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A Gradient of Nuclear Localization of the *dorsal* Protein Determines Dorsoventral Pattern in the Drosophila Embryo

Siegfried Roth, David Stein, and Christiane Nüsslein-Volhard
Max-Planck-Institut für Entwicklungsbiologie
Spemannstrasse 35
7400 Tübingen
Federal Republic of Germany

Summary

The dorsoventral axis of the Drosophila embryo is determined by a morphogen gradient established by the action of 12 maternal-effect genes: the dorsal group genes and *cactus*. One of the dorsal group genes, *dorsal* (*dl*), encodes the putative morphogen. Although no overall asymmetry in the distribution of *dorsal* protein is observed, a gradient of nuclear concentration of *dl* protein is established during cleavage stages, with a maximum at the ventral side of the egg. At the dorsal side of the egg, the protein remains in the cytoplasm. Nuclear localization of the *dl* protein, and hence gradient formation, is blocked in dorsalizing alleles of all of the other dorsal group genes, while in ventralizing mutants nuclear localization extends to the dorsal side of the egg. A correlation between *dl* protein distribution and embryonic pattern in mutant embryos indicates that the nuclear concentration of the *dl* protein determines pattern along the dorsoventral axis.

Introduction

Axis determination in the Drosophila embryo is mediated by four systems defined genetically by groups of maternal genes with similar or identical phenotypes. Three of the systems provide positional information for regions along the anteroposterior axis, while pattern along the dorsoventral axis is determined by a single system (Nüsslein-Volhard et al., 1987; Anderson, 1987; Nüsslein-Volhard and Roth, 1989). For each of the systems, the asymmetric distribution of the active product of at least one gene is decisive for the pattern controlled by the system. The four systems use strikingly different biochemical mechanisms, both to establish asymmetry in the distribution of components and to translate that asymmetrical distribution into the patterns of expression of zygotic target genes.

Of the four systems of axis determination, the dorsoventral system is the most complex. The pattern elements defined by this system are, from ventral to dorsal: the mesoderm; the ventral ectoderm, which gives rise to central nervous system and ventral hypoderm; the dorsolateral ectoderm, from which the tracheae and the dorsal hypoderm are derived; and the amnioserosa. The order and size of these regions on the fate map are determined by the concerted action of 12 maternal-effect genes. Eleven of these, the dorsal group, give rise to the same phenotypes, and lack-of-function alleles in any of these loci cause a complete dorsalization of the embryo, with cells at all positions along the dorsoventral axis developing according to a dorsal fate. These 11 genes are: *gastrulation-defective*, *dorsal*, *windbeutel*, *nudel*, *tube*, *pipe*, *snake*, *easter*, *Toll*, *spätzle*, and *pelle* (Nüsslein-Volhard, 1979a; Anderson and Nüsslein-Volhard, 1984a, 1984b, 1986; Schüpbach and Wieschaus, 1989; Nüsslein-Volhard and Roth, 1989). In contrast, lack-of-function alleles at the *cactus* (*cact*) locus cause partial ventralization (Schüpbach and Wieschaus, 1989; Nüsslein-Volhard and Roth, 1989).

In addition to lack-of-function alleles there exist partial lack-of-function alleles of the dorsal group genes that exhibit principally the same phenotype: a partial dorsalization. In such embryos, the dorsal and lateral anlagen are expanded and shifted toward a more ventral position at the expense of ventral and ventrolateral anlagen (Nüsslein-Volhard, 1979a, 1979b; Nüsslein-Volhard et al., 1980; Anderson and Nüsslein-Volhard, 1984b, 1986). For most of the dorsal group genes, mutant alleles of different strength give rise to a continuous series of partially dorsalized phenotypes in which increasingly larger portions from the ventral pattern are eliminated. The similarity in phenotype indicates that each of the genes is participating in a common pathway. The continuous spectrum of phenotypes suggests that the action of the dorsal group genes culminates in the establishment of a gradient of a morphogen. This morphogen gradient is thought to determine the size and fate of the subregions along the dorsoventral axis in a concentration-dependent manner.

Genetic analysis and transplantation experiments have helped to specify the roles of the individual dorsal group genes and to order them in a tentative hierarchy of functions (Anderson et al., 1985a, 1985b). These studies identified *dorsal* as the candidate gene encoding the morphogen for the following reasons. First, the *dorsal* gene exhibits a strong gene dosage sensitivity of phenotype, consistent with the concentration-dependent action of a morphogen. Second, in transplantation experiments, in which cytoplasm from various regions of wild-type embryos was transplanted into mutant embryos at syncytial blastoderm stages, only *dorsal*-rescuing activity showed a weak but significant enrichment at the ventral side of the donor embryo (Santamaria and Nüsslein-Volhard, 1983; Nüsslein-Volhard and Roth, 1989). Finally, in double mutants with the ventralizing mutation *cactus*, only *dl cactus* embryos show a dorsalized phenotype, while double mutants between *cact* and any of the other dorsal group loci confer some potential to form lateral and ventral pattern elements (Roth and Nüsslein-Volhard, unpublished data). This result suggests that only *dl* is absolutely required for the definition of dorsolateral, ventrolateral, and ventral regions and points to a late and direct function of *dorsal* in the determination of pattern along the dorsoventral axis.

The *dorsal* gene is the candidate to encode the postulated morphogen; the function of the other dorsal group genes seems to be the establishment of the morphogen

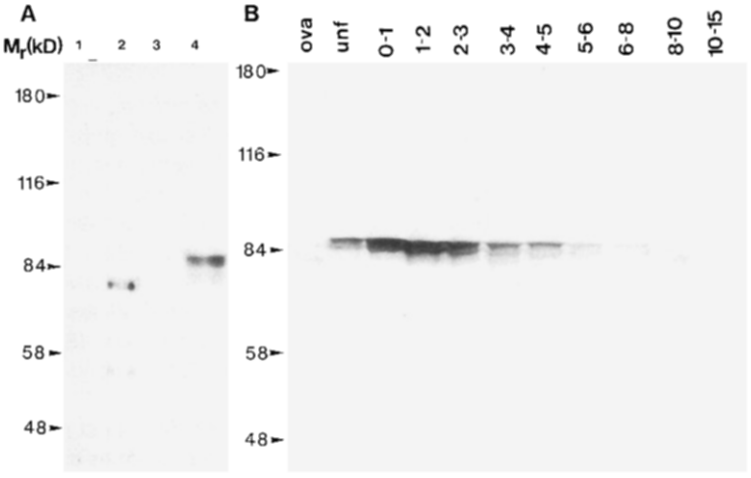

Figure 1. Specificity of the Anti-*dl* Antibody and Developmental Profile of the *dl* Protein

(A) A *dl* cDNA containing the complete open reading frame was cloned into a T7 expression construct (pAR3038; Studier and Moffat, 1986) to allow the production of full-length *dl* protein in bacteria. E. coli and embryonic extracts were separated by SDS–PAGE (7.5% acrylamide) and transferred to nitrocellulose. The Western blot was incubated with anti-*dl* antibodies. Lane 1, extract from an induced E. coli culture that contains the plasmid pAR3038 without an insert; lane 2, extract from E. coli that expresses *dl* under the control of the T7 phi 10 promoter (plasmid pAR-*dl*); lane 3, extract from *dl* mutant embryos (*In(2L)dl^{H}/Df(2L)TW119*); lane 4, extract from 0–3 hr old wild-type embryos.

(B) Extracts from ovaries (ova), unfertilized eggs (unf), and staged embryos were separated by SDS–PAGE (7.5% acrylamide) and treated as in (A). Ages of the embryos are indicated at top in hr after egg deposition (at 22°C). Apparent molecular masses of marker proteins in kd are indicated at left.

gradient in the correct orientation. *Toll* (*Tl*) and *cact* have unique roles in this process. *Tl* is required for orientation of the dorsoventral axis relative to the polarity of the outer egg shell pattern. *Tl*$^{-}$ embryos do not have any intrinsic polarity, and the polarity of the pattern that can be induced by the transplantation of wild-type cytoplasm is determined by the site of injection (Anderson et al., 1985a, 1985b). Recent molecular analysis (Hashimoto et al., 1988) suggests that the *Tl* product could function as a receptor located in the outer egg membrane, which responds to an external signal triggering the orientation of the morphogen gradient. The other dorsal group genes presumably have individual functions in the process, providing and transmitting the external signal via the *Tl* function to the final establishment of the morphogen gradient. *cact* is unique because its absence results in a ventralization of the dorsoventral pattern.

