Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7861–7865, September 1992
Developmental Biology

In vivo self-association of the *Drosophila* rel-protein dorsal

(morphogen/gradient/dorsoventral/signal transduction)

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Communicated by Allan Spradling, May 15, 1992 (received for review April 2, 1992)

ABSTRACT **The *Drosophila* morphogen dorsal, KBF1, NF-κB, and the proto-oncogene c-*rel* belong to the rel family of transcription factors whose function is regulated post-translationally by selective nuclear import. In the early *Drosophila* embryo, dorsal protein is proposed to be retained in the cytoplasm through its interaction with cactus protein. The maternal dorsal group genes constitute a signal transduction pathway, which results in targeting cytoplasmic dorsal protein into the nuclei of the syncytial blastoderm embryo, in a ventral-to-dorsal gradient. The asymmetric transcriptional regulation of zygotic genes along the dorsoventral axis by the dorsal morphogen gradient establishes embryonic dorsoventral polarity. In the lymphocytes, the functional equivalent of cactus is IκB, which appears to retain NF-κB in the cytoplasm. This retention is relieved by extracellular signals in tissue culture. NF-κB and rel proteins each are known to function as oligomeric complexes. Here we present genetic and biochemical evidence for the existence and functional importance of an oligomeric dorsal complex *in vivo*.**

The dorsal (dl) protein specifies ventral and lateral cell fates along the dorsoventral axis. In the syncytial blastoderm embryo, the cytoplasmic dorsal protein is partitioned into nuclei such that the ventral nuclei have the highest levels, whereas the lateral and dorsal nuclei have progressively lower to undetectable amounts of dorsal protein. dl protein functions as a transcriptional activator and a repressor of zygotic genes, which are sensitive to different levels of nuclear dorsal protein. The ability of nuclear dorsal protein to differentially control target zygotic genes, as a function of its nuclear concentrations, enables it to confer positional identities along the embryonic dorsoventral axis (1–7).

Eleven maternal components of the embryonic dorsoventral polarity pathway have been genetically identified and have been found to control the subcellular distribution of dorsal protein. Whereas cactus retains dorsal protein in the cytoplasm (5), a group of 10 "dorsal group" genes function to counteract this retention and stimulate ventral nuclear uptake of cytoplasmic dorsal protein (2–4). Dorsoventral asymmetry is clearly apparent in later stage egg chambers during oogenesis. This asymmetry is transmitted to the embryo through an extracellular ventral signal, matured in the perivitelline space. This signal is thought to asymmetrically activate the transmembrane Toll receptor (8, 9). The selective import of dl into ventral and ventrolateral nuclei is the terminal step of this maternal signal transduction pathway (1–9).

The dorsal protein shares similarity with two DNA-binding subunits of the vertebrate transcription factor NF-κB and the products of the *rel* oncogene family (recent reviews, refs. 10–13). The homology extends over 300 amino acids in the N terminus, whereas the carboxyl-terminal ends of all of these proteins are divergent (14–19). In addition to structural similarities, these proteins also share likeness in their regulation and mode of action. Like dorsal, NF-κB, and possibly also the rel proteins, are regulated by selective nuclear transport. NF-κB is retained in the cytoplasm by IκB and this retention is also thought to be relieved due to extracellular signals. Once in the nucleus, dorsal/NF-κB/rel bind to similar sequence motifs within enhancer and promoter elements of target genes and regulate their cell-specific expression (10–13).

rel forms multiprotein complexes within cells (20–22). Biochemical experiments have shown that NF-κB is a heterodimer of p50 and p65 subunits (23) and that p50 can also form heterodimers with c-rel and v-rel (17, 24, 25). Given the fact that NF-κB and dorsal share significant structural similarity and are regulated by similar post-translational selective nuclear import events, we investigated whether dorsal also exists in a complex. Here we show that dl self-associates, probably forming a dimer, and that this self-association is important for its transcriptional activities.

METHODS

Constructs and Injections. The *Bgl* II site within the *dl* cDNA corresponding to amino acid 668 in dl (wild-type dl has 678 amino acids) was used to make an in-frame fusion at the *Bam*HI site located at the 5′ end of the *lacZ* coding region in pCaSpeR-β-Gal vector (26). Expression of the fusion gene was driven by the constitutive and maternally active heat shock 83 (*hs*) promoter (27). The simian virus 40 (SV40) polyadenylylation signal [SV40poly(A)] was added at the 3′ end of the fusion to ensure proper polyadenylylation of the fusion transcript (14). DNA was microinjected into embryos (28), heterozygous for (Δ *2-3*) and homozygous for the *white*^{1} (w^1) mutation. Twenty stably transformed lines (w^+) were obtained. No major zygotic effects of the fusion gene were observed.

***Drosophila* Strains.** The *dl* null stocks *al dp b Df(2L)TW119 cn bw/b Cy* and *b pr cn sca In(2L)dl*^{T}*/b Cy* have been described (29). The dominant alleles of dorsal (dl^{D4}, dl^{D5}, dl^{D6}, dl^{D7}) were isolated by Szabad *et al.* (30).

Cuticle Preparations and Staining of Embryos. β-Galactosidase activity determination and antibody staining of embryos were performed as described (refs. 31 and 4, respectively). Monoclonal anti-lacZ antibody was purchased from Promega and secondary goat anti-mouse antibody was obtained from Vectastain. Primary rabbit anti-dl (4) and anti-twist (5) antibodies were detected by anti-rabbit secondary antibodies (Vectastain, Vector Laboratories). Cuticle preparations were done as described (32).

