 2nd chromosome were recombined with the $In(2L)dl^T$ or the *Df(2L)TW119* chromosomes. Number of inserts was determined on the basis of eye color.

quently, the w^+ *hsp83dl* insert was introduced, either by ordinary crosses or by meiotic recombination. Females of the desired genotype were selected on the basis of markers in the stock.

Morphology of embryos

Cuticle preparations of embryos were performed after dechorionating them as described by Wieschaus and Nüsslien-Volhard (1986). Analysis of cellular movements during early gastrulation was done by using trans-illumination and time-lapse recording (Merrill et al., 1988). Embryos were fixed in formaldehyde and devitellinized essentially as for antibody staining as described in Steward, 1989. They were processed for scanning electron micrography according to the procedure described in Sweeton et al., 1991. Antibody stained embryos were sectioned according to the procedure of Wieschaus and Nüsslein-Volhard (1986).

A classification of phenotypes resulting from complete or partial loss of dl protein function was proposed by Anderson et al. (1985a), and has been modified by Roth et al. (1991). These phenotypes have been defined on the basis of analysis of cuticle structures, gastrulation pattern and use of cell-type markers such as anti-twist and anti-zen antibodies (for details see Roth et al., 1991). According to this classification, weak to progressively stronger dorsalization is described by the D3, D2 and D1 phenotypes. Embryos deficient in the dl protein, or those unable to localize it to the nuclei, show the D0 phenotype. In these embryos, all cells develop to form the extraembryonic amnioserosa and the dorsal epidermis, structures that do not require the function of dl protein. In the ventralized embryos (V4 to V0), the ventral and lateral structures are expanded at the expense of the dorsal pattern elements. In an analogous series, weak ventralization to complete ventralization is represented by the V4, V3, V2, V1 and the V0 phenotypes. Lateralized embryos show a loss of dorsal and ventral structures. Instead they gain lateral structures at different positional levels resulting in L1 to L3 (ventrolateral-dorsolateral) embryos. We adhere to the classification of Roth et al., 1991 to describe the rescuing activities of the *hsp83dl* cDNA constructs and those of overexpression of dl in wild-type and mutant backgrounds.

RESULTS

Increasing levels of nuclear dl protein are required for the production of structures with progressive ventral positional values. Genetic studies and antibody staining experiments have suggested that the dl protein is sequestered in the cytoplasm through its interaction with cact protein. The nuclear import of dl protein, and therefore its function, is promoted by the action of the signal transduction pathway mediated by the Toll receptor and pelle and tube proteins (Roth et al., 1989, 1991; Steward, 1989; Letsou et al., 1991). One

important step in nuclear targeting of dl relies on the specific dissociation of dl from cact. To understand how this process is regulated such that the opposing effects of *cact* and the dorsal group signal result in the graded import of cytoplasmic dl protein, we manipulated the levels of the different gene products in the pathway. We specifically increased dl level in wild-type and different mutant backgrounds and analyzed their phenotypes. A correlation between the nuclear gradient of dl, the expression pattern of twist and zerknüllt proteins as well as the corresponding mutant phenotypes has been established by S. Roth (Roth, 1990; Roth et al., 1989; 1991). Because of this correspondence, we relied primarily on phenotypic analysis to evaluate our results.

The *dl*⁻ phenotype is completely rescued by *hsp83dl* constructs

In order to manipulate the levels of dl protein in vivo, we needed to establish that a *dl* transgene could substitute for the endogenous *dl* gene. The *hsp83* promoter was used to drive the expression of a *dl* cDNA. Unlike *hsp70*, which shows little, if any, constitutive expression (Lindquist, 1986), the *hsp83* promoter shows constitutively high maternal expression largely similar to that of *dl* (Zimmerman et al., 1983; Mason et al., 1984, Ambrosio and Schedl, 1984; Steward et al., 1985; Xiao and Lis, 1989). Two constructs containing the entire *dl* coding region, and the partial 5′- and 3′-non-coding regions, under the control of the *hsp83* promoter (*hsp83dl* or a fragment of this promoter called *hsp83dlSpe*; see Materials and methods and Fig. 1A for details), were injected into embryos. To assess the degree of rescue of the *dl*⁻ phenotype, embryos derived from transgenic females that are null for endogenous *dl*, were examined for hatching and cuticular phenotypes.

A wide range of rescue (Fig. 2 and Table 1) was observed among the different lines. In two transformed lines (lines 2 and 38), one copy of the *hsp83* driven *dl* cDNA rescued a majority of the embryos completely, and partially rescued the remaining embryos. While line 38 is homozygous lethal, line 2 shows almost complete rescue (80% hatching) when present in two copies in a *dl* null background. This 80% rescue is roughly equal to that conferred by a single wild-type *dl* gene (Table 1). Since at room temperature, the *dl* null chromosomes show a back-

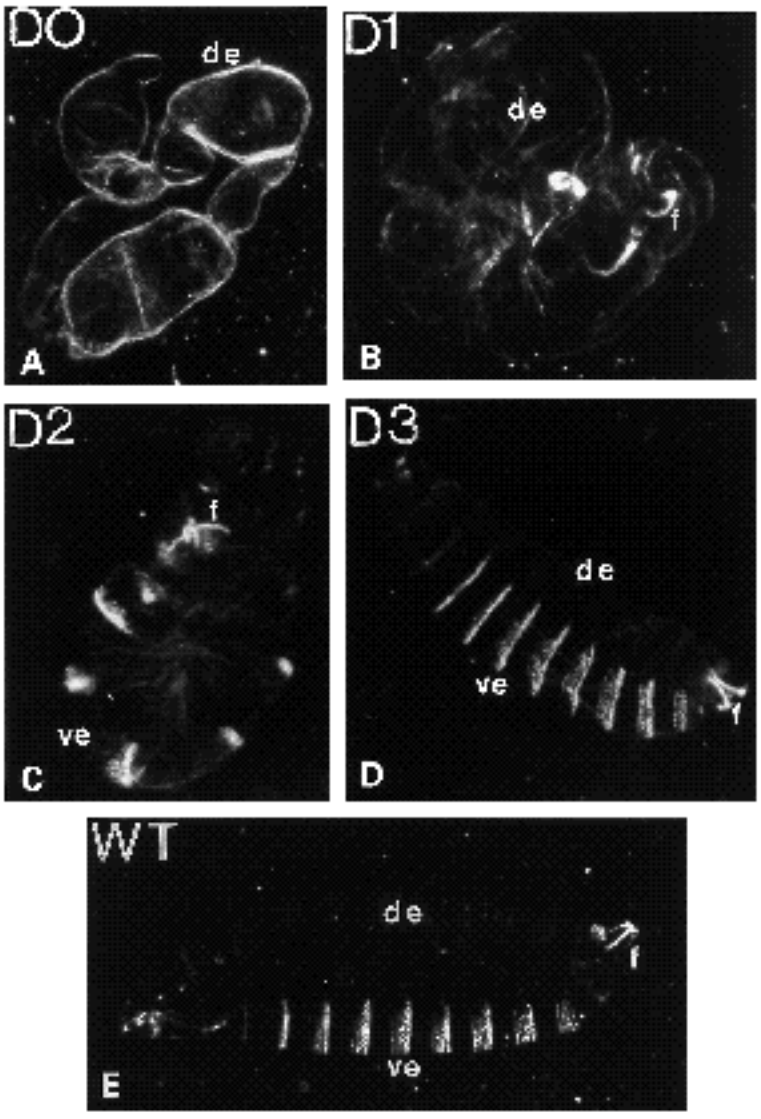

Fig. 2. Rescue of *dl*⁻ phenotype by *dl* cDNA constructs. Phenotypes of the unhatched embryos ranged from almost normal looking larvae that did not hatch (D3 phenotype), to strongly dorsalized (D1) embryos. In transformant lines that showed intermediate rescue (about 50% hatch), a greater fraction of the cuticles exhibited lateral and ventral structures of the D2 and D3 phenotypes. Percentage hatch correlated with phenotypic strength. A majority of the embryos showed the D1 phenotype, where only the more dorsally derived structures, such as the filzkörper, are formed. (A-E) Cuticle preparations of embryos from *dl*⁻ mother, transformed lines in *dl*⁻ background photographed under dark-field optics. de, dorsal epidermis; f, filzkörper; ve, ventral epidermis marked by the ventral setae. Cuticles show the control *dl*⁻ D0 phenotype (A) and the progressively weaker dorsalized phenotypes (D1, D2, and D3 in B, C and D, respectively). E shows a wild-type larva.

Table 1. Rescue of *dl*⁻ embryos by *hsp83dl* cDNA constructs

Construct	No. of inserts	Linkage	% Hatch
hsp83dlSpe			
2*	1	3	63
2	2	3	80
4	2	3	0
13	2	3	0
18	1	2	28
18	2	2	40
hsp83dl			
38	1	2	66
51	1	3	57
51	2	3	68
Untransformed controls			
$In(2L)dl^T/Df(2L)TW119;w^1/w^1$			0
$In(2L)dl^T/In(2L)dl^H;w^1/w^1$			0
$In(2L)dl^T/+;w^1/w^1$			10
$In(2L)dl^H/+;w^1/w^1$			16
$Df(2L)TW119/+;w^1/w^1$			83

*Genetic background for line 2 for testing rescue is $In(2L)dl^T/In(2L)dl^H$. It is $In(2L)dl^T/Df(2L)TW119$ for all the other lines.

