The functional domains of the 
*Drosophila* morphogen *dorsal*: evidence from the analysis of mutants

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The *dorsal* (*dl*) protein is a member of the Rel family of transcription factors. It is distributed in a nuclear concentration gradient along the dorsoventral axis of *Drosophila* embryos and activates or represses a discrete set of zygotic genes in a concentration-dependent manner. The nuclear uptake of the *dl* protein is stimulated by products of the dorsal group genes but inhibited by the *cactus* (*cact*) product. To analyze the functional domains of the *dl* protein, we sequenced 11 *dl* alleles and studied their interaction with *cact*. Four of these alleles were found to result in carboxy-terminal truncations of the protein. A deletion of 80 carboxy-terminal amino acids abolishes the ability of *dl* protein to activate the expression of mesodermal genes. Larger deletions also affect the repressor function of *dl*. However, a protein consisting only of the Rel homologous region still acts as a weak repressor of *zerkniillt* transcription. A missense mutation in the presumptive DNA-binding domain causes a complete lack-of-function phenotype in *trans* to a deficiency but exerts a dominant-negative effect in *trans* to a wild-type copy of *dl*. These and genetic data with the alleles that produce truncated proteins indicate that *dl* oligomerizes. The proteins truncated at the carboxy-terminal end show increased levels of nuclear uptake dorsally, but they still respond to the *cact*-mediated inhibition of nuclear transport. Therefore, carboxy-terminal sequences influence the cytoplasmic retention, although a domain of *dl-cact* interaction resides in the amino-terminal portion.

[Key Words: *cactus*, dorsoventral polarity, NF-κB, nuclear localization, *rel*, transcription factor]

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The *dorsal* (*dl*) protein is a morphogen that forms a nuclear concentration gradient along the dorsoventral axis of *Drosophila* embryos [Roth et al. 1989; Rushlow et al. 1989; Stewart 1989]. It belongs to the Rel family of transcriptional regulators [Steward 1987], which includes the protein encoded by the *c-rei* proto-oncogene, among others [Stephens et al. 1983; Wilhelmsen et al. 1984; Bull et al. 1990; Capobianco et al. 1990], and the p50 and p65 subunits of NF-κB [Baueuerle and Baltimore 1988; Ghosh et al. 1990; Kieran et al. 1990; Nolan et al. 1991; Ruben et al. 1991]. A region of ~300 amino acid residues in the amino-terminal half, the Rel homology (RH) domain, is conserved in the various Rel family proteins.

The DNA-binding region of Rel family proteins resides in the RH domain [Gilmore 1991]. DNA-binding sites have been characterized for NF-κB [Baueuerle 1991]. They reveal an imperfect palindrome, the κB motif, as a consensus binding sequence for NF-κB. Homodimerization has been found for most Rel-related proteins and seems to be a necessary prerequisite for their function as transcriptional regulators [Kieran et al. 1990; Inoue et al. 1991; Logcat et al. 1991; Nolan et al. 1991; Urban et al. 1991].

*dl* protein also binds DNA via its RH domain [Ip et al. 1991]. The identified binding sites in the *zerkniillt* (*zen*) and *twist* (*twi*) promoters resemble those of NF-κB [Ip et al. 1991; Jiang et al. 1991; Pan et al. 1991; Thissie et al. 1991]. However, although NF-κB binding so far has been found to lead only to the activation of target genes, the *dl* protein is thought to act as an activator of some and a repressor of other genes in the same nucleus. In the ventral-most region of the embryo where the *dl* protein reaches its highest nuclear concentrations, it stimulates the expression of *twi* and *snail* (*sna*), two genes required for mesoderm formation, and represses the expression of *zen* and *decapentaplegic* (*dpp*), both involved in the specification of dorsally derived structures [Irish and Gelbart 1987; Rushlow et al. 1987; Roth et al. 1989; Ray et al. 1991].

The activity of the Rel-related proteins is not regulated primarily at the level of transcription or translation but, rather, depends on the controlled nuclear uptake of protein constitutively present in the cytoplasm [Baueuerle 1991]. Cytoplasmic NF-κB and c-Rel are complexed with κB and pp40, respectively, which function as inhibitors of nuclear transport and DNA binding. κB and pp40
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probably block the use of the nuclear localization signal (NLS) found at identical positions in all known Rel-related proteins [Baueuerle and Baltimore 1988; Davis et al. 1991; Haskell et al. 1991; Kerr et al. 1991; Nolan et al. 1991]. The NF-xB/IkB complex dissociates (or undergoes a structural change), presumably upon phosphorylation of IkB, so that nuclear transport and DNA binding can occur [Ghosh and Baltimore 1990]. Thus, the activity of the Rel family proteins appears to be linked to extracellular signals via the modification of inhibitor proteins.

The formation of the nuclear dl protein gradient also occurs by regulation of nuclear uptake [Roth et al. 1989; Rushlow et al. 1989; Steward 1989]. The dl protein is first distributed uniformly in the embryonic cytoplasm. During the syncytial blastoderm stage, the ventral nuclei accumulate the highest dl protein amounts, lateral nuclei gain intermediate levels, and no (detectable) uptake of the dl protein into dorsal nuclei occurs. Because the nuclear dl protein activates and represses zygotic dorsoventral genes in a concentration-dependent fashion, the shape of the nuclear dl protein gradient determines the spatial pattern of cell differentiation along the dorsalventral axis. Eleven factors are known to be required for the regulation of the nuclear uptake of the dl protein. Ten of these constitute [together with dl] the dorsal group (Anderson and Nüsslein-Volhard 1986), whose products act to promote nuclear localization ventrally. Only one component, encoded by the gene cactus [cact], so far is known to act as an inhibitor of nuclear localization [Roth et al. 1991].