A number of zygotically expressed genes that are required for the formation of subregions of the dorsoventral pattern have been identified as possible targets for the maternal morphogen gradient. *twist*, required for formation of the ventralmost (mesoderm) part of the dorsoventral pattern, is expressed in a longitudinal stripe in the ventralmost 20% of the egg circumference (EC) (Nüsslein-Volhard et al., 1984; Simpson, 1983; Thisse et al., 1988). The zygotic genes *zerknüllt* and *decapentaplegic* are required for the development of dorsal pattern elements (Wakimoto et al., 1984; Irish and Gelbart, 1987). Both are expressed at the dorsal 40% of the EC in blastoderm embryos (Doyle et al., 1986; St. Johnston and Gelbart, 1987). The expression patterns of these genes are under the control of *dl* (Rushlow et al., 1987; Thisse et al., 1987b).

The *dorsal* gene has been cloned and sequenced (Steward et al., 1984, 1985; Steward, 1987) and shows strong homology to the avian oncogene c-*rel*. Although the mRNA is distributed evenly in early Drosophila embryos, the protein product has been reported to appear in the syncytial blastoderm stage embryo and to be distributed in a gradient in the nuclei with a maximum at the ventral side of the egg (Steward et al., 1988). Here, we show that the *dl* protein is already present in freshly laid eggs, where it is evenly distributed. Although no overall asymmetry in the distribution of *dl* protein is observed, a gradient of nuclear localization of the *dorsal* protein is established during cleavage stages, with a maximum of *dl* nuclear concentration present at the ventral side of the egg. On the dorsal side, the protein remains in the cytoplasm. The other dorsal group genes and *cact* regulate nuclear uptake of *dl* protein. We show that the local concentration of the *dl* protein in the nuclei determines cell fate along the dorsoventral axis.

Results

Identification of the *dl* Protein in Embryonic Extracts

Based on sequence analysis (Steward, 1987), the product of the *dl* gene is predicted to contain 677 amino acids and to have a molecular weight of 75,000. The amino-terminal region of the protein contains a region homologous to the proto-oncogene c-*rel* (Wilhelmsen et al., 1984), and the carboxy-terminal half of the molecule contains several *opa* repeat sequences (McGinnis et al., 1984; Wharton et al., 1985). Antibodies were generated against a *lacZ–dl* fusion protein that contains most of the c-*rel* homologous region but lacks the *opa* repeats. The antibodies were purified by affinity chromatography.

The specificity of the antibodies was confirmed by Western blot analysis of embryonic extracts and of Escherichia coli extracts containing full-length *dl* protein produced under the control of the T7 promoter. While the antibodies detected several bands in the range of 84–90 kd in extracts of embryos derived from wild-type females, no protein bands were detected in extracts of embryos derived from females that do not produce *dl* mRNA (Steward et al., 1984) (Figure 1A, lane 3; see also Figure 5 and Figure 8,

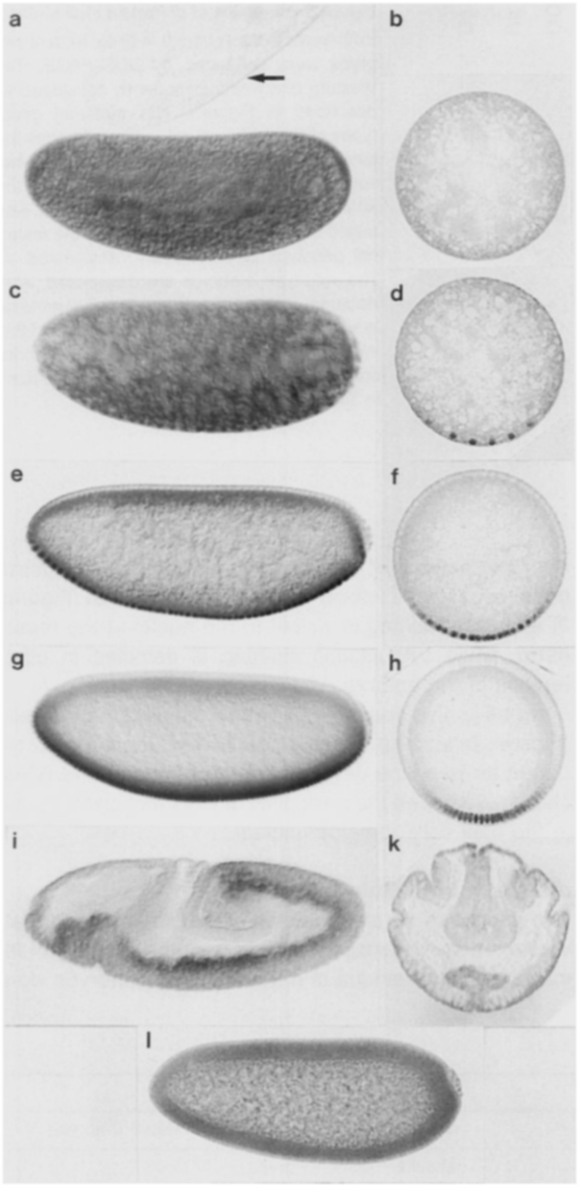

Figure 2. *dl* Protein in Whole Mounts and Transverse Sections of Wild-Type Embryos

Wild-type embryos of different ages were stained using anti-*dl* antibody as described in the Experimental Procedures. Whole mount preparations were photographed using Normaski optics (a, c, e, g, i, l) or embedded in durcupan-ACM (Fluka), sectioned, and photographed using bright field optics (b, d, f, h, k).

(a) and (b) Early cleavage stage embryo (stage 2) and dl^- embryo ([a], see arrow at top).

(c) and (d) Embryos at pole cell formation (stage 3).

(e) and (f) Syncytial blastoderm embryos (stage 4).

(g) and (h) Cellular blastoderm embryos (stage 5).

(i) and (k) Embryos at germband extension (stage 7).

(l) Syncytial blastoderm embryo during mitosis.

The orientation of the embryos in (a), (c), (e), (g), and (i) is anterior at left and dorsal at top, and in (b), (d), (f), (h), and (k), dorsal at top. Staging is according to Campos-Ortega and Hartenstein (1985).

which show the wild-type banding pattern with slightly higher resolution). *dl* protein expressed in E. coli under the control of the T7 promoter has an apparent molecular weight of 75,000, the predicted molecular weight (Figure 1A, lane 2). No protein is detected in extracts from bacteria that do not contain the *dl* expression vector. The molecular weight difference between bacterial-derived and embryonic *dl* protein suggests that the *dl* protein becomes modified in embryos.

Figure 1B shows a developmental Western blot analysis of extracts obtained from ovaries, unfertilized eggs, and wild-type embryos at various stages of development. The *dl* protein appears during oogenesis and persists throughout early embryogenesis. After egg deposition, *dl* protein is detected as several bands in the range of 84–90 kd in both unfertilized and fertilized eggs. The overall abundance of *dl* protein remains fairly constant throughout embryonic development until about 3 hr after egg deposition, the onset of gastrulation, when the protein begins to disappear. We can detect protein until 10–15 hr after egg deposition.

Distribution of *dl* Protein in Wild-Type Embryos

The distribution pattern of *dl* protein during embryonic development was studied by staining whole embryos with anti-*dl* antibodies. In cleavage-stage embryos (stage 2; Campos-Ortega and Hartenstein, 1985; Figures 2a and 2b) *dl* protein is homogeneously distributed throughout the egg; control embryos that do not produce a *dl* mRNA and contain no *dl* protein (see Western blot, Figure 1A) are unstained. Protein is present in cortical regions and in the central cytoplasmic islands surrounding the nuclei. *dl* protein is also present in unfertilized eggs and remains homogeneously distributed for several hours after egg deposition (data not shown).

An apparently asymmetric distribution of *dl* protein is first seen when the somatic nuclei reach the periphery, after the pole cells have formed (stage 3, nuclear cycle 10; Figures 2c and 2d). At this time, *dl* protein staining becomes concentrated such that the nuclei in the ventralmost 25% of the EC appear more darkly stained than the cytoplasm immediately surrounding them. The nuclear staining is graded, with the nuclei close to the ventral midline staining most strongly. During the syncytial blastoderm stage a nuclear *dl* protein gradient is established that extends over the ventral half of the EC (Figures 2e and 2f). The nuclei of the ventralmost 25% of the EC are stained more intensely than the surrounding cytoplasm. In the ventrolateral 10%–15% of the EC the nuclei and the cytoplasm are stained with similar intensity, which makes the nuclei hard to distinguish from the surrounding cytoplasm. In the adjacent lateral 10% of the EC the nuclei appear more lightly stained than the surrounding cytoplasm, but nuclear staining above background levels is visible. The dorsal half of the EC shows no nuclear *dl* staining above background level, but *dl* protein is present in the cytoplasm.