The fraction of rescued embryos derived from lines containing either the *hsp83dl* or the *hsp83dlSpe* construct. Numbers represent average rescue of a large number of embryos (300-800) derived from several females. Unfertilized eggs were excluded. Number of inserts was determined by intensity of eye color. Transformed flies were crossed into *dl*⁻ background according to steps shown in Fig. 1B.

ground-dependent *dl* haploinsufficient phenotype, the precise activity derived from the *hsp83dl* inserts in these backgrounds is difficult to assess. Only about 10% of the eggs laid by *b pr cn sca* $In(2L)dl^T/+$ females and 16% of those laid by *b pr cn sca* $In(2L)dl^H/+$ females hatch at room temperature and the unhatched embryos show partial dorsalization (Table 1).

Cell fates of rescued embryos shift along a dorsoventral continuum such that with increasing dl protein, increasing rescue of the *dl* null phenotype is observed (Fig. 2A-E). This was true of all lines that showed at least some rescue, except line 38 which resulted in embryos with a weakly ventralized phenotype. When embryos from different lines were stained with the anti-dl antibody, a gradient of nuclear dl protein of varying intensities was seen in the syncytial blastoderm stage embryos (Fig. 3A-C). A majority of embryos from line 2 showed strong staining, indistinguishable from wild-type embryos.

Protein extracts from transformed embryos in *dl*⁻ and wild-type backgrounds were found to correlate with rescue levels. As seen in Fig. 4, the level of dl protein derived from 1 insert of line 2 in *dl*⁻ background is roughly between 1/3 and 1/2 of that observed in w^1/w^1; dl^+/dl^+ embryos (compare lanes 1 and 3). dl level is much higher in line 38, where 1 insert codes for more dl protein than that derived from 2 endogenous *dl* genes (compare lanes 1, 4 and 6), consistent with the phenotype of line 38 in both wild-type and *dl*⁻ backgrounds. Based on the rescue and Western blot experiments, we established that two copies of the transgene in *hsp83dlSpe* line 2 are equivalent to one copy of the endogenous *dl* gene. The transgenic lines provided us with the tools to specifically modify the endogenous levels of dl protein.

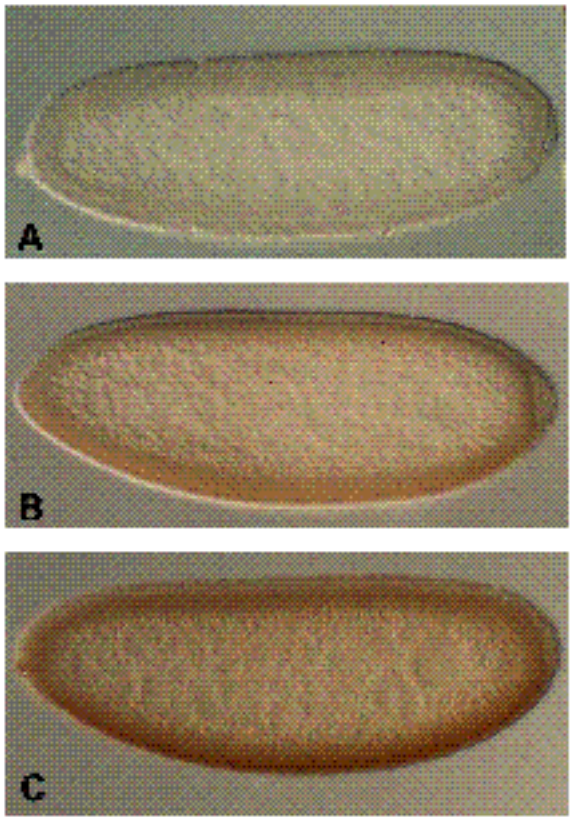

Fig. 3. Dorsal protein distribution in embryos derived from *hsp83dlSpe* line 2 transformed females that are deficient in endogenous dorsal protein. Embryos from blastoderm stage were stained with anti-dl antibody. (A-C) Staining intensity ranged from undetectable protein levels to those found in wild-type embryos and correlated with rescue levels. The ventral-to-dorsal gradient of nuclear staining and cytoplasmic dorsal staining is like that found in wild-type embryos (e.g., Fig. 6A).

Overexpression of dl protein copies the loss-of-function mutant *cact* phenotype

The observation that one insert in line 2 does not exhibit any phenotypic abnormalities in a wild-type background suggests that wild-type *cact* activity is at least in some excess of that required for inhibition of wild-type dl levels. However, since *cact* shows a maternal haploinsufficiency (Roth et al., 1991), this excess is not expected to be large. Thus, a substantial overexpression of dl is predicted to saturate the cytoplasmic retention capacity of cact.

While the phenotypic effects of dl produced from homozygous transgenic lines 2, 51 and 2.51 (chromosome carrying both inserts 2 and 51) is only slight (80-91% embryos hatch), one insert in line 38 showed a clear effect and only 71% of the embryos hatched. The percentage hatch was further reduced when one insert of line 38 was combined with an insert in lines 2 or 2.51, where only 38% or 36% of the embryos hatched (Table 2 and Fig. 5). A correlation between percentage hatch and phenotype of unhatched embryos was observed. Whereas cuticles from embryos of lines 2 and 51 showed the weak V4 phenotype, those derived from embryos of heterozygous line 38 or line 38 with 2 or 2.51 showed loss of dorsal structures and

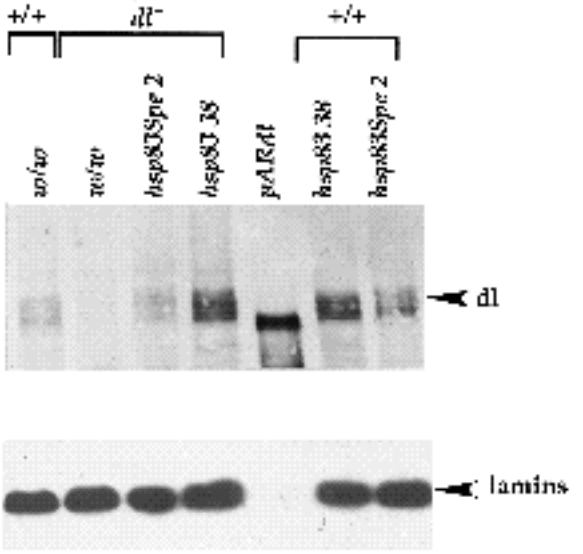

Fig. 4. *dl* protein levels in the transformed lines correlate with the extent of rescue. Western analysis of protein extracts obtained from transformed and wild-type embryos was done according to Steward, 1989. Genotypes of females from which the embryonic extracts were made are indicated on the top of the blot. *w/w* is the extract that serves as the control for wild-type (+/+) dl protein. dl^- indicates extract from embryos produced by the dl protein deficient *In(2L)*dl^T*/Df(2L)TW119* females. *hsp83 38* and *hsp83Spe 2* are extracts from embryos expressing 1 copy each of the corresponding insert in the dl^- background. *pARdl* refers to dl protein expressed in bacteria. At least 4 different forms of dl protein are visible in these embryonic extracts.

Table 2. Additional dl protein causes ventralization of wild-type embryos

Line	Number of *hsp83dl* inserts	% Hatch	Phenotype
w^1/w^1	0	95	no d/v defects
51	2	91	no d/v defects
2	2	80	V4
2.51	4	86	V4
38*	1	71	V3-V4
38 and 2	2	38	V3-V4
38 and 2.51	3	36	V3-V4

*Zygotic lethal.

Phenotypes of embryos in wild-type background derived from those lines that independently showed medium to high level of rescue in dl^- background (see Table 1) were examined. One insert of line 38 expressed high level of dl protein and also showed some ventralization even in dl^- background. Lines 2 and 51 carry insertions on the third chromosome and were recombined to generate line 2.51. Recombinants were selected on the basis of eye color. Lines 2, 38 and 51 were also combined. 300-500 eggs were scored for larvae hatching at room temperature. Unfertilized eggs were excluded. The differentiated cuticle of unhatched embryos showed weakly ventralized phenotype (Fig. 5).

dorsoventral pattern defects corresponding to both the V4 and V3 phenotypes (Fig. 5A,B; for description of phenotypes, see legend to Fig. 5 and Roth et al., 1991). The V4 phenotype is comparable to the maternal *cact* haploinsufficient phenotype shown by embryos from $cact^{A2}$/+ females (Fig. 5C), while V3 phenotype is similar to that seen in embryos derived from homozygous $cact^{PD}$ females (Fig. 5D). Embryos laid by females overexpressing dl protein were also stained with anti-dl antibody. The staining intensity ranged from near wild-type levels (Fig. 6A,B) of a spa-