The dorsal group genes control a complicated sequence of events, which includes the formation of an extracellular signal and its transmission to the cytoplasm by a transmembrane receptor, the product of the Toll gene [Hashimoto et al. 1988; Stein et al. 1991]. Only two of the dorsal group genes, pelle [pll] and tube [tub], function like dl inside the embryo [Lescou et al. 1991; K.V. Anderson, pers. comm.], suggesting that they are more directly involved in the nuclear uptake of the dl protein. The products of pll and tub must counteract the inhibition of nuclear transport exerted by cact.

To define functional domains of the dl protein, we have sequenced 11 dl alleles and studied their phenotypes alone and in double mutants with cact. The correlation of sequence alterations with phenotypic features of these dl alleles is consistent with the idea that dl, like the other Rel family proteins, has an amino-terminal DNA-binding domain and a carboxy-terminal domain required for transcriptional activation/repression. The phenotypes resulting from truncated proteins indicate that both amino- and carboxy-terminal regions of dl protein are involved in the regulation of nuclear transport. Furthermore, we describe dominant effects that indicate a self-association of the dl protein.

Results

Of 17 ethylmethane sulfonate [EMS]-induced dl alleles, 6 are complete loss-of-function [amorphic] alleles, as judged by genetic criteria. No protein was detected in embryonic extracts of these six alleles, indicating that their mutant phenotype is caused by a defect in transcription or translation [Roth et al. 1989]. To gain information on the functional organization of the dl protein, we sequenced the coding region of the remaining 11 alleles. Each of these 11 alleles exhibits some residual gene function, in one case detectable only as a dominant-negative [antimorphic] effect [dlD^T]. The dl protein distribution and the Western blot analysis of most of these alleles have been described earlier [Roth et al. 1989] and are summarized in Table 1.

Phenotypic classification of dl alleles

The dl alleles can be grouped according to the strength of the mutant phenotype [Nüsslein-Volhard 1979; Anderson et al. 1985; Anderson and Nüsslein-Volhard 1986]. Weak alleles [dlSc and dlSc at 22°C, dlG and dlI at 18°C] cause a deletion of mesoderm but allow the formation of some ventral and lateral epidermis (weak dorsalization, D2). The weak dorsalization is characterized by the absence of twi expression ventrally [Roth et al. 1989]. Because twi transcription is activated by the dl protein [Jiang et al. 1991; Pan et al. 1991; Thissle et al. 1991], the weakly dorsalizing alleles are defective in transcriptional activation. On the other hand, the determination of ventral and lateral epidermis found in D2 embryos occurs via the repression of genes like zen and dpp [Irish and Gelbart 1987; Ip et al. 1991; Ray et al. 1991]. The zen expression domain is only slightly expanded in D2 embryos as compared with wild type [Roth et al. 1989]. Hence, weakly dorsalizing dl alleles have normal or only slightly impaired repressor functions.

In addition to a loss of mesoderm, stronger dl alleles [dlSc, dlI, dlE, and dlI at 22°C, dlSC, dlT, dlI, and dlD] also lead to a loss of ventral epidermis [strong dorsalization, D1]. The cuticle of strongly dorsalized embryos consists only of lateral and dorsal pattern elements. The zen expression domain of D1 embryos is strongly expanded toward the ventral side of the embryo [Roth et al. 1989]. These alleles therefore code for dl proteins that not only have lost their activating function but are, in addition, only weak transcriptional repressors.

Finally, completely dorsalizing alleles [dlD^T/Df] lead to apolar embryos that differentiate only dorsal epidermis [complete dorsalization, D0]. Neither activation of twi nor repression of zen or dpp occurs. zen and dpp are expressed uniformly around the entire embryonic circumference [Rushlow et al. 1987; Ray et al. 1991]. dl mutations causing such phenotypes have lost their function as transcriptional regulators entirely.

Amino acid changes of 11 dl alleles

The results of our sequence analysis are shown in Figure 1a and Table 1. A single amino acid change was detected in each of the 11 alleles. Eight alleles, dlI^T, dlI, dlI, dlE, dlE, dlI, dlI, dlD, and dlG, show normal amounts of protein in whole embryos or in embryonic extracts [Table 1; Roth et al. 1989; Roth 1990]. Therefore, it seems that the
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| Allele | Amino acid change | Phenotype | dl protein in whole embryos* | dl protein in embryonic extracts* |
|--------|------------------|-----------|-------------------------------|----------------------------------|
| Amino-terminal group |
| D7     | Arg-63 → Cys     | D0        | normal                         | normal abundance (85 kD)         |
| 2      | Gly-68 → Glu     | D1 [22°C], D2 [18°C] | normal                         | normal abundance (85 kD)         |
| QF     | Ser-103 → Phe    | D1        | reduced abundance              | absent                           |
| SC     | Cys-124 → Ser    | D2        | normal                         | normal abundance (85 kD)         |
| 5      | Val-188 → Met    | D1        | normal                         | normal abundance (85 kD)         |
| PZ     | Arg-310 → His    | D1 [22°C], D2 [18°C] | absent                         | absent                           |
| Carboxy-terminal group |
| 011    | Gln-339 → stop   | D0/D1      | absent                         | absent                           |
| U5     | Gln-488 → stop   | D1        | nuclear gradient extended*     | normal abundance (45, 52, 60 kD) |
| QD     | Trp-524 → stop   | D1        | nuclear gradient extended*     | normal abundance (56, 60, 68 kD) |
| 3      | Ala-531 → Val    | D0-D1      | normal*                        | reduced abundance (85 kD)        |
| SG     | Gln-598 → stop   | D2        | nuclear gradient extended*     | normal abundance (70 kD)         |

*The data in these columns are cited from Roth et al. (1989) and Roth (1990).