The establishment of the nuclear gradient of *dl* protein during the syncytial blastoderm stage is accompanied by a depletion of *dl* staining in the ventral cytoplasm that sur-

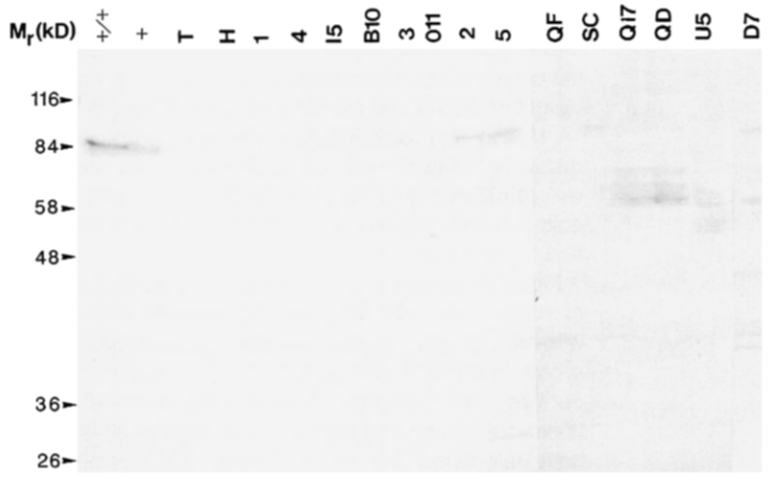

Figure 3. Detection of *dl* Protein in *dl* Mutants

Embryonic extracts from 0–4 hr old mutant embryos were separated by SDS–PAGE. The Western blot was subsequently developed as described in Figure 1. The maternal genotypes of the embryos are indicated above the lanes. Females carried the mutant chromosome in *trans* to *Df(2L)TW119*. Therefore, the abundance of the mutant protein was estimated in comparison to embryos of the maternal genotype *Df(2L)TW119/+*, designated +. The wild-type embryos are designated +/+. Apparent molecular masses of marker proteins in kd are indicated at the left. The results of this Western blot analysis together with a description of the mutant phenotypes are summarized in Table 1.

rounds the heavily stained nuclei. This ventral cytoplasmic depletion of *dl* staining becomes more apparent as cellularization begins (stage 5, Figure 2h). When nuclear cleavages occur during the syncytial blastoderm stage, the nuclear membrane partially disintegrates (Foe and Alberts, 1983; Stafstrom and Staehelin, 1984). The protein that had differentially accumulated in the nuclei apparently redistributes in the cytoplasm (Figure 2l). No asymmetry in *dl* protein distribution is visible in these stages. This indicates that the overall abundance of *dl* product is not graded along the EC. Only when nuclei are present is an apparent gradient of *dl* protein distribution visible.

Shortly before the onset of gastrulation the *dl* protein concentration in the ventral nuclei decreases (data not shown). However, consistent with the results of our Western blot analysis, *dl* protein remains visible during germband extension at least 5 hr after egg deposition (Figures 2i and 2k). Staining is visible in the nuclei of the mesoderm, while cytoplasmic staining is detected in other regions of the embryo.

We have also observed *dl* protein staining in pole cells (Figures 2e and 2g). However, the *dorsal* function is not required for germline development (Schüpbach and Wieschaus, 1986), and the possibility of any function for *dorsal* in the pole cells remains unclear.

dl Protein in *dl* Mutant Embryos

To detect possible alterations in *dl* protein, we performed Western blots on extracts derived from the progeny of females carrying various *dl* mutant alleles. Embryos were

Table 1. *dorsal* Protein in *dorsal* Mutant Embryos

Allele	Phenotypic Strength[a]	*dl* Protein in Embryonic Extracts	*dl* Protein Distribution in Whole Mount Embryos
H	D0	absent	absent
T	D0	absent	absent
1	D0	absent	absent
4	D0	absent	absent
I5	D0	absent	absent
B10	D0	absent	absent
6	D0	absent	absent
8	D0	absent	absent
011	D0–D1	absent	absent
QF	D1	absent	weak gradient
3	D0–D1	reduced abundance normal MW	weak gradient
SC	D2	reduced abundance normal MW	normal gradient
5	D1	normal	normal gradient
2	D1	normal	normal gradient
Q17	D1	normal abundance (56, 60, and 68 kd)	gradient with ectopic[b] *dl* protein
QD	D1	normal abundance (56, 60, and 68 kd)	gradient with ectopic[b] *dl* protein
U5	D1	reduced abundance (45, 52, and 60 kd)	gradient with ectopic[b] *dl* protein
D7	D0	normal abundance (54 and 90 kd)	normal gradient

[a] Classification of phenotypic strength (in *trans*-heterozygotes with the *dorsal* deficiency, *Df(2L)TW119*), according to Anderson et al., 1985a: D0: complete dorsalization, D1: strong dorsalization, no ventral epidermis, D2: weak dorsalization, ventral epidermis present, but mesoderm absent.
[b] "ectopic" refers to the presence of small amounts of *dl* protein in the dorsal region. No *dl* protein is found in this location in wild-type.

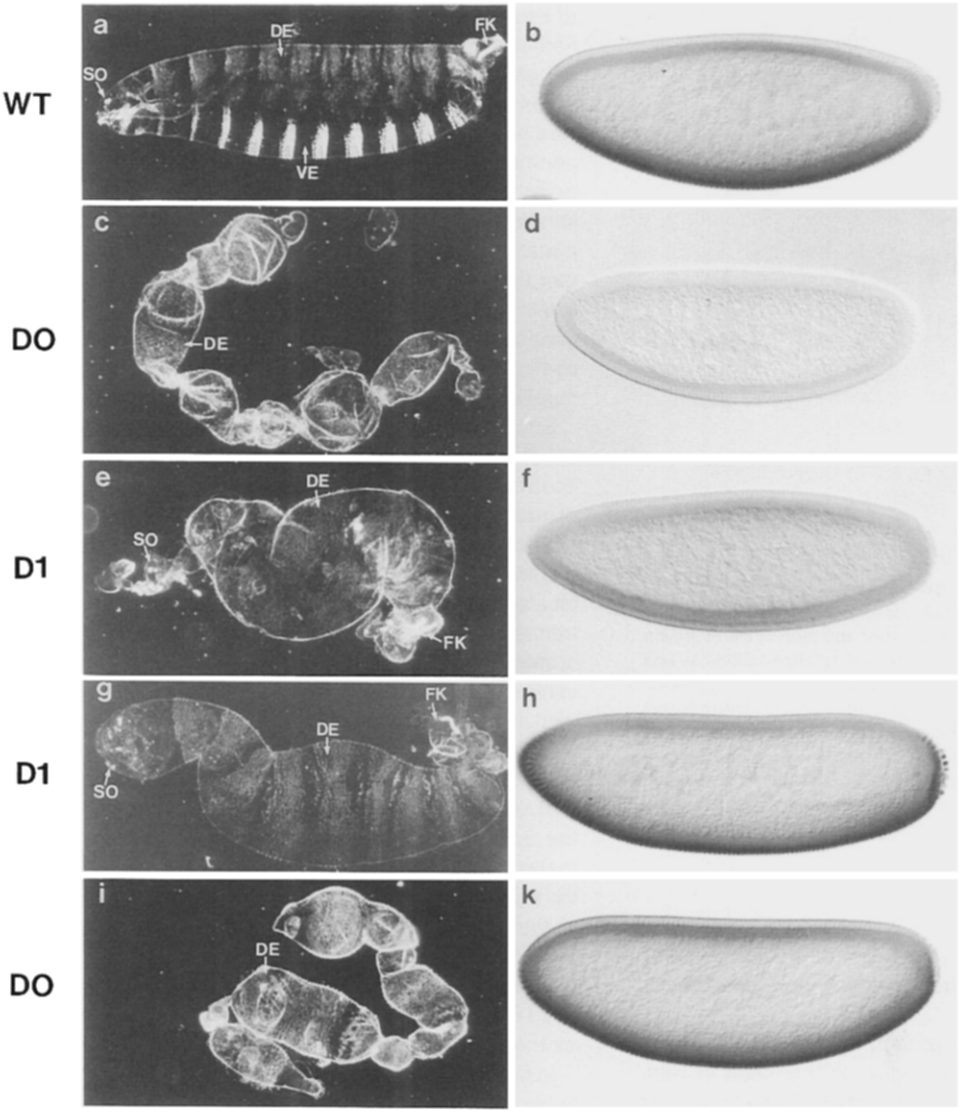

Figure 4. *dl* Protein in Whole Mounts of *dl* Mutant Embryos

Cuticles of differentiated embryos were photographed using dark-field optics (a, c, e, g, i). *dl* mutant embryos were stained using anti-*dl* antibody as described in the Experimental Procedures. Whole mount preparations were photographed using Nomarski optics (b, d, f, h, k). The maternal genotypes are: (a) and (b), wild-type; (c) and (d), dl^{1}/*Df(2L)TW119*; (e) and (f), dl^{QF}/*Df(2L)TW119*; (g) and (h), dl^{QI7}/*Df(2L)TW119*; and (i) and (k), dl^{D7}/*Df(2L)TW119*. The phenotypic strength is indicated at the left. For explanation, see Table 1. DE, dorsal epidermis; FK, filzkörper (dorsolateral structures); SO, antenno-maxillary sense organs of the head (dorsolateral structures); VE, ventral epidermis.

derived from females that were *trans*-heterozygous for mutant alleles and a deficiency for *dl*, *Df(2L)TW119*. Eight of the 16 *dl* alleles tested did not express a visible *dl* protein band (Figure 3; summarized in Table 1), and mutant embryos corresponding to these alleles are not stained by anti-*dl* antibodies. These eight alleles give rise to completely dorsalized embryos (D0, according to the terminology of Anderson et al., 1985a; see Figure 4d for a representative staining). Although it is possible that several of the mutant alleles investigated retain a *dl* protein that lacks epitopes recognized by our antiserum, the fact that all recessive alleles that give rise to completely dorsalized embryos show a complete lack of *dl* staining suggests that nonstaining embryos contain no residual *dl* protein.