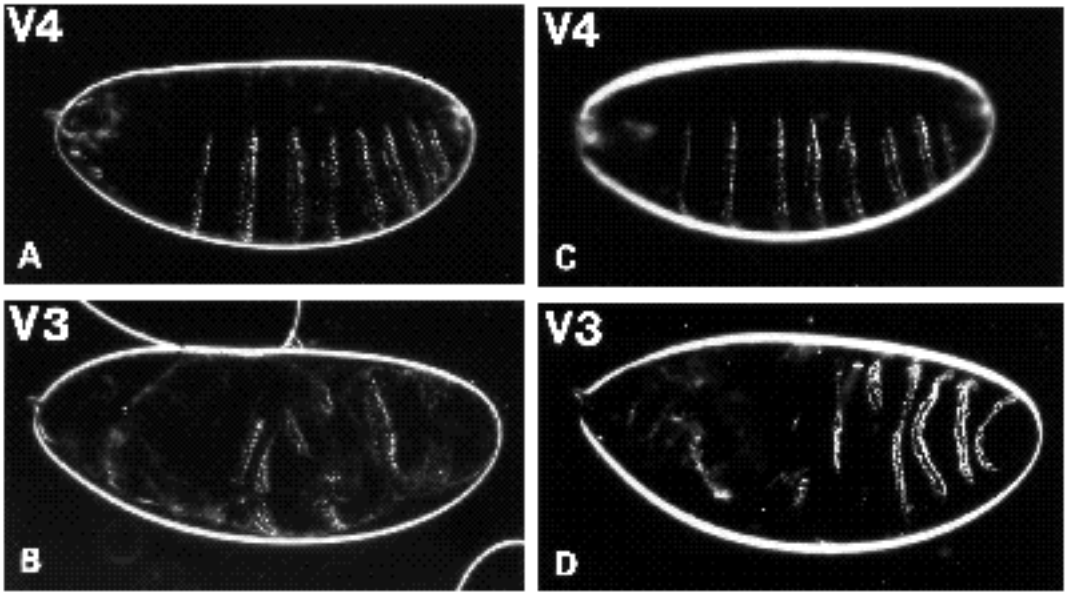

Fig. 5. Saturation of the cact inhibitory activity by elevated dl levels results in partial ventralization. In the ventralized embryos, the ventral and lateral structures are expanded at the expense of the dorsal pattern elements. The V4 embryos have reduced amnioserosa, dorsal epidermis, the filzkörper and antennal sense organ structures. In embryos that exhibit the V3 phenotype, the dorsally derived amnioserosa, dorsal epidermis, filzkörper and the antennal sense organs are absent, and the lateral epidermis and anal plates are reduced. Instead, the ventral epidermis is expanded (Roth et al., 1991). (A, B) Cuticle preparations of embryos expressing the *hsp83dl* cDNA in line 38 that failed to hatch. Cuticles show weak V4 and stronger V3 phenotypes respectively. (C, D) Cuticle preparations of embryos derived from females heterozygous for $cact^{A2}$ mutation and homozygous for $cact^{PD}$ mutations that exhibit the V4 and V3 phenotypes respectively. Note expansion of the ventral epidermis and reduction of dorsal structures.

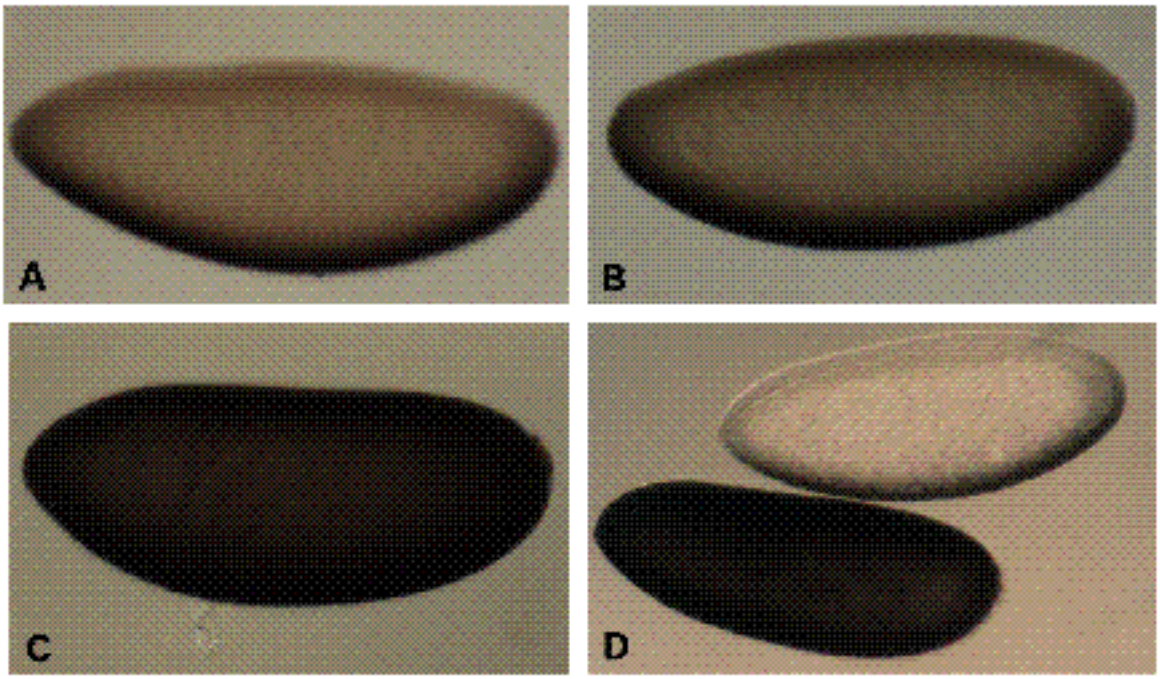

Fig. 6. Elevated dorsal protein levels in embryos derived from females transformed with *hsp83dlSpe* and *hsp83dl* in wild-type background. (A) Embryo from tud^1/tud^1 female, (B,C) embryos from females of *hsp83Spe* line 2 and (D) embryo from females of line 38 compared with those derived from tud^1/tud^1 females. tud^1/tud^1 embryos were mixed with those of line 2 or line 38 and simultaneously stained with anti-dorsal antibody. Note higher levels of dorsal protein in transformed embryos.

tially normal gradient of nuclear dl protein to more pronounced staining (Fig. 6A,C,D) where the higher levels of stain apparently obscured the gradient. As expected, this partial ventralization was achieved by partial repression of zerknüllt expression, while twist expression was not affected (data not shown).

Since 2 inserts in line 2 showed only a marginal effect on percentage hatch and phenotype (Table 2), cell movements of these transformed embryos during gastrulation were compared to those in wild-type embryos by time-lapse video recording. Three of the seven embryos analyzed, showed clear gastrulation defects, similar to those described for homozygous mutant *zerknüllt* embryos (data not shown, Rushlow and Levine, 1990).

Additional dl protein in mutant *cact* backgrounds causes ventralization

To gain further insight into the relative activities of dl and cact proteins within the embryo, dl protein was increased above wild-type levels by crossing *hsp83dlSpe* line 2 into different mutant *cact* backgrounds. Depending on the strength of the *cact* allele used, different levels of ventralization were observed. We compared percentage hatching as well as the phenotypes of embryos laid by heterozygous or hemizygous *cact* mothers to those that in the same genetic background, carry one insert in *hsp83dlSpe* line 2 (Table 3A).

Females homozygous for the weak loss-of-function allele, $cact^{PD}$ (Roth et al., 1991) produce embryos that exhibit the V3 phenotype (Fig. 5D). Females heterozygous for $cact^{PD}$ show virtually no maternal haploinsufficiency and 92% of the fertilized eggs hatch (Table 3A). In the presence of additional dl protein, the $cact^{PD}$ dominant phenotype becomes significantly more severe and only 11% of the fertilized embryos hatch. Unhatched embryos show the weaker V4 phenotype.

Females homozygous for the strong $cact^{A2}$ allele produce embryos completely lacking the lateral epidermis as well as the more dorsally derived structures, giving rise to the V2 phenotype (Roth et al., 1991). While 72% of embryos derived from $cact^{A2}$/+ females hatch, those derived from females that have additional dl in this genetic background ($cact^{A2}$/+, *hsp83dlSpe* line 2) do not hatch at all, and exhibit the V4 phenotype. Similar results were obtained when the *cact* deficiency (*DfTE116GW21*) was used instead of $cact^{A2}$. Thus, in the presence of one wild-type dose of *cact*, only a slight overexpression of dl (one insert of line 2) is sufficient to achieve a completely penetrant ventralized phenotype. This result suggests that at least some 'free' dl protein that is not retained by cact becomes nuclear, resulting in the observed ventralized phenotype.