The phenotype of dlPZ is only weakly rescued at 18°C. The mutant embryos show small amounts of ventral epidermis if they develop at 18°C. The temperature-sensitive period comprises the syncytial blastoderm stage (Anderson and Nüsslein-Volhard 1986).

**dF**PZ allows the formation of normal amounts of ventral epidermis at 18°C. In contrast to dlPZ, the temperature-sensitive period of dlPZ is restricted to late stages of oogenesis (Anderson and Nüsslein-Volhard 1986).

Twenty percent of the embryos have Filzkörper. The majority differentiate only dorsal epidermis; however, their expression and distribution are indistinguishable from the wild-type copy, and gastrulation movements are polarized (Fig. 2).

Extended refers to the dl protein distribution shown in Fig. 5c. In contrast to the wild-type distribution, the nuclei of the dorsal half contain detectable amounts of dl protein.

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Figure 1. Mutations and proposed functional organization of the dl protein. (a) A schematic drawing of the primary structure of the dl protein is shown. Solid areas indicate basic regions. (NLS) Nuclear localization sequence. Proline (P), glutamine (Q), asparagine (N), and alanine (A)-rich regions are patterned. Arrows indicate the positions of the mutations. The four open bars show the size of the four truncated proteins. (b) The model of the functional organization of the dl protein combines data published earlier (Ip et al. 1991; Richardson and Gilmore 1991) with the analysis of the dl alleles presented in this paper. (Cytoplasmic retention) Either a second cact-binding site or the site of interaction with a component not yet known.

5a, below). The mutation in dl<sup>rc</sup> changes the consensus sequence for the phosphorylation of Ser-312 by cAMP/cGMP-dependent kinase. This sequence is conserved in all known Rel-related proteins. dl<sup>rc</sup> exhibits a strong temperature sensitivity (Anderson and Nüsslein-Volhard 1986) and shows impaired protein stability. The dl<sup>rc</sup> protein is not detectable in embryonic extracts or whole embryos at 22°C (Table 1; Roth et al. 1989) in contrast to the proteins of all other hypomorphic alleles of comparable phenotypic strength. Like the dl<sup>rc</sup> protein, many known temperature-sensitive proteins are also unstable (for review, see Goldenberg 1988).

Carboxy-terminal group of dl alleles Four of the carboxy-terminal group mutants are nonsense mutations, providing a series of deletions from the carboxy-terminal end (Fig. 1a). The shorter mutant proteins cause the more severe mutant phenotypes: Whereas dl<sup>sc</sup> (stop at amino acid 598) causes a weak dorsализation (D2), dl<sup>d3</sup> (stop at amino acid 524) and dl<sup>d5</sup> (stop at amino acid 488) lead to strongly dorsialized embryos (D1, Fig. 3c, below). The strongest mutant phenotype is caused by dl<sup>o11</sup> (stop at amino acid 339), whose product consists only of the RH domain. The nonsense mutation of dl<sup>o11</sup> is located in the region of the NLS. Only a small portion of the embryos (20%) derived from dl<sup>o11</sup>/dl<sup>+</sup> females differentiate lateral pattern elements visible in cuticle preparations (Fig. 3e, below). However, ventral repression of zen and a weak polarization of the gastrulation movements were detectable in all mutant embryos (Fig. 2). This indicates that dl<sup>o11</sup> has residual function as a transcription factor and is subject to some degree of spatial regulation.

The product of dl<sup>o11</sup> was not detectable in embryonic extracts or whole embryos (Roth et al. 1989). Because the antibodies that we used were raised against a fusion protein containing only amino acids 145–570 of dl (Roth et al. 1989), they may not detect the protein derived from dl<sup>o11</sup>, dl<sup>us</sup>, dl<sup>d3</sup>, and dl<sup>sc</sup> produce normal amounts of truncated protein but show alterations in the protein distribution. All three mutants cause an extension of the
duced.

This indicates that the carboxy-terminal region of the wild-type protein, contain low levels of mutant protein. As a result, the dorsally located nuclei, which exclude the embryo (Table 1; Fig. 5c, bclow; Roth ct al. 1989). As in the absence of cact function more wild-type dl protein enters the nuclei at the dorsal side of the embryo (Roth et al. 1991), one would expect that homozygous double mutant combinations of the hypomorphic dl alleles with cact similarly cause an increased import of the mutant protein at dorsal positions. Therefore, the embryos from the doubly mutant females (cact dl*/cact dl*) should show a loss of dorsally derived structures and a compensating expansion of structures found only ventrally in the single mutant (dl*/dl*). We observed this result with dlP, dlD, and dlPG (Table 3). The homozygous double mutants of cact and dlP, dlD, and dlPG cause a lateralized phenotype: Ventral or lateral pattern elements are present around the entire embryonic circumference; however, two of the amino-terminal group alleles, dlP and dlD, behave differently. Instead of an expansion, they show a reduction of lateral and ventral pattern elements when cact is re-

nuclear concentration gradient toward the dorsal side of the embryo (Table 1, Fig. 5c, below; Roth ct al. 1989). As a result, the dorsally located nuclei, which exclude the wild-type protein, contain low levels of mutant protein. This indicates that the carboxy-terminal region of the dl protein influences the inhibition of nuclear transport.