Several *dl* alleles are hypomorphic, giving rise to embryos that produce a partial dorsoventral pattern lacking only the mesoderm (D2, Anderson et al., 1985a) or both the mesoderm and the ventral epidermis (D1, Anderson et al., 1985a). These embryos develop only dorsolateral (head sense organs, trachea, and filzkörper) and dorsal structures. The hypomorphic alleles can be divided into two groups based on Western blot and embryo staining patterns. In the first group (e.g., dl^{QF}, see Figures 4e and 4f), the hypomorphic character is reflected in an embryonic staining pattern in which only low nuclear *dl* protein levels are reached ventrally in comparison with the wild-type control. These alleles give a weak or complete absence of signal on Western blots (Figure 3: dl^{011}, dl^{QF},

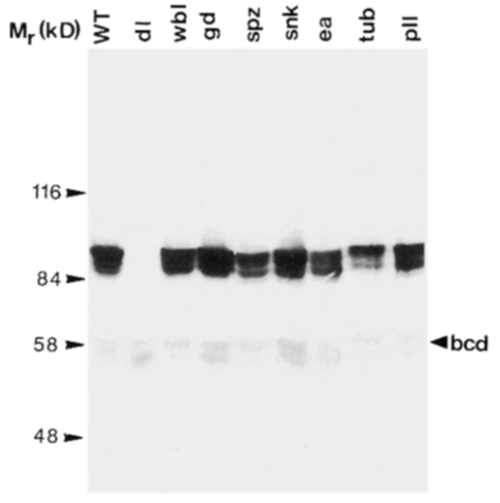

Figure 5. Detection of *dl* Protein in Mutants of the Dorsal Group Genes

Embryonic extracts from 0–4 hr old mutant embryos were separated by SDS–PAGE and transferred to nitrocellulose. The Western blot was first incubated with anti-*dl* antibodies. After detection of the *dl* protein bands, a second incubation with monoclonal anti-*bcd* antibodies was performed to visualize differences in the amount of protein applied to each lane, employing *bcd* signal as an internal standard. The maternal genotype of the embryos is indicated above the lanes. The following allele combinations were used: *In(2L)dl^{Tl}/Df(2L)TW119* (negative control); *wbl^{RP}/wbl^{T6}*; *gd^{7}/gd^{7}*; *spz^{197}/spz^{rm7}*; *snk^{073}/snk^{229}*; *ea^{1}/ea^{2}*; *tub^{118}/tub^{238}*; and *pll^{078}/pll^{rm8}*. Apparent molecular masses of marker proteins in kd are indicated at the left.

dl^{3}, and *dl^{SC}*). The mutant phenotype of the first group of *dl* alleles is likely to result from reduced production or stability of the gene product.

Embryos of the second group of hypomorphic alleles exhibit protein distributions similar to that seen in wild-type embryos; some also show weakly stained nuclei at the dorsal side of the embryo (e.g., *dl^{Ql7}*, Figures 4g and 4h). The *dl* proteins from this second group of hypomorphic alleles migrate with either wild-type or increased electrophoretic mobility on Western blots, depending upon the allele under study. The second group of alleles may encode gene products with a defective function that are expressed and localized normally but are not functional. An extreme example of this class is the completely penetrant dominant allele *dl^{D7}* (Szabad et al., 1989) (see Figures 4i and 4k). *Trans*-heterozygotes of this allele with a deficiency give rise to completely dorsalized embryos (see Figure 4i), demonstrating that the gene product is completely nonfunctional. In these embryos the mutant gene product is expressed and localized normally (Figure 4k). *Trans*-heterozygotes of this allele with a wild-type copy of the *dl* gene give rise to weakly dorsalized embryos (data not shown). The dominant effect of this allele may result from an interference of the mutant gene product with the function of the wild-type protein. Among the ten alleles producing detectable *dl* protein, none was found that exhibits a cytoplasmic gradient in the absence of nuclear localization.

dl Protein in Mutants of the Dorsal Group and *cactus*

Females homozygous for strong alleles of any of the dorsal group genes give rise to completely dorsalized embryos (Anderson and Nüsslein-Volhard, 1984a, 1986). It was previously shown in cytoplasmic transplantation experiments that the cytoplasm of dorsal group mutant embryos contains *dl* rescuing activity (Anderson and Nüsslein-Volhard, 1986). To investigate the effects of these mutations on the distribution of *dl* protein we stained mutant embryos and prepared Western blots of embryonic extracts from mutants of the dorsal group. For all of the genes tested, Western blot analysis revealed the presence of a protein of similar abundance and electrophoretic mobility to wild-type *dl* product (Figure 5).

Embryos derived from all members of the dorsal group are stained with anti-*dl* antibodies. However, the *dl* protein does not become localized within nuclei, and the gradient of nuclear localization seen in wild-type embryos is not established. Figures 6a–6d show, as an example, whole mount stainings of *Tl^{-}* embryos at different developmental stages. As seen in wild-type, the protein is evenly distributed in cleavage stage embryos. However, as development proceeds, *dl* protein never accumulates in the ventral nuclei of *Tl^{-}* embryos. The distribution of *dl* protein around the entire circumference of a *Tl^{-}*embryo is similar to that seen on the dorsal side of a wild-type embryo. To rule out the possibility of minor differences in pattern and abundance of *dl* protein in different members of the dorsal group, we stained embryos derived from females mutant for different members of the dorsal group in the same batches (see legend to Figure 6). No differences in the distribution or abundance of *dl* protein were observed.

Figure 6e also shows an unstained control embryo derived from a *dl* female that was stained in the same batch as the *Tl*-derived embryos.

In summary, these data show that in the absence of any of the other dorsal group gene products, a *dl* protein is produced that is indistinguishable from that seen in wild-type embryos with respect to its electrophoretic mobility and abundance. However, the mutant embryos never showed any nuclear localization or asymmetric distribution of *dl* protein. This suggests that the function of the dorsal group gene products is to regulate the asymmetric nuclear uptake of *dl* protein.

Ventralized embryos derived from females carrying dominant gain-of-function alleles of *Tl* or recessive lack-of-function alleles of *cact* show an expansion of the lateral and ventral parts of the dorsoventral pattern at the expense of dorsal regions. This phenotype is accompanied by increased nuclear *dl* protein levels in the dorsal half of the EC. Figure 7 shows sections of a completely dorsalized and a strongly ventralized embryo that were stained in the same batch. In the dorsalized embryo the cytoplasm is strongly stained by anti-*dl* antibodies and the nuclei are not stained, whereas cytoplasmic *dl* staining of the ventralized embryo is strongly reduced and the nuclei are intensely stained. The depletion of the *dl* protein from the cytoplasm in the ventralized embryo is most apparent in the small cytoplasmic space between the egg mem-