In addition to the loss-of-function alleles, two gain-of-function alleles of cact $cact^{E10}$ and $cact^{BQ}$ have been described (Roth et al., 1991). Females homozygous for $cact^{E10}$ or $cact^{BQ}$, or in trans to *cact* deficiency result in strongly dorsalized or dorsolateralized phenotypes (Fig. 7A and Table 3A; also see Roth et al., 1991). Females transheterozygous for $cact^{E10}$ or $cact^{BQ}$ and a loss-of-function *cact* allele result in embryos in which ventral structures are progressively lost at the expense of dorsal structures as the strength of the loss-of-function *cact* allele increases. Thus, while $cact^{E10}/cact^{PD}$ females produce weakly dorsalized

Table 3A. Additional dorsal protein in mutant *cactus* backgrounds causes ventralization

	No construct		Construct: *hsp83dl* line 2	
Genetic background	%H	Phenotype	%H	Phenotype
Loss-of-function alleles				
$cact^{PD}$/+	92	wt	11	V4
$cact^{PD}/cact^{PD}$	0	V3	nd	
$cact^{A2}$/+	72	V4	0	V4/V3
$cact^{A2}/cact^{A2}$	0	V2	nd	
DfTE116GW21/+	70	V4	0	V3
Gain-of-function alleles				
$cact^{E10}$/+	72	wt/D3	77;82†·*	wt/D3
$cact^{E10}$/*Dfcact*	0	L2	Zygotic lethal	
$cact^{E10}/cact^{PD}$	0	D2	0	V4
$cact^{E10}/cact^{A2}$	0	L2-D1	0	L1
$cact^{E10}/cact^{E10}$	0	L2, D0	nd	
$cact^{BQ}$/+	65	wt	nd	
$cact^{BQ}/cact^{PD}$	0	D2	0	V2
$cact^{BQ}/cact^{A2}$	0	D1	0	L1
$cact^{BQ}/cact^{BQ}$	0	L2-D0	nd	
$cact^{BQ}/cact^{E10}$	0	D0	no significant effect	
Controls				
b Tft/CyO; w^1/w^1	97			

*% hatch control (*b Tft/CyO;* w^1/w^1) for corresponding genetic background $cact^{E10}$/+.

†Numbers for one and two copies of *hsp83dlSpe* line 2 respectively. nd=not done.

Weak ($cact^{PD}$), strong ($cact^{A2}$) and null (*DfTE116GW21*) alleles of *cact* were used to titrate the extra dl protein produced from one copy of *hsp83dlSpe* line 2. In addition, the transgene in line 2 was introduced into genetic backgrounds with homozygous gain-of-function *cact* alleles ($cact^{E10}$ and $cact^{BQ}$), or gain-of-function in trans to the loss-of-function *cact* alleles. %H refers to percentage of embryos that hatched after excluding unfertilized eggs.

Table 3B. Expression of dorsal protein from the *dl* transgene rescues the $cact^{E10}/dl^1$ phenotype

	No construct	Construct: *hsp83dlSpe* line 2	
	%Hatch and	%Hatch	
Genetic background	phenotype	1 copy	2 copies
$cact^{E10}$ +/+ dl^1	0; D2	65	87
controls			
$cact^{E10}$/+	72; wt/D3	77	82
al dp b dl^1 *pr cn sca sp*/+	46; D3	−	−

One or two copies of the *hsp83dlSpe* line 2 were crossed into the $cact^{E10}/dl^1$ background by using stocks with balancers for the 2nd and the 3rd chromosomes. $cact^{E10}$ is a neomorphic allele of *cact*. Embryos from homozygous dl^1 females are completely dorsalized (D0) and the dl protein in these embryos is not detected (Roth et al., 1989).

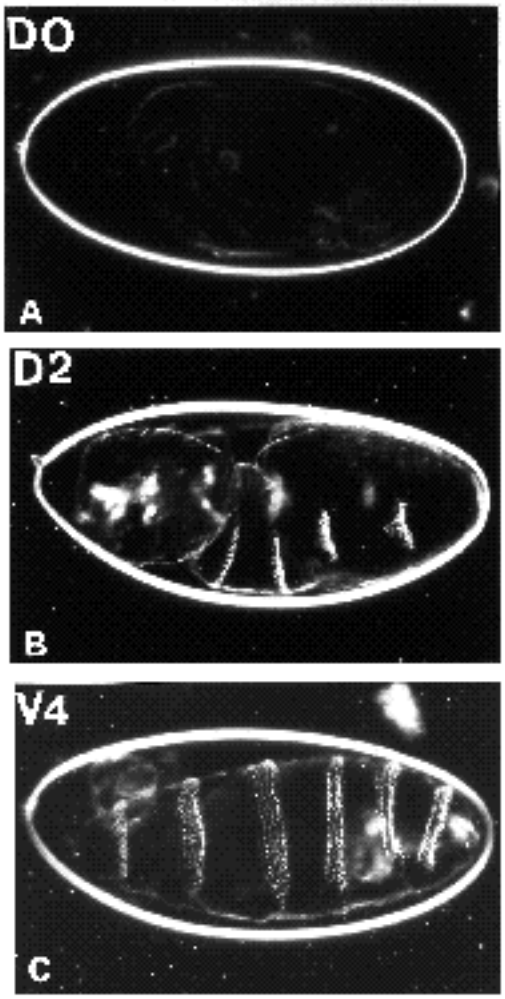

Fig. 7. Effect of overexpression of dl protein in females transheterozygous for loss-of-function and gain-of-function alleles of *cact*. Cuticle preparations of embryos derived from $cact^{E10}/cact^{E10}$ (A), $cact^{E10}/cact^{PD}$ (B) and $cact^{E10}/cact^{PD}$, with one copy of *hsp83dlSpe* line 2 (C) females. These embryos show the strongly dorsalized D0, weakly dorsalized D2 and the weakly ventralized (V4) phenotypes respectively. For explanation of the V4 phenotype, see legend to Fig. 5. Dorsalization results in progressive loss of ventral pattern elements; D2 embryos have no mesoderm and reduced ventral epidermis. Embryos deficient in the dorsal protein, or those unable to localize it to the nuclei, show the D0 phenotype. In these embryos, all cells develop to form the extraembryonic amnioserosa and the dorsal epidermis, structures which do not require the function of dorsal protein.

embryos (Fig. 7B and Roth, 1990), those derived from $cact^{E10}/cact^{A2}$ or $cact^{E10}/Dfcact$ females result in strongly dorsalized or dorsolateralized embryos (Roth, 1990). Extra dl protein derived from one copy of *hsp83dlSpe* line 2 shifted the dorsolateralized (L2-D1) phenotype of embryos derived from $cact^{E10}/cact^{A2}$ females to the ventrolateralized (L1) phenotype (Table 3A). The D2 phenotype of embryos derived from $cact^{E10}/cact^{PD}$ females was changed to a weakly ventralized (V4) phenotype (Fig. 7C and Table 3A). Similar effects were observed for the $cact^{BQ}$ allele (Table 3A). Thus, as in the case of wild-type and loss-of-*cact* function backgrounds, increasing amounts of dl protein shifts positional information ventrally in the gain-of-function *cact* backgrounds.

In trans to amorphic *dl* alleles, $cact^{E10}$ results in dorsalized embryos that show the D2 phenotype (Table 3B and Roth et al., 1991). This transheterozygous combination was rescued to the level of that of $cact^{E10}$/+ background in a dose dependent manner ($cact^{E10}$ +/+ dl^1; *hsp83dlSpe* line 2/+, Table 3B), when 1 or 2 inserts of line 2 were introduced.

Table 4. Effect of increasing the dose of *cactus* relative to *dorsal*

Genetic background	Number of *dl* genes	Number of *cact* genes	%Hatch
In(2L)dl^{T}/+;Dp (osp^{3}) Ki/+	2	3	95
In(2L)dl^{T}/Df(2L)TW119;Dp(osp^{3})Ki/+	1	3	92

The *Dp(osp^{3})* marked with *Ki* was used to increase *cact* activity over wild-type levels. This duplication contains both *cact* and *dl* genes. *In(2L)dl^{T}/In(2L)dl^{H}* chromosomes, null for dl protein, were used in conjunction with the duplication to create hemizygous *dl* flies containing 3 doses of *cact*.

Females with three copies of *cact* in hemizygous *dl* background produce normal embryos

To determine the effect of overexpression of cact protein in a hemizygous *dl* background, a duplication containing both the *dl* and the *cact* genes on the third chromosome (*Dp(2;3) osp^{3}*, Mohler and Wieschaus, 1986) was used. This duplication was crossed into a *dl*$^{-}$ background so that the resulting flies contained three *cact* gene doses but only one *dl* gene. This stock was viable and greater than 90% of the embryos hatched (Table 4) suggesting that in contrast to the >2*dl*:1*cact* condition described above, which gives a completely penetrant weakly ventralized phenotype, the 1*dl*:3*cact* condition is permitted by the system. These and other experiments allow us to define the range of dl and cact protein levels, within which the wild-type phenotype is realized (see Table 6 and Discussion).

Additional dl protein in gain-of-function dorsal group backgrounds shifts the nuclear dl gradient ventrally

Experiments described so far establish that the relative levels of *dl* and *cact* are relevant to regulating the slope of the nuclear dl gradient. Since the gradient of nuclear dl protein is formed in response to the ventral signal, perhaps a similar relative importance between the level of the signal to that of its target, i.e., the dl-cact complex exists. The existence of both gain-of-function and loss-of-function alleles of *ea* and *Tl* already point to the importance of the level and distribution of the signal to the formation of the gradient. A dominant lateralizing allele of *ea*, *ea$^{5.13}$* and the strongly ventralizing *Tl* allele, *Tl^{10b}* show polarity (Roth, 1990). We wondered if this polarity is due to a limitation in the signal, or in the target. Thus, would an increase in dl protein in these backgrounds alter the phenotypes of these mutants?