The other carboxy-terminal group mutant, dlP, is located in an alanine-rich region, which may form an α-helix as predicted by the method of Garnier (1978). dlP causes a strong dorsalization. In whole embryos, the dlP protein gradient appears normal; however, in embryonic extracts we detected only reduced amounts of protein.

Interactions with cact

On the basis of genetic experiments, a direct interaction of cact and dl products was proposed (Roth et al. 1991). Both cact and dl are dosage sensitive. Females that have only one wild-type copy of dl produce weakly dorsIALIZED embryos at 29°C (Nüsslein-Volhard 1979). In contrast, females hemizygous for cact produce weakly ventralized embryos [50–90% of the embryos die with reduced dorsal anlagen; Roth et al. 1991]. The cact haploinsufficiency is suppressed if the dl dosage is reduced simultaneously with the cact dosage: Females lacking one copy of cact and one copy of dl (cact−/+/+ dl−) have normal numbers of progeny at 22°C. This suppression of the cact haploinsufficiency was observed with all amorphic, but only one group of hypomorphic, dl alleles (Table 2).

Table 2. dl mutants transheterozygous to a strong cact mutation

| dorsal allele | Percent nonhatching larvae derived from females of the genotype |
|---------------|---------------------------------------------------------------|
| dl*/CyO + dl*/cact42 + (phenotype) | Amorphic alleles |
| 1 | 
| 4 | NT |
| 6 | NT |
| 8 | NT |
| 15 | NT |

Hypomorphic alleles: amino-terminal group

| QF | NT |
| SC | NT |
| 5 | NT |
| 2 | NT |
| PZ | NT |

Hypomorphic alleles: carboxy-terminal group

| 3 | 50 |
| 011 | 55 |
| QD | 20 |
| US | 10 |
| SG | 5 |

Eggs (300–500) were scored at room temperature to determine the number of nonhatching larvae. D2 embryos have lost the mesoderm completely, D3 embryos have a reduced mesoderm. L1 refers to lateralized embryos that have deleted both ventral and dorsal anlagen. (NT) Not tested.
Figure 3. The cuticle pattern of \(dl\) mutants transheterozygous for a strong \(cact\) mutation. Maternal genotypes: (a) \(dl^{F}/Df(2L)TW119\). (b) \(+dl^{F}/cact^{F2}+\). (c) \(dl^{F}/Df(2L)TW119\). (d) \(+dl^{F}/cact^{F2}+\). (e) \(dl^{F}/Df(2L)TW119\). (f) \(+dl^{F}/cact^{F2}+\). (Fk) Filzkörper; (DE) dorsal epidermis; (VE) ventral epidermis. Dorsal is up, anterior is to the left. The lack of ventral epidermis in a and c is characteristic of strongly dorsalized phenotypes (D1). In addition, the embryo in e has lost the Fk, indicating that it is almost completely dorsalized. In transheterozygous combinations with \(cact\), \(dl^{F}\) leads to wild-type embryos and to a small portion (30%) of embryos with slight head or telson defects (b). In contrast, transheterozygous combinations with \(cact\) and \(dl^{F}/dF^{P}\) [d] or \(dl^{P}/dF^{P}\) [f] cause an expansion of ventral epidermis at the expense of dorsal and dorsolateral structures in all mutant embryos. This fate map alteration leads to strong defects in the head, thorax, and telson region. Fk are absent or reduced in size and pulled inside the embryo.

moved. This is most striking with \(dl^{F/2}\). Whereas \(dl^{F/2}/dl^{P/2}\) leads to embryos that possess nearly all elements of the wild-type pattern (D2) at 18°C, \(cact^{A2}dl^{F/2}/cact^{F1}dl^{P/2}\) causes a complete dorsalization at 18°C. Thus, \(dl^{F/2}\) (and \(dl^{P/2}\)) show a positive requirement for \(cact\) activity. Their residual function is stabilized in the presence of \(cact\) but lost when \(cact\) is absent. Given the observation that the mutant proteins of both alleles could not be detected in embryonic extracts [Table 1], we propose that \(dl^{G2}\) and \(dl^{F2}\) form unstable proteins that acquire increased stability in complexes with the \(cact\) protein.

**Carboxy-terminal group of \(dl\) alleles** In contrast to the \(dl\) alleles of the amino-terminal group, the hypomorphic \(dl\) alleles of the carboxy-terminal group become completely (or almost completely) dominant if \(cact\) activity is reduced. Of the embryos derived from transheterozygous females \([cact^{F2}+\times dl^{+}]\), 100% (or nearly 100%) die [Table 2]. The missense mutation, \(dl^{P}\), causes a weak...
The embryos of the four truncation mutations in trans to cact and the amino-terminal group of hypomorphic dl alleles

| Genotypes                  | Epidermis              |
|----------------------------|------------------------|
|                           | mesoderm ventral lateral dorsal |
| dl^2/d^2                  |                        |
| cact^Ace dl^2/cact^Ace dl^2|                        |
| dl^5/d^5                  |                        |
| cact^Ace dl^5/cact^Ace dl^5|                        |
| dl^O^2/dl^O^2             |                        |
| cact^Ace dl^O^2/cact^Ace dl^O^2|                        |
| dl^O^2/dl^O^2 (18°C)      |                        |
| cact^Ace dl^O^2/cact^Ace dl^O^2 (18°C) | |

The bold rules indicate the pattern elements present in embryos derived from mutant females. Asterisks indicate the pattern elements present in embryos derived from the dorsal margin of the ventral epidermis.