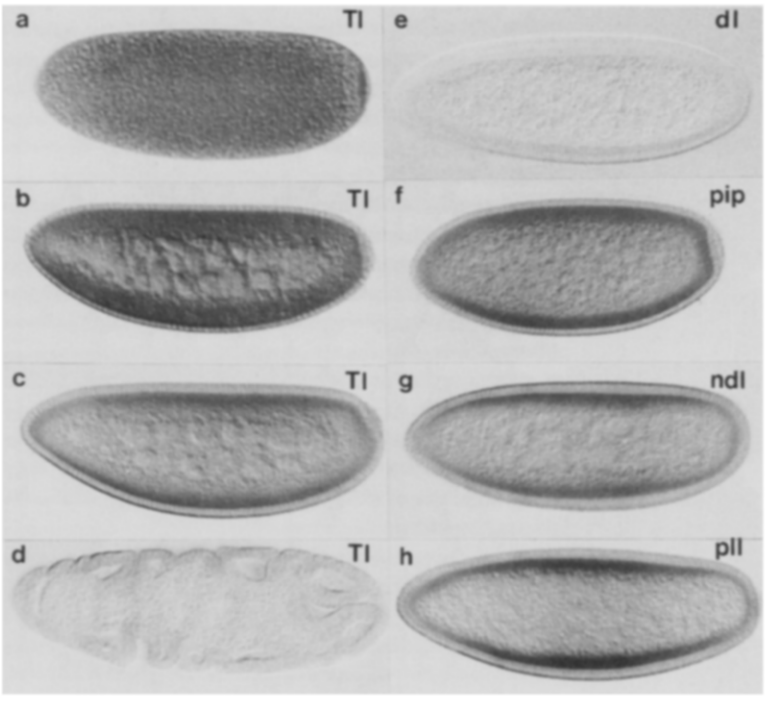

Figure 6. *dl* Protein in Whole Mounts of Dorsal Group Embryos

(a), (b), (c), and (d) Developmental series of *Tl* mutant embryos derived from females of the genotype $Tl^{5BRE}/Df(3R)ro^{XB3}$.
(a) Cleavage stage (stage 2).
(b) Syncytial blastoderm (stage 4).
(c) Cellular blastoderm (stage 5).
(d) Stage corresponding to germband extension in wild-type (stage 7).
(e) Negative control embryos derived from females mutant for both dl^{1} and $stau^{D3}$. They can be recognized by the absence of pole cells.
(f) Maternal genotype pip^{386}/pip^{664}.
(g) Maternal genotype $ndl^{o46}osk^{166}/ndl^{093}osk^{166}$. The embryos can be identified by the absence of pole cells and were stained in the same batch with *Tl* and *pipe* mutant embryos.
(h) Maternal genotype pll^{078}/pll^{rm8}.

brane and the nuclear layer. As shown by Western blot analysis, dorsalized and ventralized embryos appear to contain the same overall amount of *dl* protein (Figure 8). We do, however, observe a difference in the electrophoretic mobility of the *dl* protein bands from ventralized vs. dorsalized embryos. The *dl* protein derived from ventralized embryos migrates slightly more slowly, and the lower molecular weight species found in wild-type and dorsalized embryos has almost disappeared. This effect is consistently observed, irrespective of the genotype causing the ventralization (*cact* or Tl^{D}).

The Nuclear Concentration of the *dl* Protein Determines the Pattern of Zygotic Gene Expression

To investigate further the relationship between nuclear *dl* protein concentration and cell fate, we manipulated the nuclear *dl* protein concentration gradient using genetic conditions that cause various degrees of dorsalization and ventralization. Such embryos can be produced by mutant alleles of *dl* group genes and *cact*. As markers for cell fate we used antibodies against the products of the

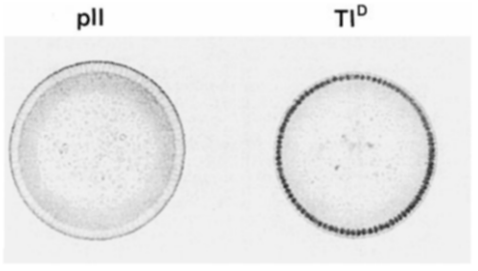

Figure 7. Cytoplasmic vs. Nuclear *dl* Protein Content of Dorsalized and Ventralized Embryos

Dorsalized and ventralized embryos were stained for *dl* protein in the same batch, embedded together, and sectioned. The maternal genotypes are pll^{078}/pll^{rm8} (left) and $Tl^{10b}/+$ (right).

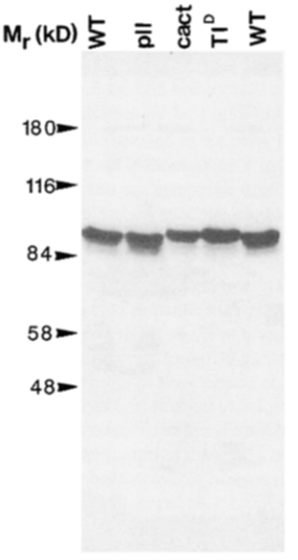

Figure 8. Detection of *dl* Protein in Ventralizing Mutants

Embryonic extracts from 500 0–4 hr old mutant embryos were separated by SDS–PAGE. The Western blot was subsequently developed as in Figure 1. The maternal genotype of the embryos is indicated above the lanes. The following allele combinations were used: pll^{078}/pll^{rm8}, $cact^{A2}/cact^{A2}$, and $Tl^{10b}/+$. Apparent molecular masses of marker proteins in kd are indicated at the left.

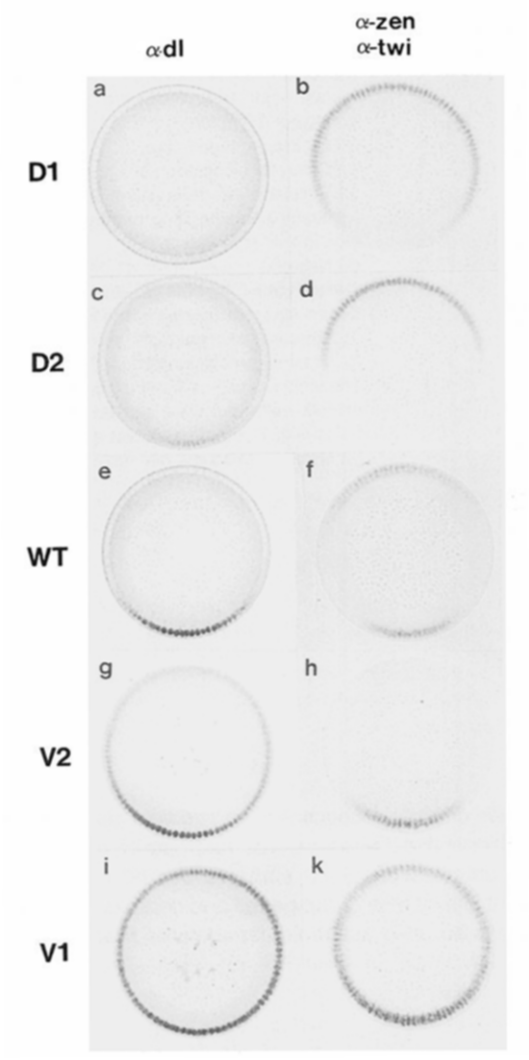

Figure 9. *dl* Protein Distribution and the Expression Pattern of *zen* and *twi* in Partially Dorsalizing and Ventralizing Mutants

Mutant embryos of syncytial or cellular blastoderm stage were stained for *dl* (a, c, e, g, i) or simultaneously for *zen* and *twi* proteins (b, d, f, h, k), embedded, and sectioned as described in the Experimental Procedures. Transverse sections shown are derived from 40%–50% egg length. The maternal genotypes are: (a) and (b), Tl^{r632}/Tl^{9QRE} at 18°C; (c) and (d), spz^{67}/spz^{rm7} at 18°C; (e) and (f), wild-type; (g) and (h), $cact^{A2}/cact^{A2}$; and (i) and (k), $Tl^{10b}/+$. The phenotypic strength is indicated at the left. The classification of the dorsalized phenotypes is provided in the legend to Table 1. For ventralized phenotypes, we introduce a terminology different from that used by Anderson et al. (1985a). V2 refers to phenotypes in which all dorsal and dorsolateral structures are completely deleted and replaced by ventral epidermis, although the mesoderm is not significantly increased in the middle region of the embryo. V1 refers to phenotypes that show, in addition to the fate map shifts seen in V2, increased mesodermal anlagen in the middle part of the mutant embryos.

zygotic genes *twist* (*twi*) and *zerknüllt* (*zen*). In the wild-type embryo, the early *zen*-expressing area covers the dorsal 40% of the EC, and the *twi*-expressing domain comprises 20% of the EC across the ventral midline (Doyle et al., 1986; Thisse et al., 1987b, 1988). Neither of the two genes is expressed in the ventrolateral region (Figure 9f). Thus, the distribution of *twi* and *zen* protein in the early blastoderm defines a simple pattern of three regions, one expressing only *twi*, one expressing neither of the two, and one expressing only *zen*. This pattern is under the control of the dorsal group: in dl^- embryos, *zen* is expressed all around the EC (Rushlow et al., 1987) while *twi* expression is abolished (Thisse et al., 1987b). We describe the *dl* protein distribution (left column of Figure 9) as well as the expression pattern of *twi* and *zen* in partially dorsalized or ventralized mutant embryos. The expression of *twi* and *zen* was monitored simultaneously in the same embryo, using double staining with the two antibodies (right column of Figure 9).