Wild-type *ea* activity is not only required for the development of ventral and lateral structures, but is also involved in controlling spatial distribution of these structures (Chasan and Anderson, 1989). The dominant lateralizing allele of *easter*, *ea$^{5.13}$*, results in the polar lateralized (L1) embryonic phenotype, in which lateral epidermis is expanded at the expense of the more dorsal and ventral structures (Chasan and Anderson, 1989). When one insert of *hsp83dlSpe* line 2 was crossed into the *ea$^{5.13}$* background, the embryos now showed a strongly ventralized phenotype (mostly V2, some V1, Table 5) in which all dorsal and dorsolateral elements are absent, and the ventral pattern elements are expanded.

Table 5. Effect of additional dorsal protein in mutant dorsal group backgrounds

Genetic background	No construct Phenotype	Construct : *hsp83dl* line 2* Phenotype
Gain-of-function alleles		
ea$^{5.13}$/+	L1	V2-V1
Tl^{10b}/+	V1	V0
Tl^{10b}/+;cactDfTE116GW21/+	V1-V0	nd
Tl^{10b}/Df(3R)ro^{80b}	V1	nd
Loss-of-function alleles		
spz^{rm7}/spz^{rm7}	D0	D0
Tl^{r444}/Tl^{r444} 29^{0}C	D1	D1
pll^{122}/pll^{122}	D2	D2
Tl^{rm9}/Tl^{rm10}	L1	L1
spz^{rm7} +/+ Tl^{r444} 29^{0}C	D2-D3	86% hatch; D3

*One insert of *hsp83dlSpe* line 2 was used for experiments in the dominant *ea* and *Tl* backgrounds, whereas two inserts of the same line were introduced in females with the recessive dorsal group mutations.

The cuticle phenotype was observed as described before. For strongly ventralized (V1 and V0) phenotypes, the effect of additional dl protein was analyzed by observing cellular movements during gastrulation (see Materials and methods). Formation of the ventral furrow, cephalic furrow, posterior midgut as well as germband extension were used as indicators of polarity.

Similar results were obtained when one insert of line 2 was crossed into females carrying the strongly ventralizing *Tl^{10b}* allele. Embryos derived from *Tl^{10b}/+* females show the V1 phenotype with mesoderm expanded at the expense of more dorsal structures (Roth et al., 1989). When one copy of line 2 is crossed into this background, polarity of the V1 embryos is lost and no ventral furrow is observed (Table 5).

The formation of the ventral furrow in these embryos was examined by observing cell morphology at higher magnification by scanning electron microscopy. In embryos derived from *Tl^{10b}/+* females, a ventral furrow is clearly

Table 6. Limits of the system

dl	cact	%Hatch	Phenotype	dl/cact	cact/dl
0	2	0	D	0	–
1	3	92	wt	0.3	3.0
1	2	0*	D at 29^{o}C	0.5	2.0
1	2	83*	wt at 25^{o}C	0.5	2.0
2	3	95	wt	0.7	1.5
1	1	95**	wt	1.0	1.0
2	2	95	wt	1.0	1.0
3	3	98	wt	1.0	1.0
2.5	2	91	wt	1.25	0.8
3	2	80	wt/V	1.5	0.6
2	1	70	V	2.0	0.5
2.5	1	0	V	2.5	0.4

*% Hatch is strongly dependent on the genetic background.

**From Isoda et al., 1992.

Tabulation of data to demonstrate limits of the cact and dl proteins that are acceptable by the embryo for achieving wild-type phenotype. The level of dl produced from insert in *hsp83dlSpe* line 2 was estimated from its ability to rescue the *dl*$^{-}$ phenotype and was confirmed by western analysis. Approximately 63% and 80% of the embryos laid by *dl*$^{-}$ females expressing 1 or 2 copies of insert 2 respectively hatched. 80% of embryos hatch when 2 copies of this insert are present in addition to the endogenous *dl* genes. Thus, 2 copies of insert 2 are equivalent to 1 endogenous copy of *dl*.

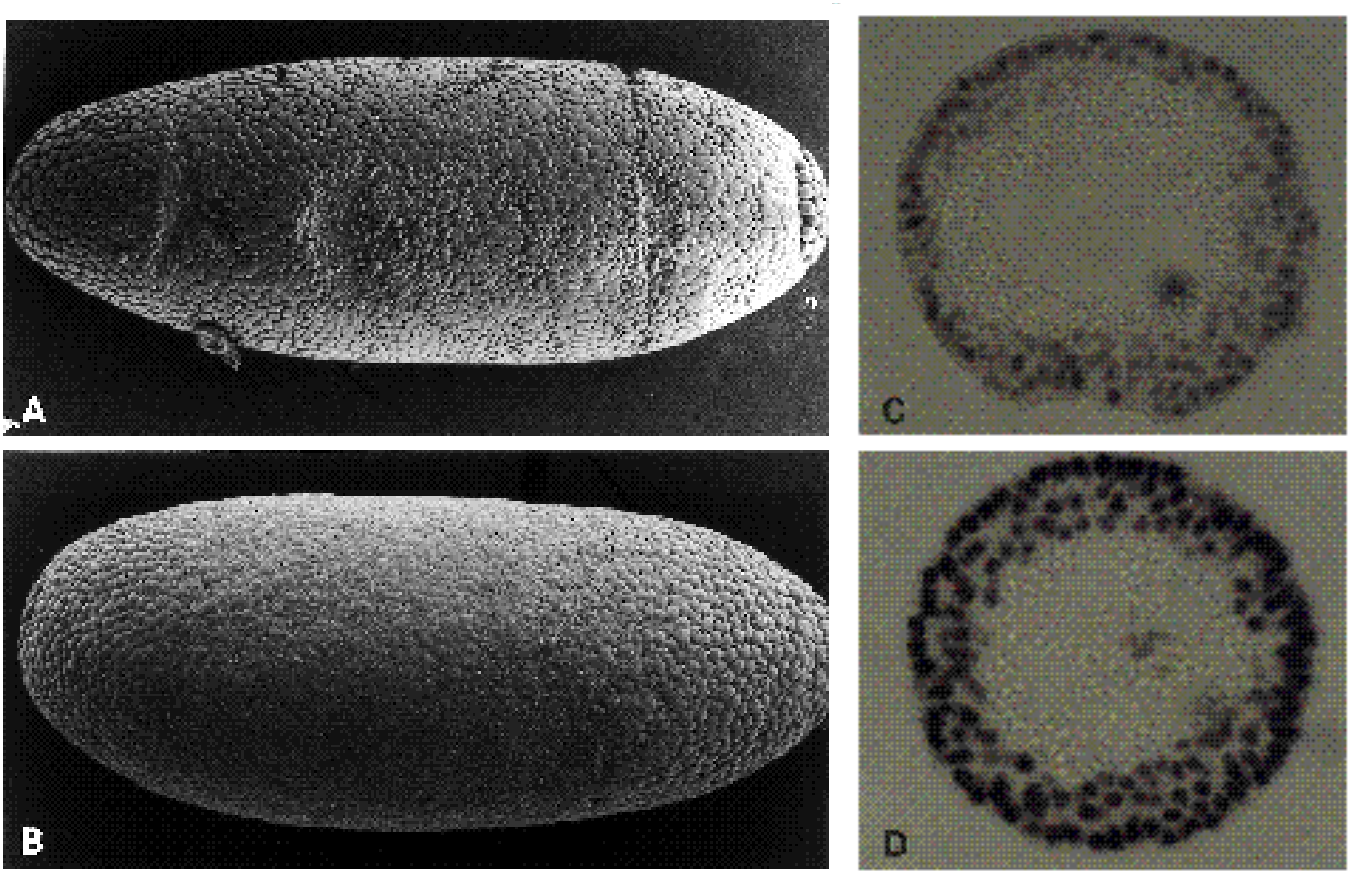

Fig. 8. Additional dorsal protein in embryos derived from *Toll^{10b}/+* females abolishes polarity. Scanning electron micrographs (A, B) and anti-twist antibody-stained 10 μm cross-sections (C, D). Gastrulae in A and C were derived from *Toll^{10b}/+*, and those in B and D from *Toll^{10b}/+; hsp83dl* mothers. While embryos laid by the *Toll^{10b}/+* females are strongly ventralized (V1), they do retain polarity as seen by the ventral furrow. With the addition of extra dl protein, this polarity is lost and the ventral furrow is initiated along the entire dorsoventral axis. In wild-type embryos, the ventral furrow is initiated as a result of shape changes in the cells that constitute the 12-cell wide midventral domain (Leptin and Grunewald, 1990; Sweeton et al., 1991; Costa et al., 1992). In embryos from *Tl^{10b}/+* females, a ventral furrow is clearly initiated, and in some regions, it extends further laterally than in the wild-type (A). The blebs of the apical membrane obscure the constricted apices. With additional dl protein, most of these embryos become fully ventralized and lose polarity. A ventral furrow is symmetrically initiated around the entire dorsoventral circumference, but perhaps due to the absence of lateral cell fates only isolated cells throughout the primordium are able to complete invagination. The entire surface of these embryos is therefore covered with apical membrane blebs (Fig. 8B).

initiated, and in some regions, it extends further laterally than in the wild-type (Fig. 8A). However, in the presence of additional dl protein, a ventral furrow is symmetrically initiated along the entire dorsoventral axis (Fig. 8B) but perhaps due to the absence of lateral cell fates only isolated cells throughout the primordium are able to completely invaginate.