However, the reduction of twi expression resulting from the same transheterozygous combinations demonstrates that the truncated proteins exert an opposite effect ventrally: They lead to an inactivation of the wild-type dl protein. Therefore, at the ventral side they act in a manner similar to the dominant-negative mutation dl^O^2.

In embryos derived from cact^Ace dl^O^2/+ + dl^O^2 females no wild-type dl protein is present. Using these embryos, we could study directly the effect of the reduced cact dosage on the distribution of mutant dl proteins. In the following text, we compare the truncation allele dl^O^2 to the amino-terminal group allele dl^5, which exhibits no dominant interaction with cact. As mentioned earlier, dl^O^2/dl^− leads to a wild-type-like nuclear protein gradient, whereas dl^O^2/dl^− causes an extension of the nuclear gradient toward the dorsal side (Fig. 5a,c). If one cact copy is removed using +dl^O^2/cact^− dl^− females, this extension of the nuclear dl protein gradient toward the dorsal side is even more pronounced (Fig. 5c,d). In contrast, we could not detect a change in the distribution of dl^− protein in embryos derived from +dl^O^2/cact^− dl^− females as compared to those derived from dl^5/dl^− females (Fig. 5a,b). Therefore, the distribution of dl^O^2 protein, but not the distribution of dl^− protein, is sensitive to a reduction of the cact dosage. These observations indicate that the reduced inhibition of the nuclear transport exhibited by the dl^O^2 protein is accompanied by an impaired interaction with the cact product. dl^O^2 and dl^O^2 behave similarly to dl^O^2 (data not shown). In summary, the truncated proteins appear to be less susceptible to the inhibition cact exerts on the nuclear transport of the dl protein. However, they clearly maintain some ability to interact with cact.

**Discussion**

The correlation of protein defects with the mutant phenotypes of 11 dl alleles is informative with respect to the functional organization of the dl protein. In particular, we can address the following aspects of dl function: (1) DNA binding and transcriptional activation or repression, (2) control of nuclear transport and interaction with cact, and (3) self-association of the dl protein.

**DNA binding and transcriptional activation/repression**

It has been demonstrated that the dl protein binds to DNA by its RH domain. A truncated protein that contains only residues 1–378 was as efficient as the full-length dl protein in DNA-binding assays (Ip et al. 1991). However, our data show that a truncated protein containing residues 1–488 (dl^O^2) already causes a strongly dosed phenotypic effect. It has lost the ability of transcriptional activation completely and possesses only a residual repression function. This suggests that the dl protein is composed of at least two domains, both required for its function as a transcription factor. The RH domain is involved in DNA binding, and the carboxy-terminal part is required for transcriptional activation and repression. Richardson and Gilmore (1991) have shown that residues...
Figure 4. twi and zen expression of dl mutants transheterozygous for a strong cact mutation. Embryos at blastoderm stage were stained simultaneously with anti-tw7 and anti-zen antibodies. (a,b) Transverse section. Dorsal is up, ventral is down. (c,d) Optical sagittal section. Anterior is to the left, ventral is down. Maternal genotypes: (a, c) Df(2L)TW119/ Df(2L)E10RN2. (b) +dP''/cact''^+. (d) +d''/cact''^+. Df(2L)TW119 is a dl deficiency; Df(2L)E10RN2 is a cact deficiency. The zen and twi expression patterns in embryos a and c resemble closely that of wild-type embryos. The terminal zen expression, which is unchanged in the embryo shown in d, is not controlled by dl [Rushlow et al. 1987].

431–678 of the dl protein, bound to target DNA through the heterologous DNA-binding domain of LexA, can activate gene expression in chicken embryo fibroblasts. In these experiments the carboxy-terminal dl sequences behaved similarly to the carboxy-terminal domains of chicken, mouse, and human c-Rel, even though they are not homologous or only weakly homologous to each other.

The series of truncated dl proteins that we have studied show that transcriptional activation of twi and sna requires a longer carboxy-terminal region than transcriptional repression of dorsally required zygotic genes. The d1^{Nsc} protein that is missing only 80 carboxy-terminal residues [25% of the carboxy-terminal half] is already unable to activate twi or sna expression while its repression of dorsally required zygotic genes is similar to wild type [as inferred from the cuticle phenotype]. If ≈40% of the carboxy-terminal region is deleted, the truncated proteins show a reduced repressor function in addition to the lack of activation. However, even if the carboxy-terminal half is deleted completely and the protein consists only of the RH domain [d1^{p21}], the repressing function is not abolished totally.

In addition to truncated proteins, we found only one missense mutation, d1^{p3}, that affects the activation/repression function. d1^{p3} may break a putative α-helix formed by an alanine-rich region of the carboxy-terminal half. It causes an almost complete dorsalization, indicating that some higher-order structure in addition to glutamine and proline richness might be required for the function of the activation/repression domain of dl.