In partially dorsalized embryos (D1, D2), the reduction in nuclear *dl* protein levels at the ventral side is paralleled by a lack of expression of *twi* and an expansion of the *zen*-expressing areas. In weakly dorsalized embryos (D2) exhibiting low *dl* protein concentration in the ventralmost nuclei, the *zen*-expressing area is only slightly expanded (Figures 9c and 9d). In D1 embryos, where hardly any *dl* protein can be detected in the ventral nuclei, the *zen*-expressing area covers more than half of the EC (Figures 9a and 9b). In partially ventralized embryos (V1, V2) the region of *dl* protein nuclear localization is expanded toward the dorsal egg side. This expansion is correlated with a deletion of the *zen*-expressing area and, in V2 embryos, a concomitant expansion of the region expressing neither *twi* nor *zen* over the dorsal side of the egg (Figures 9g and 9h). The *twi*-expressing region is only slightly expanded in V2 embryos. In the strongly ventralized V1 embryos, *dl* protein is present in the nuclei all around the EC. These embryos retain only a slight asymmetry with respect to the nuclear *dl* content. The *twi*-expressing area of these embryos surrounds the entire egg (Figures 9i and 9k), but the highest levels of expression are reached only ventrally, suggesting a sensitive dependence of *twi* expression on high nuclear *dl* protein levels.

The correlation of *dl* nuclear concentration and cellular determination as monitored in the expression pattern of *twi* and *zen* also holds for embryos that are apolar, as revealed in their gastrulation pattern and final cuticular phenotypes. These embryos have only one level of *dl* nuclear protein concentration all around the EC and have only one of the three regions defined by *twi* and *zen* expression (Figure 10). In completely dorsalized embryos (D0), the absence of *dl* protein in the nuclei around the entire EC is accompanied by a uniform expression of *zen* and a complete absence of *twi* expression (Figures 10l and 10m). Apolar embryos displaying a ventrolateral pattern are characterized by the complete absence of *zen* and *twi* expression (lateralization L1; Figures 10g and 10h). They exhibit an even *dl* protein distribution with nuclear levels similar to cytoplasmic *dl* protein levels (Figures 10e and 10f). Finally, embryos that are completely ventralized (V0) express *twi* at equal levels at all positions of the EC. They display the highest nuclear *dl* protein levels uniformly around their entire EC (Figures 10a and 10b).

In summary, there is a strong correlation between the pattern of expression of *twi* and *zen*, and thus the embryonic fate map, with that of the *dl* protein nuclear con-

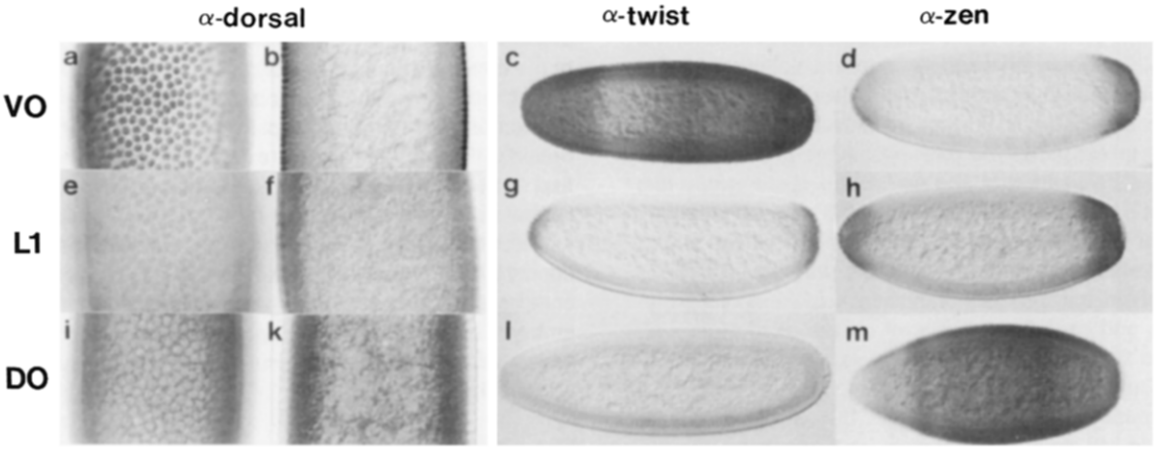

Figure 10. *dl* Protein Distribution and Expression Pattern of *zen* and *twi* in Completely Dorsalized, Lateralized, and Ventralized Mutants

Mutant embryos were stained for *dl* (a, b, e, f, i, k), *twi* (c, g, l), or *zen* protein (d, h, m).

(a), (e), and (i) Surface views of the middle part of syncytial blastoderm embryos.

(b), (f), and (k) Optical midsections of the same embryos shown in (a), (e), and (i).

(c), (d), (g), (h), (l), and (m) Optical midsections of cellular blastoderm embryos. The maternal genotypes are: (a)–(d), $cact^{A2};Tl^{rm9}/cact^{A2};Tl^{rm9}$; (e)–(h), Tl^{rm}/Tl^{rm}; and (i)–(m), $Tl^{5BRE}/Df(3R)ro^{XB3}$. The classification of the phenotypes is indicated at the left. D0: complete dorsalization, L1: lateralization at a ventrolateral level (according to Anderson et al., 1985a), V0: complete ventralization. In correlating *zen* and *twi* expression patterns with nuclear *dl* protein levels, we have considered only the nonterminal portions of the embryo (30%–70% egg length). In the case of *zen*, it has been shown that expression in the terminal regions is subject to additional regulatory inputs (Rushlow et al., 1987).

centration. High concentrations promote the expression of *twi*. Intermediate levels, in which the nuclear *dl* protein concentration is similar to that of the cytoplasm, are not sufficient to promote *twi* expression but are sufficient to repress *zen*. Finally, an apparent absence of nuclear *dl* protein is correlated with *zen* expression.

Discussion

The Formation of the Nuclear *dl* Protein Gradient

The correspondence between pattern and nuclear *dl* protein concentration suggests that *dorsal* acts as a morphogen that determines size and fate of regions of the dorsoventral axis. Strikingly, a graded distribution is only observed for the *dl* protein localized in the nuclei, while the sum of nuclear and cytoplasmic protein content is equal all around the egg, and at no stage of early development is an asymmetry of total *dl* protein observed. Several lines of evidence support this notion: First, no gradient is observed in embryos undergoing blastoderm mitotic divisions, whereas the nuclear gradient is reestablished as soon as the nuclei are reconstituted after mitosis. Second, in the ventral region of blastoderm embryos, the high nuclear concentration of *dl* protein correlates with a depletion in the cytoplasm. Such a depletion is also observed in ventralized embryos; dorsalized embryos lacking *dl* nuclear staining show relatively high *dl* protein concentration in the cytoplasm. Finally, the total amount of *dl* protein, as determined in Western blots, does not differ in ventralized as compared with wild-type and dorsalized embryos.

We conclude that the polarity of the dorsoventral axis is determined by the differential distribution of the *dl* protein between the nuclear and cytoplasmic compartments of the embryo. The cytoplasmic *dl* protein content does not appear to have a function in pattern determination, since dl^- mutant embryos that completely lack the *dl* protein have a phenotype indistinguishable from that of dorsal group mutants containing cytoplasmic *dl* protein. The nuclear accumulation of the *dl* protein in the ventral egg side apparently occurs as the consequence of a gradient of nuclear uptake, with the cytoplasmic protein serving as a source. The *dl* protein contains a sequence that shows homology to nuclear targeting signals (Steward, 1987; Silver and Hall, 1988). This signal may be necessary for the nuclear uptake of the *dl* protein, but it cannot be sufficient. Our studies of mutants of the dorsoventral system demonstrate that external factors, both of an inhibitory (*cact*) and a permissive (dorsal group) nature, are involved in this process.

cactus

In the absence of *cactus* function, the *dl* protein is taken up into the nuclei on the dorsal as well as the ventral side (Figures 9g and 9h). This suggests that the wild-type function of the *cact* gene product is to inhibit nuclear localization of the *dl* protein. The proposed *dl–cact* interaction shows some similarity to the regulation of nuclear localization of the transcription factor NF-κB (Baeuerle and Baltimore, 1988). The nuclear accumulation of NF-κB is specifically inhibited by complex formation with a cytoplasmic inhibitor, IκB. The release of NF-κB occurs as a consequence of a modification of the inhibitor protein. By analogy, the *cact* protein could bind to the *dl* protein to form a complex that cannot be taken up into the nuclei.