Embryos from *Tl^{10b}/+* and *Tl^{10b}/+* with one insert of *hsp83dlSpe* line 2 females were stained with anti-twist antibody and sectioned transversely. As seen in Fig. 8C, embryos from *Tl^{10b}/+* females show staining all along the dorsoventral axis and invaginate a ventral furrow. However, the embryo in Fig. 8D from a *Tl^{10b}/+* female with extra dl protein does not exhibit a ventral furrow and the invaginated mesodermal cells around the entire circumference show more intense staining with anti-twist antibody.

To determine if *cact* haploinsufficiency would alter cellular movements in embryos of *Tl^{10b}/+* females, embryos from *Tl^{10b}/+; Dfcact/+* females were also studied. These embryos showed gastrulation movement patterns that were qualitatively intermediate between embryos from *Tl^{10b}/+* and *Tl^{10b}/+* with *hsp83dlSpe* line 2 females. Of the 7 embryos filmed, 4 showed complete or almost complete loss of polarity as measured by gastrulation movements (data not shown).

Additional dl protein in loss-of-function dorsal group backgrounds

Overexpression of dl protein in complete and partial loss-of-function dorsal group backgrounds e.g., embryos derived from homozygous *spz^{rm7}*, *Tl^{rm9}*, *Tl^{r444}*, or *pll^{122}* females did not change cell identities (Table 5). However, a strong effect was observed in embryos from transheterozygous mutant *Tl* and *spz* females. Embryos derived from homozygous *Tl^{r444}* females show the temperature-sensitive D1 phenotype, whereas those produced by females homozygous for the *spz^{rm7}* allele exhibit the amorphic D0 phenotype (Table 5). At 29°C, females transheterozygous for

Tl^{r444} and *spz^{rm7}* result in embryos with a D2-D3 phenotype. Increasing the level of dl protein by introducing 2 inserts of *hsp83Spe* line 2 rescued the dorsalized phenotype. At 29ºC, 86% of all embryos produced by these females hatched and the unhatched embryos showed a weak D3 phenotype (Table 5).

DISCUSSION

The maternal dorsoventral polarity pathway unfolds in multiple steps initiated within the egg-chamber, resumed within the perivitelline space and terminated within the syncytial blastoderm embryo (St. Johnston and Nüsslein-Volhard, 1992). The continuous embryonic phenotypes, ranging from complete dorsalization (D0) to complete ventralization (V0) is reflected in the nuclear concentration of dl protein. Formation of this nuclear dl gradient is controlled by the antagonistic functions of *cact* and the dorsal group genes. Previously proposed models (Roth et al., 1991; Govind and Steward, 1991) postulate that cact must inhibit the nuclear import of dl protein by a direct interaction and that this inhibition is countered by the activity of the extracellular ventral signal synthesized and mediated by the dorsal group genes. Upon dissociation from the dl-cact complex, free dl protein is imported into the nucleus, where it controls transcription of zygotic genes.

Transplantation experiments suggest that cact activity is uniformly distributed in the cytoplasm of early embryos (Roth et al., 1991). Thus, cact protein distribution is expected to be cytoplasmic and uniform along the dorsoventral axis. The total level of dl protein is also constant along the dorsoventral axis; however, in early development, dl protein is partitioned into nuclei ventrally, while it remains cytoplasmic on the dorsal side. To begin with a uniformly distributed cytoplasmic dl-cact complex and to subsequently achieve the precision in the shape of the nuclear dl gradient at the very least requires that the activity of either the signal or its target be limiting. We established the parameters necessary for forming the dl nuclear gradient by varying the doses of *dl*, *cact* and the ventral signal relative to each other.

The dl-cact concentration is restricted in the wild-type embryo

In wild-type embryos, the *cact* activity is somewhat in excess of its absolute requirement. Increasing *dl* by one dose over wild-type had only a slight effect in a wild-type background (Table 6). The effect was very strong when one dose of *cact* was removed: 1 copy of line 2 together with the endogenous *dl* was sufficient to saturate 1 dose of *cact*, resulting in ventralization of all the embryos. These saturation experiments suggest that cact levels are roughly twice what is required for inhibition of wild-type dl function. Upon adding dl over wild-type levels, cact is initially saturated and the absolute number of the dl-cact complexes increases. Once all cact molecules are 'occupied', any additional dl protein is unbound and behaves like 'free' dl in embryos with reduced *cact* activity. It is not clear if import of 'free' dl is unregulated along the dorsoventral axis and/or if it responds to the ventral signal.

While in hemizygous *cact* condition, additional dl protein gave a strong phenotypic effect, the system seemed to be tolerant of excess cact in wild-type and hemizygous *dl* backgrounds (Table 6). Since an increase in cact does not 'dilute' the ventral signal, we propose that either the signal does not go through cact, or only bound cact is a target for the signal (Fig. 9A). Thus, the dl-cact complex, target of the ventral signal, is regulated by the level of dl. This excess cact activity available in wild-type embryos may act as a buffer for varying levels of dl protein, *dl* expression being sensitive to genetic background.

The haploinsufficiency of both *dl* and *cact* genes, as well as the ventralization caused by overexpression of dl protein, clearly suggests that the ratio of cact and dl proteins is important for attaining the precise shape of the gradient (Table 6). The *dl/cact* ratio should be larger than 0.5 but less than 1.5 for maintaining the wild-type phenotype. A ratio of 1*dl*:1*cact* results in a wild-type phenotype even if each gene is present in 1, 2 or 3 copies. Thus, dl-cact interaction does not depend on a strict stoichiometric constraint to respond to the ventral signal. Instead, the relative amounts of dl and cact seem to be critical as long as all the dl protein is retained by cact protein.

The dorsal-cactus complex and the ventral signal

A shift in cell fates towards more ventral positional values was observed upon increasing dl in *Tl^{10b}/+* and *$ea^{5.13}$/+* backgrounds, as well as in the transheterozygous *+ Tl^{r444}/spz^{rm7} +* combination, but not in homozygous loss-of-function dorsal group backgrounds. These results suggest that the signal provided by these mutant dorsal group proteins is not used maximally to target all wild-type dl protein. The amount of dl was increased only to a level that does not saturate the retention activity of cact. Thus, the observed change in phenotype must be due to the signal acting through the additional dl-cact complex, and therefore it appears that increasing the level of the complex can enhance the effect of the ventral signal. Apparently for the effect of the ventral signal to be realized, both the dl-cact complex and the signal itself must be above a certain threshold. Since the amount of the dl-cact complex is dependent on the level of dl protein, it is, in effect, the level of the signal relative to the concentration of the dl protein that is essential for the elaboration of the gradient.

The phenotypic effects observed in our experiments, where extra doses of dl were added in the *$ea^{5.13}$* and *Tl^{10b}* backgrounds were similar to those observed in embryos where one copy of *cact* was removed in the same genetic background (this study and Roth, 1990). It is likely that the mechanisms leading to the change in nuclear dorsal protein are different in the two situations. When a copy of *cact* is removed, dl is free to enter the nuclei. In contrast, extra dl results in the increase of the dl-cact complex levels which is responsive to the ventral signal.

Transplantation rescue experiments have shown that the steps between Tl activation and selective dl nuclear uptake are critical for the slope of the nuclear dl gradient. When a ventral high point is experimentally induced on the dorsal side of the embryo, proportionate dorsoventral pattern elements in reversed polarity are formed (Anderson and Nüsslein-Volhard, 1984; Anderson et al., 1985b; Schneider