Ip et al. [1991] have further restricted the DNA-binding domain of dl protein to residues 47–244, using internally deleted proteins in DNA-binding assays. Thus, only the amino-terminal part of the RH domain is required for DNA binding. Our calculation of the local amino acid composition shows that a region between residues 40 and 120 is more basic than the rest of the protein [with the exception of the NLS]. Four of the six amino-terminal group alleles map inside or close to that
Molecular dissection of the dorsal morphogen

Figure 5. The dl protein distribution of dl mutants transheterozygous for a strong cact mutation. Embryos at blastoderm stage were stained with anti-dl antibody and sectioned. Dorsal is up; ventral is down. Maternal genotypes: [a] dl^/+ Df(2L)TW119. (b) +dP/cact^dV. (c) dP''/Df(2L)TW119. (d) +dP''/cact^dV. Df(2L)TW119 is a dl deficiency, and dP is an amorphic allele. The arrowheads indicate the region of the dl gradient where the concentration of the nuclear dl protein becomes higher than that of the cytoplasmic dl protein. Embryos derived from dF'/Df(2L)TW119 females [a] have a wild-type-like dl protein distribution, which is unchanged if cact activity is reduced [b]. dP''/dl causes a shift of the nuclear dl protein gradient toward the dorsal side [c], which is significantly enhanced if the cact activity is reduced [d].

Control of nuclear transport and interaction with cact

Some of our data suggest the formation of protein complexes between cact and dl proteins. Two of the amino-terminal group mutations form unstable proteins [dl^23, dl^22], which are apparently stabilized by the presence of cact. This positive effect on dl protein stability exerted by cact strongly suggests a direct and stable interaction between cact and dl proteins.

If we assume complex formation with cact, it should be possible to define those parts of the dl protein that are required to interact with cact. Our results indicate that sequences in both the amino- and the carboxy-terminal half are involved in the cact-mediated inhibition of nuclear transport. All of the truncated proteins are less well retained in the cytoplasm than is the wild-type dl protein. They lead to nuclear dl protein gradients similar to those caused by weak cact mutations [Roth 1990]. However, the nuclear uptake of the truncated proteins is enhanced additionally if the cact amount is reduced. Thus, these proteins are less susceptible to the cact inhibition but still interact with cact. Because of the observed genetic interaction, we assume that this applies even to the smallest truncated protein [dl^011], although this protein was not detectable using our antibody preparation. dl^011 consists only of the RH domain. Therefore, the RH domain alone appears to interact with cact.

IkB interacts with NF-xB by the p65 subunit. Similar to our results with dl and cact, progressive carboxy-terminal deletions of p65 demonstrate that IkB binds to the RH domain of p65 [Nolan et al. 1991]. If we assume that cact interacts primarily with the RH domain of dl protein, the question arises why carboxy-terminal sequences are involved in the inhibition of nuclear transport. Two explanations are possible. The presence of the carboxy-terminal region may stabilize the dl-cact interaction or may represent a second site of cact binding. Alternatively, the carboxy-terminal region could interact with a cytoplasmic anchoring protein [not yet identified], whereas cact would be required to strengthen this interaction.

The implication of carboxy-terminal sequences for the cytoplasmic retention of the dl protein had been proposed earlier on the basis of tissue culture experiments [Rushlow et al. 1989]. It might be a general feature of Rel family proteins, because it was also observed for avian c-Rel. Chicken c-Rel is cytoplasmic in chicken embryo fibroblasts. If 103 carboxy-terminal residues are removed, the truncation protein enters the nucleus [Caponi Bianco et al. 1990].
Self-association of the dl protein

One of the mechanisms known to be responsible for the dominant-negative action of mutant transcriptional regulators (for review, see Herskowitz 1987) applies to proteins that are able to self-associate. In such proteins the DNA-binding domain can be inactivated, whereas the oligomerization domain is not affected. An inactivation of wild-type protein results when multimers between mutant and wild-type proteins are formed.

Logeat et al. (1991) have shown that mutant Rel-related proteins can cause dominant-negative effects by this mechanism. They have constructed internal deletion mutants of p50, which are unable to bind DNA but can form homo- or heterodimers with full-length Rel family proteins [p50, c-Rel]. These deletion mutants inhibit the transcriptional activation caused by the wild-type proteins, because the heterodimers of mutant and wild-type protein are apparently unable to bind DNA. The deletion mapping of p50 restricts the dimerization region to the carboxy-terminal part of the RH domain. This region corresponds approximately to residues 180–350 of the dl protein.

A dimerization of the dl protein by the carboxy-terminal part of the RH domain would readily explain the phenotype of dl<sup>DO7</sup>, which is nonfunctional in trans to a deficiency but dominant negative in trans to a wild-type copy of dl. dl<sup>DO7</sup> is probably unable to bind DNA owing to a single amino acid change in the proposed DNA-binding domain. However, it should still form heterodimers with the wild-type dl protein because the potential dimerization domain is unaltered. These heterodimers might be nonfunctional or only partially functional. Thus, the heterodimerization would cause an inactivation of the wild-type protein.

There is one other strongly dorsalizing allele, dl<sup>5</sup>, which, like dl<sup>DO7</sup>, produces normal amounts of full-length protein and leads to the formation of a normal nuclear gradient. Unlike dl<sup>DO7</sup>, dl<sup>5</sup> is not dominant. Interestingly, dl<sup>5</sup> does not map to the putative DNA-binding domain but to the region corresponding to the dimerization domain in p50. A mutation that disrupts dimerization should be unable to inactivate the wild-type copy.