In one model, the function of the dorsal group genes would be required to modify the *cact* protein in the ventral egg region such that it no longer binds to *dl*, and the free

form of the *dl* protein would enter the nucleus. A prediction of this model is that, in the absence of *cact* function, the *dl* protein should be taken up in the nuclei of the dorsal as well as the ventral side at a high rate, resulting in apolar, ventralized embryos. Further, an exclusive function of the dorsal group genes via *cact* postulates that the double mutants between *cact* and any of the dorsal group loci (with the exception of *dl*) would exhibit a *cact* phenotype. Neither of these predictions are fulfilled, however: strong *cact* embryos display polarity and a partially ventralized phenotype, and double mutants of any of the dorsal group genes and *cact* show low level uptake of *dl* protein all around the EC, resulting in an apolar, lateralized phenotype (Roth and Nüsslein-Volhard, unpublished data). The discrepancy could be explained if the strongest *cact* alleles do not completely abolish *cact* function. Alternatively, the function of the dorsal group genes in promoting *dl* nuclear uptake could be, at least in part, independent of *cact*.

Dorsal Group Genes

All ten members of the dorsal group genes are required for the nuclear uptake of the *dorsal* protein in a spatially restricted manner. In lack-of-function mutants of all of the dorsal group genes, *dl* protein remains in the cytoplasm, and the embryos show an apolar, dorsalized phenotype. An important function of the dorsal group genes is to orient the *dorsal* nuclear gradient, thus defining the ventral side of the embryo. The process of establishing polarity takes place in the absence of *dl*, as evident from transplantation experiments in which *dl* embryos display a strong intrinsic dorsoventral polarity: wild-type cytoplasm (containing dl^+ RNA and protein) only rescues dl^- embryos if placed in a ventral (as opposed to a dorsal) position, and the rescued pattern shows a polarity coinciding with that of the outer egg shell (Santamaria and Nüsslein-Volhard, 1983). This experiment indicates that the function of the dorsal group genes is to provide the asymmetric environment required for the ventral nuclear localization of the *dl* protein.

Tl is an important factor in the establishment of polarity. It encodes a putative membrane-bound receptor that could be activated by a local signal coming from the outside of the embryo. Genetic analysis has revealed that most of the dorsal group genes act upstream of *Tl* (three of the loci were not at that time tested)(Anderson et al., 1985a). The genes encoding *easter* and *snake* have been cloned and are predicted, on the basis of sequence analysis, to encode secreted serine proteases (DeLotto and Spierer, 1986; Chasan and Anderson, 1989). It is possible that the dorsal group functions exclusively via *cact*, as described above. Alternatively, the dorsal group genes may act upon *dl* directly and lead to a local modification of the *dl* protein that promotes its capacity to enter the nucleus. In this model, the unmodified form of *dl* protein would enter the nucleus at an intermediate rate, while the modified protein would be rapidly taken up into the nucleus. As in the previous model, *cact* protein bound to the unmodified form of *dl* would inhibit nuclear uptake, but the modification of *dl* would prevent *cact* from binding, resulting in quantitative nuclear uptake at the ventral side.

Our data suggest posttranslational modification of the *dl* protein, although it is not clear whether this is relevant to the differential nuclear localization. The assumption of a posttranslational modification of the *dl* protein implies that although we cannot detect a net asymmetry of distribution of the *dl* protein along the dorsoventral axis, a modified nuclear form may constitute a gradient spanning the ventral side of the egg. Such a modification could explain why in cytoplasmic transplantation experiments a higher concentration of *dl* activity could be detected in ventral, as compared with dorsal, cytoplasm of syncytial blastoderm embryos (Santamaria and Nüsslein-Volhard, 1983; Nüsslein-Volhard and Roth, 1989). However, other explanations are possible.

The *dl* Protein Is a Morphogen

In this paper, we present evidence supporting the notion that the *dl* protein is the morphogen of the dorsoventral axis. There is a strong correlation between regional nuclear *dl* protein concentration and the developmental fate of blastoderm cells. Embryos genetically determined to develop an apolar pattern always display an even *dl* protein nuclear distribution, while in polar embryos the *dl* protein is distributed in a nuclear concentration gradient. Comparison of *dl* protein distribution and embryonic phenotype in a number of different mutant conditions makes it possible to define a fate map of regions of different *dl* nuclear concentrations: High *dl* concentrations are required for the formation of the ventralmost structures, the mesoderm. Intermediate levels define the neuroectoderm at a ventrolateral region. Even lower nuclear concentrations give rise to dorsolateral structures, while absence of *dl* protein from the nuclei is required to permit the development of dorsal ectoderm.

The investigation of apolar embryos that display only one type of pattern shows that the determination of cell fate by the nuclear *dorsal* protein concentration occurs in a largely autonomous manner and is independent of adjacent regions. Using different mutant combinations, it is possible to generate at least four types of apolar embryos. Such embryos may display an exclusively dorsal, dorsolateral, ventrolateral, or ventral pattern (Anderson et al., 1985a; and this work). This means that at least four regions are autonomously defined by different levels of *dl* protein. Therefore, the three subregions defined by the expression patterns of the zygotic genes *twi* and *zen*, which we used as monitors for the blastoderm fate map, still reflect a simplified pattern of response of the zygotic genome to the maternal morphogen gradient. We do not know, however, how many more regions of the fate map are autonomously defined by the local *dl* concentration and to what extent the exact position of the boundaries between regions depends upon the local interaction of zygotic target genes.

In addition to *twi* and *zen*, several other zygotically expressed genes are involved in the establishment of pattern along the dorsoventral axis (Simpson, 1983; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984; Zusmann and Wieschaus, 1985; Irish and Gelbart, 1987; Mayer and Nüsslein-Volhard, 1988; Arora and Nüsslein-Volhard, un-

published data). The ventralmost pattern depends upon *snail* in addition to *twi*. For the dorsal and dorsolateral pattern, *decapenteplegic* (*dpp*) plays a pivotal role. *dpp* is initially expressed in a broad dorsal domain like *zen* and is required for the establishment of the entire dorsal half of the blastoderm fate map (Irish and Gelbart, 1987; St. Johnston and Gelbart, 1987).

Although *dl* does not contain any of the protein sequence elements known to confer sequence-specific DNA binding properties, such as a homeobox or zinc fingers (Struhl, 1989), it is possible that the *dorsal* protein acts as a transcriptional regulator. *dl* is homologous to c-*rel*, a proto-oncogene for which an involvement in transcriptional regulation has been proposed (Gelinas and Temin, 1988). The genes *twi*, *zen*, and *dpp* may be direct target genes of *dl*. In this case, *dl* would function as an activator of *twi* and as a repressor of both *zen* and *dpp* (Thisse et al., 1988).

Anteroposterior vs. Dorsoventral Determination

The dorsal group and *cact* constitute a system of 12 genes whose concerted actions culminate in the graded nuclear localization of a morphogen, the product of the *dl* gene. In contrast, the anteroposterior pattern is determined by three independent systems specifying largely nonoverlapping portions of the pattern along the axis (Nüsslein-Volhard et al., 1987). Therefore, it is perhaps not surprising that each of the anteroposterior systems appears to be less complex than that of the dorsoventral axis, with respect both to the number of genes and to the biochemical mechanisms involved.

In terms of action, the greatest similarity to *dl* is found in the gene *bicoid*, a morphogen that determines anterior pattern via a concentration gradient (Frohnhöfer and Nüsslein-Volhard, 1986; Driever and Nüsslein-Volhard, 1988a and 1988b). Like *dl*, the *bicoid* protein is located in the nucleus. However, in the case of *bicoid*, the distribution in the nuclei parallels that in the cytoplasm. The origin of the *bicoid* gradient is strikingly different from that of *dl*. It is the prelocalized *bicoid* RNA at the anterior tip of oocyte and early embryo (Berleth et al., 1988) that provides a source for the protein gradient, which is probably simply formed by diffusion and dispersed decay and does not require other factors for its establishment. In the case of *dl*, no asymmetry in distribution of RNA or protein is observed, and the graded nuclear uptake of the *dl* protein requires an elaborate mechanism involving 11 genes. The means by which the dorsal group genes receive and transmit a spatial signal from the outside is reminiscent of the terminal system in which the gene *torso*, as a putative receptor tyrosine kinase, plays an important role in receiving an external signal, presumably from specialized follicle cells (Klingler et al., 1988; Sprenger et al., 1989).

Finally, the orientation and asymmetry in each of the four systems can be traced to the establishment of polarity of the follicle in early oogenesis. The polar arrangement of nurse cells and oocyte determines asymmetry in the anterior and posterior system, while recent data suggest that for the terminal (Frohnhöfer and Stevens, unpublished data) and dorsoventral systems (Schüpbach, 1987; Stein et al., unpublished data), asymmetries in the layer of the somatic follicle cells provide the spatial cues that, in the case of the dorsoventral system, lead to the stable orientation of the *dl* gradient.