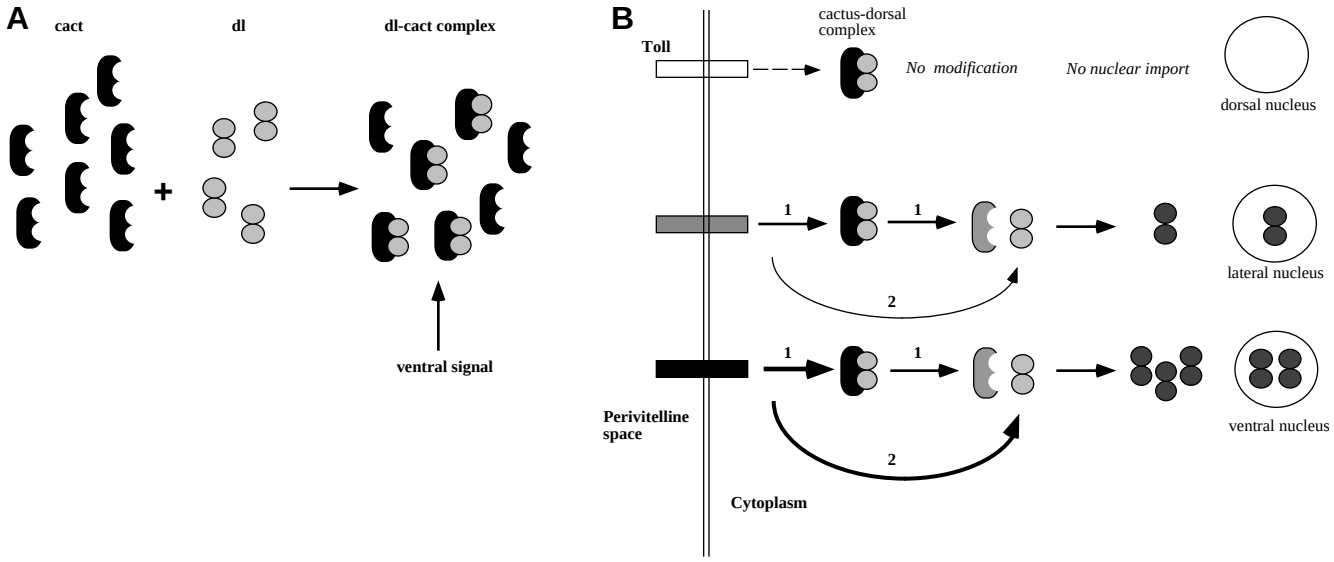

Fig. 9. (A) dl protein levels determine the concentration of the dl-cact complex: a homeostatic balance between dl and cact proteins. (A) 1.5-fold overexpression of dl protein results in wild-type phenotype, while higher levels saturate cact and result in ventralization. In contrast, even a 3-fold excess of *cact* dose does not affect the wild-type phenotype. We propose that dl protein is the limiting component of this complex and that dl levels determine the concentration of the dl-cact complex. While additional cact activity can be convoked by adding dl protein to the embryo, this 'excess' cact activity cannot be interpreted by the ventral signal unless complexed with dl protein. (B) Molecular steps involved in the graded nuclear uptake of cytoplasmic dl protein. The putative extracellular ligand interacts with the Toll receptor in order to generate a graded signal which sets up quantitative differences between ventral and ventro-lateral positions. These differences are mediated by dorsal group gene products downstream of Toll, and are sufficient to account for the quantitative intracellular differences in the nuclear import of dl protein within the corresponding dorsoventral regions. In this model we show that for dissociation and selective nuclear import of dl, both dl and cact are 'modified' (also see text). Release of dl from cact by a modification or a conformational change targeted on cact protein (arrow 1) followed by a change in free dl protein (arrow 2) which results in an enhanced ventral uptake. While in this model two distinct steps are depicted, it is also possible that 'modification' of dl alone (arrow 2) results in both its dissociation and the nuclear uptake.

and Anderson, 1991; Stein et al., 1991). These observations suggest that the concentration of the dl-cact complex is uniform along the dorsoventral axis and that regardless of the polarity of the embryo, this concentration has the same effect on the slope of the gradient.

Molecular mechanism for gradient formation

The molecular steps that lead to peak ventral, lower lateral and undetectable dorsal levels of dl protein are unclear. We propose that two distinct molecular events, both dependent on a graded ventral signal are required for gradient formation (Fig. 9B). In the first step, cact is dissociated from dl and then, in step two, dl is imported into the nucleus. This idea is based on the observation that homozygous double mutant combinations of $cact^{A2}$ and the known dorsal-group genes (except *dl*), result in lateralized phenotype in which intermediate dl levels corresponding to those found in the lateral nuclei of wild-type embryos were observed (Roth et al., 1991). This observation implies that that nuclear uptake of free cytoplasmic dl protein to lateral levels is independent of the signal mediated by the dorsal group genes, whereas uptake of dl protein ventrally requires an activation event.

The minimum requirement to achieve both dissociation and nuclear uptake of dl protein is that dl be 'modified' (arrow 2 in Fig. 9B). The term 'modification' could be a biochemical effect or a conformational change. Alternatively, cact could be the target for 'modification' by the ventral signal (arrow 1 in Fig. 9B) which results in release of dl and subsequent 'modification' of dl (arrow 2 in Fig. 9B). After 'modification', dl is primed for nuclear import at a rate higher than that observed for the unmodified free dl.

The 'modification' of cact appears important for dissociation of the dl-cact complex. The gain-of-function *cact* alleles ($cact^{E10}$ and $cact^{BQ}$) either encode proteins that show a substantial increase in affinity for dl binding or proteins that are unable to respond to the ventral signal. Our results show that, as in the case of the wild-type and hypomorphic *cact* alleles, retention capacity of the cact gain-of-function proteins can also be saturated with additional dl protein. The simplest interpretation of these results is that the binding or retention capacity is probably not increased and the gain-of-function phenotype is due to the inability of the mutant proteins to respond to the ventral signal. If this mechanistic interpretation is valid, then the ventral signal must 'modify' cact that is occupied by dl protein. Thus, the available genetic data suggest that both dorsal and cact must be modified by the ventral signal in order to result in the nuclear gradient of dl protein.

The dorsoventral system compared to the anterior system

A dissection of pattern formation has revealed that the anteroposterior and the dorsoventral patterns are under independent genetic control. Determination of both these axes requires gradient systems as well as deployment of a number of subordinate zygotic genes. The bicoid (bcd) protein is the morphogen for the anterior system (Driever and Nüsslein-Volhard, 1988a), and dosage experiments similar to those described for dl have been performed for bcd (Driever and Nüsslein-Volhard, 1988b). Unlike the case of dl, where its overexpression led to ventralization and embryonic lethality, a 4-fold increase of bcd protein led to a shift in the gradient towards the posterior and the displacement of the cephalic furrow towards the posterior, but not in embryonic lethality. Despite the shift in the bcd gradient and that of the cephalic furrow, the embryo, apparently has the capacity to compensate later in development. This does not seem to be the case for the dorsoventral axis, perhaps because the entire axis is determined by one integrated pathway, unlike the anteroposterior axis which is determined by three relatively independent pathways.

Despite the identification of several components in the dorsoventral signal transduction pathway, important questions remain. Is the ligand generated in a graded manner, does Toll respond in a graded manner, or do the steps involved in transducing the extracellular signal create an intracellular gradient of information that signals cytoplasmic dl to undergo selective nuclear import? Further work will clarify the mechanistic details of how the gradient of nuclear dl protein is elaborated.

We thank P. Schedl for suggesting the use of the *hsp83* promoter, R. Bopp for help with histology, M. McConnell and P. Fisher for affinity-purified anti-lamin antibody and G. Gray for fly food. K. V. Anderson, Y. Hiromi, S. Roth, T. Schüpbach and E. Wieschaus provided fly stocks. We also thank K. V. Anderson and D. Morisato for communicating results before publication and suggesting the use of some dorsal group mutants. We especially thank M. Costa for help with video-taping developing embryos, helping with interpreting gastrulation phenotypes and scanning electron micrography of embryos. S. Roth's help with interpretation of mutant phenotypes is gratefully acknowledged. We thank our colleagues at Princeton, especially E. C. Cox, H. Gyurkovics, Y. Hiromi, T. Schüpbach, as well as our reviewers for critical reading of the manuscript. S.G. was supported by fellowship from New Jersey Commission on Cancer Research and L.B. was supported by an American Cancer Society fellowship. This work was also supported by grants from NSF and NIH and the Horace W. Goldsmith Foundation.

REFERENCES

Alberga, A., Boulay, J.-L., Kempe, E., Dennefeld, C. and Haenlin, M. (1991). The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers. *Development* **111**, 983-992.

Ambrosio, L. and Schedl, P. (1984). Gene expression during *Drosophila melanogaster* oogenesis; analysis by *in situ* hybridization to tissue sections. *Dev. Biol.* **105**, 80-91.

Anderson, K. V. and Nüsslein-Volhard, C. (1984). Information for the dorsal-ventral pattern of *Drosophila* is stored as maternal mRNA. *Nature* **311**, 223-227.

Anderson, K. V., Jurgens, G. and Nüsslein-Volhard, C. (1985a). Establishment of dorsal-ventral polarity in the *Drosophila* embryo genetic studies on the role of the *Toll* gene product. *Cell* **42**, 779-789.

Anderson, K. V., Bokla, L. and Nüsslein-Volhard, C. (1985b). Establishment of dorsal-ventral polarity in the *Drosophila* embryo: the induction of polarity by the *Toll* gene product. *Cell* **42**, 791-798.

Anderson, K. V. and Nüsslein-Volhard C. (1986). Dorsal-group genes of *Drosophila*. In *Gametogenesis and the Early Embryo*, (ed. J.G. Gall) pp. 177-194. New York: Alan R. Liss.

Boswell, R. E. and Mahowald, A.P. (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster. Cell* **43**, 97-104.

Chasan, R. and Anderson, K. V. (1989). The role of *easter*, an apparent serine protease, in organizing the dorsal-ventral pattern of the *Drosophila* embryo. *Cell* **56**, 391-400.

Chen, I. S. Y., Wilhelmsen, K. C. and Temin, H. M. (1983). Structure and expression of c-*rel*, the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. *J. Virol.* **45**, 104-113.