The dominant effect exhibited by the truncated proteins when the cact amount is reduced may also be attributed to the dimerization of the dl protein. In trans-heterozygous combinations with cact, the truncated proteins lead to a lateralized phenotype simultaneously deleting both dorsal-most and ventral-most structures. To explain this phenotype we postulated that the truncated proteins increase the wild-type activity of dl at the dorsal side but inactivate wild-type dl ventrally. This complicated effect might be the result of the formation of heterodimers between truncated and full-length proteins. Because the nuclear import of truncated proteins is less inhibited by cact, we assume that this applies also to heterodimers of truncated and full-length proteins. Therefore, increased nuclear concentrations of the heterodimer would result when cact activity is reduced. If the heterodimers still function as transcriptional repressors, their increased nuclear concentration would result in the observed expansion of ventral epidermis at the expense of dorsal structures. If, on the other hand, the heterodimers are impaired transcriptional activators, their increased nuclear concentrations would explain the reduced twi expression and thus the loss of ventral-most structures. This model requires the assumption that the dimerization occurs by the RH domain, because dl<sup>DP11</sup>, which exhibits the strongest phenotypic effects in trans to cact, codes only for the RH domain.

In summary, our data obtained with 11 EMS-induced dl alleles support the recent models proposed for the functional organization of Rel family proteins (Fig. 1b). In particular, it seems that several functions reside in the RH domain of dl. The DNA-binding region is probably confined to an amino-terminal subregion, followed by sequences important for dimer formation. Some part of the RH domain is also required for cact binding. We have located two functions to the carboxy-terminal half of the dl protein: transcriptional activation/repression and cytoplasmic retention. Our results on both the RH domain and the carboxy-terminal half suggest that dl protein contains regions with overlapping functions.

Materials and methods

Fly strains

The wild-type stock was Oregon-R. All of the dl alleles are described in Roth et al. (1989), and the cact alleles are described in Roth et al. (1991). Flies were grown and eggs were collected under the standard conditions [Nüsslein-Volhard et al. 1984]. Staging of embryos was according to Campos-Ortega and Hartenstein (1985).

DNA sequencing

Genomic DNA was extracted from the flies transheterozygous for dl alleles and the deficiency Df(2L)TW119. We amplified the DNA of the coding region of each dl mutant gene in four pieces of DNA by polymerase chain reaction (PCR). The first piece includes 1–988 bp, the second includes 989–1096 bp, the third includes 1097–1545 bp, and the last includes 1309–2037 bp. The PCR products were subcloned into M13mp10 and sequenced by the dideoxy chain-termination method. All of the mutant genes were sequenced over the whole coding region. When we found a nucleotide change that caused a change of amino acid residue, two to three different clones of the PCR products were sequenced to confirm it. All of the reactions were made according to Sambrook et al. (1989).

Some deletions and insertions were observed in more than one mutant or in some clones of a certain mutant and not others. We show them in terms of amino acid residues in the following: the deletion of QQ between residue 479 and 482 [in dl<sup>5</sup>, dl<sup>DP11</sup>, dl<sup>DP5</sup>, and dl<sup>P4</sup>], Q between residue 495 and 503 [in some dl<sup>DP2</sup> and not in some dl<sup>DP5</sup>], Q between residues 600 and 606 [in dl<sup>5</sup>, dl<sup>DP11</sup>, dl<sup>DP5</sup>, and dl<sup>P4</sup>], N between residue 614 and 615 [in dl<sup>DP7</sup>, dl<sup>DP9</sup>, dl<sup>DP8</sup>, dl<sup>DP11</sup>, dl<sup>DP5</sup>, dl<sup>P4</sup>, and dl<sup>DP5</sup>], and the insertions of QQ and Q between residue 495 and 503 [both in dl<sup>P1</sup>], one of the same as the previous one, that is, QQ between residue 495 and 503 [in dl<sup>P4</sup>]. NPNNG between residue 615 and 616 [in dl<sup>5</sup>, dl<sup>DP11</sup>, and dl<sup>DP5</sup>]. All of them are located in glutamine (Q) or asparagine (N)-rich regions in the carboxy-terminal half.
of the \( dl \) protein. Because they are very common to many alleles with different phenotypes or they are observed only in a part of the clones from the same mutants, we interpret them to be polymorphism independent of the mutant phenotypes.

**Cuticle preparations of embryos**

Differentiated embryos were dissected from the vitelline membrane and mounted directly in a 1:1 mixture of lactic acid and Hoyer's medium [Van der Meer 1977].

**Antibody staining of embryos**

Immunological staining of whole-mount embryos, using the ABC system [Vector Laboratory], was carried out as described by Roth et al. (1989). The sections shown in the figures are derived from 40% to 50% egg length.

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**References**

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

Anderson, K.V., G. Jürgens, and C. Nüsslein-Volhard. 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: Genetic studies on the role of the Toll gene product. *Cell* 42: 779–789.

Baeverle, P.A. 1991. The inducible transcription activator NF-\( \kappa \)B: Regulation by distinct protein subunits. *Biochem. Biophys. Acta* 1072: 63–80.

Baeverle, P.A. and D. Baltimore. 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-\( \kappa \)B transcription factor. *Cell* 53: 211–217.

Bull, P., K.L. Morley, M.L. Hoekstra, T. Hunter, and I.M. Verma. 1990. The mouse \( c-rel \) protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. *Mol. Cell. Biol.* 10: 5473–5485.

Campos-Ortega, J.A. and V. Hartenstein. 1985. *The embryonic development of Drosophila melanogaster*. Springer Verlag, Berlin, Germany.

Caponbianco, A.J., D.L. Simmons, and T.D. Gilmore. 1990. Cloning and expression of a chicken \( c-rel \) cDNA: Unlike p59\(^{\text{src}} \), p68\(^{\text{rel}} \) is a cytoplasmic protein in chicken embryo fibroblasts. *Oncogene* 5: 257–265.