Experimental Procedures

Fly Strains

The wild-type stock was Oregon R. dl^1 is described in Nüsslein-Volhard (1979a), and dl^2 in Nüsslein-Volhard (1979b). The alleles dl^{011}, dl^{B10}, and dl^{U5} were recovered by virtue of their dominant phenotype in a screen for new *cact*, *spätzle*, *windbeutel*, *va*, and *staufen* alleles, carried out by Mayer, Lehmann, and Nüsslein-Volhard (unpublished data). *Df(2L)TW119* (Wright et al., 1976), *In(2L)*dl^T, *In(2L)*dl^H, dl^5, dl^6, dl^8, and dl^{15} are described in Steward and Nüsslein-Volhard (1986). dl^{QF}, dl^{SC}, dl^{SG}, dl^{QI7}, and dl^{QD} were obtained from T. Schüpbach (Schüpbach and Wieschaus, 1989). dl^{D7} was obtained from J. Szabad (Szabad et al., 1989). The alleles of dorsal group genes spz^{rm7}, pll^{rm8}, Tl^{rm9}, Tl^{rm10} (designated as *mel(3)7*, *mel(3)8*, *mel(3)9*, *mel(3)10* by T. Rice, 1973); Tl^{5BRE}, Tl^{9QRE}, Tl^{r632}, *Df(3L)ro*^{XB3}, ndl^{046}, ndl^{093}, ea^1 (former designation ea^{IIR65}), tub^{118}, tub^{238}, snk^{073}, snk^{229}, pip^{386}, pip^{664}, pll^{078}, spz^{197}, and spz^{67} are described in Anderson and Nüsslein-Volhard (1984a, 1986) and Anderson et al. (1985a). Tl^{10b} was obtained from J. Szabad (Erdélyi and Szabad, 1989). The *cact* allele $cact^{A2}$ was obtained in an mutant screen carried out by Mayer, Lehmann, and Nüsslein-Volhard (unpublished data). The mutants lacking pole cells were osk^{166} (Lehmann and Nüsslein-Volhard, 1986) and $stau^{D3}$.

All mutant chromosomes carried visible markers allowing genotypic identification. Flies were grown and eggs were collected under standard conditions (Nüsslein-Volhard et al., 1984). Staging of embryos was according to Campos-Ortega and Hartenstein (1985).

DNA Manipulations

The *dl* cDNA clones were obtained from R. Steward. They have been described in Steward et al. (1984, 1985) and Steward (1987). The *lacZ–dl* fusion gene (pUR*dl*) was constructed by inserting a 1.4 kb XhoI–PstI fragment derived from the *dl* cDNA designated c1 into the PstI–SalI-digested pUR290 (Rüther and Müller-Hill, 1983). The ligation mix was introduced by transformation into E. coli strain JM109 as described in Maniatis et al. (1982), allowing the isolation of the *lacZ–dl* fusion construct denoted pUR*dl*.

Production and Purification of Polyclonal Antibodies

After introduction of pUR-*dl* into E. coli strain XL-1 by transformation, *lacZ–dl* fusion protein was isolated according to Rio et al. (1986) and Driever and Nüsslein-Volhard (1988a). Following dialysis of a urea extract containing the hybrid protein, precipitated material was sedimented (10,000 × g for 10 min), and the supernatant and precipitate were analyzed by SDS–PAGE. The precipitate was subjected to preparative SDS–PAGE. Protein bands were visualized by incubating the gel with 0.25 M KCl in H_2O. Gel slices containing the hybrid protein band were excised, pressed through very fine metal sieves, and suspended in 2 vol of complete Freund's adjuvant. Rabbits were injected at four subcutaneous and two intramuscular sites, boosted after 4 weeks and again after an additional 2 weeks. The rabbits were bled 1 week after boosting and then on a weekly schedule. Two injected rabbits produced *dl*-specific antibodies with a similar titer.

The supernatant obtained in the last step of the hybrid protein isolation was dialyzed against 0.1 M HEPES (pH 7.5), 0.5 M NaCl and used to prepare an affinity column (Affigel 10/15, 3:1 ratio; Biorad) according to the manufacturer's protocol. A second affinity column contained β-galactosidase coupled to the Affigel 10/15.

Ten milliliters of antiserum was diluted 1:1 with PBS containing 0.1 M NaCl and loaded onto the β-galactosidase-coupled affinity column. The flowthrough was loaded onto the hybrid protein-coupled affinity column. The antibody was eluted with 4 M $MgCl_2$ (pH 3.8). Fractions containing protein were pooled and dialyzed against PBS. The antibody solution was concentrated by membrane filtration (Amicon) and extensively preabsorbed using embryos derived from *In(2L)*dl^H/*Df(2L)TW119* females, which lack the *dl* mRNA (Steward et al., 1984).

We obtained a *twi* cDNA cloned in pUC92A from F. Perrin-Schmitt (Thisse et al., 1988). The plasmid was transformed into E. coli strain

XL-1, and hybrid protein production was induced for 3 hr by the addition of 1 mM isopropyl thiogalactoside (IPTG). The washed bacterial pellet was dissolved in sample buffer (Laemmli, 1970) and subjected to preparative SDS–PAGE. The hybrid protein bands were visualized, and the immunization was performed as described for *dl*. Two rabbits were injected. Both produced high titers of *twi*-specific antibodies. The anti-*twi*-containing serum was extensively preabsorbed using staged embryos (6–18 hr after egg deposition), which express only small amounts of the antigen (Thisse et al., 1988). The specifity of the antibodies was confirmed by the complete absence of immunostaining in embryos mutant for *twi* (twi^{ID96}: Nüsslein-Volhard et al., 1984, or $Df(2R)twi^{S060}$: Simpson, 1983; Thisse et al., 1987a).

Western Blot Analysis

The Western blot analysis was carried out as described by Driever and Nüsslein-Volhard (1988a), with minor modifications. The embryos were collected and counted, or the volume of settled embryos was estimated. Five hundred embryos (corresponding to 10 μl of settled embryos) or 10 μl of settled ovaries were frozen in liquid nitrogen. The material was thawed while being homogenized by sonication in 100 μl of 2× sample buffer (Laemmli, 1970), containing 8 M urea, and frozen in liquid nitrogen for storage. Prior to electrophoresis samples were incubated for 5 min at 100°C, and undissolved material was sedimented by centrifugation. The entire supernatant (approximately 100 μl) was applied to one slot of a 3 mm thick SDS–polyacrylamide gel containing either 10% or 7.5% polyacrylamide. Subsequent steps were performed as described (Driever and Nüsslein-Volhard, 1988a).

Expression of *dl* Protein in E. coli

For the expression of *dl* protein in E. coli, a plasmid containing the *dl* cDNA was subjected to oligonucleotide site-directed mutagenesis to generate an NdeI site at the initiator ATG of the open reading frame. An NdeI–ClaI fragment containing the open reading frame was then inserted into the expression vector pAR3038 (Studier and Moffat, 1986), yielding plasmid pAR-*dl*.

dl protein was expressed from pAR-*dl* in E. coli BL 21 DE3. Plasmid-carrying cells were grown in 2× YT medium at 37°C to an optical density of 0.5. IPTG was then added to a concentration of 2 mM. After 45 min of incubation, rifampicin was added to 200 μg/ml. After a further 90 min incubation the cells were harvested by centrifugation and prepared for SDS–PAGE by boiling the cell pellets in cracking buffer for 3 min. In Western blot analysis, protein from cells containing pAR3038 served as a negative control for *dl* production in E. coli.

Staining of Embryos

Immunological staining of whole mount embryos with biotinylated HRP-avidin complexes bound to biotinylated second antibody (Vector Laboratories, Avidin/Biotin ABC system) was carried out as described by Macdonald and Struhl (1986), with the modification that, during the washes, 100 mM NaCl was added to the solutions. Antibodies against *zen* protein were obtained from C. Rushlow (Rushlow et al., 1987). For sectioning, stained embryos were dehydrated (10 min in 70% ethanol, 2 × 10 min in 100% ethanol, 2 × 10 min in 100% acetone) and mounted in Durcupan-ACM (Fluka). A complete series of transverse sections (10 μm) was prepared for each embryo to study potential changes of the staining pattern along the anteroposterior body axes. The sections presented are derived from 40%-50% egg length (0% egg length = posterior tip).

Cuticle Preparations of Embryos

For the observation of cuticular structures, differentiated embryos were dissected from the vitelline membrane and mounted directly in a mixture of Hoyer's medium (Van der Meer, 1977) and lactic acid (1:1).

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