Costa, M., Sweeton, D. and Wieschaus, E. (1992). Gastrulation in *Drosophila*: Cellular mechanisms of morphogenetic movements. In *The Development of Drosophila*, (ed. M. Bate and A. Martinez Arias), Cold Spring Harbor, In Press.

Driever, W. and Nüsslein-Volhard, C. (1988a). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**, 83-93.

Driever, W. and Nüsslein-Volhard, C. (1988b). The bicoid protein determines position in the *Drosophila* embryo in a concentration dependent manner. *Cell* **54**, 95-104.

Ferguson, E. L. and Anderson, K. V. (1991). Dorsal-ventral pattern formation in the *Drosophila* embryo: the role of zygotically active genes. *Curr. Top. Dev. Biol.* **25**, 17-43.

Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P. and Baltimore, D. (1990). Cloning of the p50 DNA-binding subunit of NF-κB:homology to *rel* and *dorsal*. *Cell* **62**, 1019-1029.

Govind, S. and Steward, R. (1991). Dorsoventral polarity in the *Drosophila* embryo: Signal transduction and nuclear targeting. *Trends Genet*. **7**, 119-125.

Govind, S., Whalen, A. M. and Steward, R. (1992). In vivo self-association of the *Drosophila* rel protein dorsal. *Proc. Natl. Acad. Sci. USA* **89**, 7861-7865.

Hashimoto, C., Hudson, K. L. and Anderson, K. V. (1988). The *Toll* gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269-279.

Hashimoto, C., Gerttula, S. and Anderson, K. V. (1991). Plasma membrane localization of the *Toll* protein in the syncytial *Drosophila* embryo: importance of transmembrane signaling for dorsal-ventral pattern formation. *Development* **111**, 1021-1028.

Ip, Y. T., Kraut, R., Levine, M., and Rushlow, C. (1991). The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila. Cell* **64**, 439-446.

Isoda, K., Roth, S. and Nüsslein-Volhard, C. (1992). The functional domains of the *Drosophila* morphogen *dorsal*: evidence from the analysis of mutants. *Genes Dev.* **6**, 619-630.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The *dorsal* morphogen gradient regulates the mesoderm determinant *twist* in the early *Drosophila* embryos. *Genes Dev.* **5**, 1881-1891.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A. and Israel, A. (1990). The DNA-binding subunit of NF-κB is identical to the factor KBF1 and homologous to the *rel* oncogene product. *Cell* **62**, 1007-1018.

Klemenz, R., Weber, U. and Gehring, W. J. (1987). *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucl. Acids Res.* **15**, 3947-3959.

Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in *Drosophila. Development* **110**, 73-84.

Letsou, A. Alexander, S. Orth, K. and Wasserman, S. (1991). Genetic and molecular characterization of *tube*, a *Drosophila* gene, maternally required for embryonic dorsoventral polarity. *Proc. Natl. Acad. Sci. USA* **88**, 810-814.

Lienhard Schmitz, M., Henkel, T. and Baeuerle, P. A. (1991). Proteins controlling the nuclear uptake of NF-κB, rel and dorsal. *Trends in Cell Biol.* **1**, 130-137.

Lindquist, S. (1986). The heatshock response. *Ann. Rev. Biochem.* **55**, 1151-1191.

Mason, P. J., Hall, L. M. C., and Gausz, J. (1984). The expression of heat

shock genes during normal development in *Drosophila melanogaster*. *Mol. Gen. Genet.* **194**, 73-78.

Merrill, P. T., Sweeton, D. and Wieschaus, E. (1988). Requirements for autosomal gene activity during precellular stages of *Drosophila melanogaster*. *Development* **104**, 495-509.

Mohler, J. and Wieschaus, E. (1986). Dominant maternal-effect mutations of *Drosophila melanogaster* causing the production of double abdomen embryos. *Genetics* **112**, 803-822.

Nolan, G. P., Ghosh, S., Liou, H.-C., Tempst, P. and Baltimore, D. (1991). DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel* related polypeptide. *Cell* **64,** 961-969.

Nüsslein-Volhard, C. (1979). Maternal effect mutations that alter the spatial coordinates of the embryo of *Drosophila melanogaster*. *Symp. Soc. Dev. Biol.* **37**, 185-211.

Pan, D., Huang, J.-D. and Courey, A. J. (1991). Functional analysis of the *Drosophila twist* promoter reveals a dorsal-binding ventral activator region. *Genes Dev*. **5**, 1892-1901.

Pirrotta, V. (1988). Vectors for P-mediated transformation in *Drosophila*. In *Vectors: A survey of Molecular Cloning Vectors and their Uses*, (ed., R. L. Rodrigues and D. T. Denhardt), pp 437-456. Boston: Butterworths.

Robertson, H. M., Preston, C. R., Phillips, R. W., Johnson-Schlitz, D., Benz, W. K. and Engels, W. R. (1988). A stable genomic source of P-element transposase in *Drosophilamelanogaster*. *Genetics* **118**, 461-470.

Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.

Roth, S. (1990). Die rolle der maternalen gene cactus und dorsal bei der dorsoventralen musterbildung im *Drosophila* embryo. Ph.D. Thesis. Tübingen.

Roth, S., Hiromi, Y., Godt, D. and Nüsslein-Volhard, C. (1991). *cactus*, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. *Development* **112**, 371-388.

Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A. and Rosen, C. A. (1991). Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. *Science* **251**, 1490-1493.

Rushlow, C., Frash, M., Doyle, H. and Levine, M. (1987). Maternal regulation of *zerknüllt*, a homeo box gene controlling differentiation of dorsal tissues in *Drosophila. Nature* **330**, 583-586.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport. *Cell* **59**, 1165-1177.

Rushlow, C. A. and Levine, M. (1990). Role of the *zerknüllt* gene in dorsal-ventral pattern formation in *Drosophila.Adv. in Genet.* **27**, 277-307.

Schneider D. S., Hudson, K. L., Lin, T-Y. and Anderson, K. V. (1991). Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the *Drosophila* embryo. *Genes Dev.* **5**, 797-807.

Schüpbach, T. and Wieschaus, E. (1989).Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. *Genetics* **121**, 101-117.

Shimell, M. J., Ferguson, E. L., Childs, S. R. and O'Connor, M. B. (1991). The *Drosophila* dorsal-ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. *Cell* **67**, 469-481.

Spradling, A. L. and Rubin, G. M. (1982). Transformation of cloned P-elements into *Drosophila* germline chromosomes. *Science* **218**, 341-347.

Stein, D., Roth, S., Vogelsang, E. and Nüsslein-Volhard, C. (1991). The polarity of the dorsoventral axis in the *Drosophila* embryo is defined by an extracellular signal. *Cell* **65**, 725-735.

St Johnston, R. D. and Gelbart, W. M. (1987). *Decapentaplegic* transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. *EMBO J.* **6**, 2785-2791.

St Johnston, R. D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 1-20.

Steward, R., McNally, F. and Schedl, P. (1984). Isolation of the *dorsal* locus of *Drosophila. Nature* **311**, 262-265.

Steward, R., Ambrosio, L. and Schedl, P. (1985). Expression of the *dorsal* gene. *Cold Spring Harbor Symp. Quant. Biol.* **50**, 223-228.

Steward, R. and Nüsslein-Volhard, C. (1986). The genetics of the *dorsal-Bicaudal-D* region in *Drosophila melanogaster.Genetics* **113**, 665-678.

Steward, R. (1987). *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-*rel*. *Science* **238**, 692-694.

Steward, R., Zusman, S. B. and Huang, L. H. and Schedl, P. (1988). The *dorsal* protein is distributed in a gradient in early *Drosophila* embryos. *Cell* **55**, 487-495.

Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179-1188.

Sweeton, D., Parks, S., Costa, M. and Wieschaus, E. (1991). Gastrulation in *Drosophila*: The formation of ventral furrow and posterior midgut invaginations *Development* **112**, 775-789.

Thisse, B., Stoetzel, C., Messal, M. E. and Perrin-Schmitt, F. (1987). Genes of the *Drosophila* maternal dorsal group control the specific expression of the zygotic gene *twist* in presumptive mesodermal cells. *Genes Dev.* **1**, 709-715.

Thisse, C., Perrin-Schmitt, F., Stoetzel, C. and Thisse, B. (1991). Sequence specific transactivation of the *Drosophila* twist gene by the dorsal gene product. *Cell* **65**, 1191-1201.

Xiao, H. and Lis, J.T. (1989). Heat shock and developmental regulation of the *Drosophila melanogasterhsp83* gene. *Mol. Cell. Biol.* **9**, 1746-1753.

Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila*: *A practical Approach*. (ed. D.B. Roberts) pp. 199-228 IRL Press: Washington DC.

Wilmhelmsen, K. C., Eggleton, K. and Temin, H. M. (1984). Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. *J. Virol.* **52**, 172-182.

Zimmerman, J. L., Petri, W. and Meselson, M. (1983). Accumulation of a specific subset of *D. melanogaster* heat shock mRNAs in normal development without heatshock. *Cell* **32**, 1161-1170.

(Accepted 25 September 1992)