Davis, N., S. Ghosh, D.L. Simmons, P. Tempst, H.-C. Liou, D. Baltimore, and H.R. Bose Jr. 1991. Rel-associated \( pp40 \): An inhibitor of the \( rel \) family of transcription factors. *Science* 253: 1268–1271.

Garnier, J., D.J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120: 97–120.

Ghosh, S. and D. Baltimore. 1990. Activation in vitro of NF-\( \kappa \)B by phosphorylation of its inhibitor I\( \kappa \)B. *Nature* 344: 678–682.

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

Gilmore, T.D. 1991. Malignant transformation by mutant Rel proteins. *Trends Genet.* 7: 318–322.

Goldenberg, D.P. 1988. Genetic studies of protein stability and mechanisms of folding. *Annu. Rev. Biophys. Chem.* 17: 481–507.

Harrison, S.C. 1991. A structural taxonomy of DNA-binding domains. *Nature* 353: 715–719.

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

Haskill, S., A.A. Beg, S.M. Tompkins, J.S. Morris, A.D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A.S. Baldwin Jr. 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes I\( \kappa \)B-like activity. *Cell* 65: 1281–1289.

Herskowitz, I. 1987. Functional inactivation of genes by dominant negative mutations. *Nature* 329: 219–222.

Inoue, J.-I., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma. 1991. \( c-rel \) activates but v-rel suppresses transcription from \( \kappa \)B sites. *Proc. Natl. Acad. Sci.* 88: 3715–3719.

Ip, Y. T., R. Kraut, M. Levine, and C.A. Rushlow. 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.

Irish, V.F. and W.M. Gelbart. 1987. The decapentaplegic gene is required for dorsal-ventral patterning of the Drosophila embryo. *Genes & Dev.* 1: 868–879.

Jiang, J., D. Kosman, Y.T. Ip, and M. Lecvnc. 1991. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early Drosophila embryos. *Genes & Dev.* 5: 1881–1891.

Kerr, L.D., J.-I. Inoue, N. Davis, E. Link, P.A. Baeverle, H.R. Bose Jr., and M. Verma. 1991. The Rel-associated \( pp40 \) protein prevents DNA binding of Rel and NF-\( \kappa \)B: Relationship with I\( \kappa \)B and regulation by phosphorylation. *Genes & Dev.* 5: 1464–1476.

King, M., V. Blank, F. Logeat, J. Vandeukkenhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Pourilsky, P.A. Baeverle, and A. Israel. 1990. The DNA binding subunit of NF-\( \kappa \)B is identical to factor KBF1 and homologous to the \( rel \) oncogene product. *Cell* 62: 1007–1018.

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

Logeat, F., N. Israel, R. Ten, V. Blank, O. Le Bail, P. Pourilsky, A.S. Wasserman, and A. Israel. 1991. The DNA binding subunit of NF-\( \kappa \)B is identical to factor KBF1 and homologous to the \( rel \) oncogene product. *Cell* 62: 1007–1018.

Mitchell, P. and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245: 371–378.

Nolan, G. P., S. Ghosh, H. Liou, P. Tempst, and D. Baltimore. 1991. DNA binding and I\( \kappa \)B inhibition of the cloned p65 subunit of NF\( \kappa \)B, 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. In *Determinants of spatial organization* (ed. S. Subtelny and I.R. Koelingsberg), pp. 185–211. Academic Press, New York.
Nüsslein-Volhard, C., E. Wieschaus, and H. Kluding. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol.* 183: 267–282.

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

Ray, R.P., K. Arora, C. Nusslein-Volhard, and W. Gelbart. 1991. The control of cell fate along the dorsal-ventral axis of the Drosophila embryo. *Development* 113: 85–54.

Richardson, P. and T.D. Gilmore. 1991. vRel is an inactive member of the rel family of transcriptional activating proteins. *J. Virol.* 65: 3122–3130.

Roth, S. 1990. “Die Rolle der maternalen Gene cactus und dorsal bei der dorsoventralen Musterbildung im *Drosophila* Embryo.” Ph.D. thesis, University of Tübingen, Tübingen, Germany.

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

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

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

Rushlow, C., M. Frasch, H. Doyle, and M. Levine. 1987. Maternal regulation of zerknüllt: A homeobox gene controlling differentiation of dorsal tissues in Drosophila. *Nature* 330: 583–586.

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

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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

Stephens, R.M., N.R. Rice, R.R. Hiebsch, H.R. Bose Jr., and R.V. Gilden. 1983. Nucleotide sequence of v-rel: The oncogene of reticuloendotheliosis virus. *Proc. Natl. Acad. Sci.* 80: 6229–6233.

Steward, R. 1987. *Dorsal*, an embryonic polarity gene in Drosophila, is homologous to the vertebrate protooncogene, c-rel. *Science* 238: 692–694.

———. 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* 59: 1179–1188.

Thisse, C., F. Perrin-Schmitt, C. Stoeckel, and B. Thisse. 1991. Sequence-specific transactivation of the Drosophila twist gene by the dorsal gene product. *Cell* 65: 1–20.

Urban, M.B., R. Schreck, and P.A. Baeuerle. 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* 10: 1817–1825.

Van der Meer, J.M. 1977. Optical clean and permanent whole mount preparations for phase-contrast microscopy of cuticular structures of insect larvae. *Dros. Inf. Serv.* 52: 160.

Wilhelmson, K., K. Eggleton, and H. Termin. 1984. Nucleic acid sequence of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel. *J. Virol.* 52: 172–182.
The functional domains of the Drosophila morphogen dorsal: evidence from the analysis of mutants.

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