Immunoprecipitation and Western Analysis. Whole embryo extracts were made from 0- to 3-hr embryos in extraction buffer A [50 mM Tris, pH 7.5/140 mM NaCl/5 mM $Mg(CH_3COO)_2$/0.05% Nonidet P-40 (NP-40)/1 mM phenylmethylsulfonyl fluoride (PMSF)/10 mg of pepstatin A per

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Abbreviations: SV40, simian virus 40; DTT, dithiothreitol.
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ml/10 mg of aprotinin per ml/1 mg of leupeptin per ml] at 4°C. For immunoprecipitations, these extracts were diluted in 10 mM Tris, pH 7.5/140 mM NaCl/0.05% NP-40/1 mM PMSF to a final concentration of 700 mg/ml of total protein. Clarified extracts (10,000 × *g*, 10 min, 4°C) were added to specified IgG-bound to protein A-agarose beads (33) (Bio-Rad) and incubated for 12 hr at 4°C. Immunoprecipitates were boiled in an SDS loading buffer containing 100 mM dithiothreitol (DTT), fractionated on an 8% SDS/polyacrylamide gel, and transferred to a nitrocellulose filter.

RESULTS

The Dominant Alleles of *dl* Are Antimorphic. The first indication that dorsal might self-associate came from the identification of dominant negative or antimorphic alleles of *dl* (30). As shown in Table 1, these alleles, in trans to wild-type *dl*, result in complete female sterility, and the embryos exhibit weak (D3) dorsalized phenotype (degree of dorsalized phenotypes, strong-to-weak/D0-to-D3 are defined in ref. 5; see also legend to Fig. 1). This embryonic phenotype is more severe (D0) when the dominant allele is placed in trans to a deficiency of *dl*. However, the dominant negative effect is almost completely rescued by increasing dl levels. This was achieved by expressing wild-type *dl* cDNA under the control of the constitutive heat shock 83 promoter (*hsdl2*), which is maternally active (S.G., unpublished data; Table 1). The distribution of dl protein in embryos from females, hemizygous for the four *dl* dominant alleles, is identical to the wild-type dl distribution (results not shown and ref. 2). These results imply that the mutant proteins interfere with the function of wild-type dl protein. Different mechanisms have been proposed that can account for dominant negative effects (34). One interpretation of the above observations is that it is the association of mutant dl subunits with wild-type subunits that results in loss of dl function. Alternatively, the wild-type and mutant proteins may compete for binding with some other component.

A dl–lacZ Fusion Protein Is Also Antimorphic. To determine the composition of the dorsal complex in embryos, a *dl–lacZ* construct in which nucleotides encoding the last 10 amino acids of dl were replaced with the bacterial *lacZ* gene (*hsdl–lacZ*, Fig. 1*A*) was injected into embryos. Six independent transgenic lines expressing the fusion protein showed almost complete dominant female sterility. Two of these lines, *hsdl–lacZ7* and *hsdl–lacZ22*, were studied in more detail (Table 2). Although the majority of embryos from these lines showed intermediate dorsalized D2 phenotype (Fig. 1*B*), a small fraction of them exhibited a weaker D3 phenotype. As in the case of the *dl* dominant alleles, reducing the dose of wild-type *dl* resulted in a more severe dorsalized phenotype (Table 2). However, in contrast to the *dl* dominant alleles, which do not have any function, the dl–lacZ fusion protein retained some dl function since in *dl* minus background (which gives a D0 phenotype) it conferred a less severe D1 phenotype (Fig. 1*C*). This indicates that the fusion protein is able to recognize and bind to the target DNA sequences within promoters of zygotic genes.

The dl–lacZ Fusion Protein Is Distributed in a Nuclear Gradient. To characterize the antimorphic effect of the dl–lacZ fusion protein, we studied its distribution in early embryos. Embryos from transgenic females were stained for lacZ enzymatic activity as well as with anti-lacZ antibody. The lacZ activity staining pattern (Fig. 2*C*) paralleled that observed with the anti-lacZ antibody in wild-type background (Fig. 2*D*). An identical distribution of the fusion protein was observed in *dl* minus embryos (results not shown). This pattern was indistinguishable from that observed for wild-type dl distribution in cleavage-stage and syncytial blastoderm embryos (Fig. 2*A*). The lacZ protein itself was distributed uniformly in the cytoplasm (data not shown). This suggests that in a wild-type background, all information required for proper gradient formation is contained within the dorsal protein and that adding the lacZ polypeptide does not change the ability of the dl moiety to respond to signals encoded by the dorsal group and cactus genes. The dl–lacZ fusion protein seems not to interfere with the distribution of the wild-type dl protein, as embryos expressing the dl–lacZ fusion protein showed a normal nuclear gradient of the endogenous dl protein (Fig. 2*E*).

Further, the graded distribution of the dl–lacZ fusion protein in the *dl* minus background shows that the C-terminal 10 amino acids *per se* do not provide any essential information for subcellular localization of dl protein in the embryo. This result differs from the observations in transfected cultured cells, where dl protein lacking the last 8 amino acids was constitutively nuclear (3).

The dl–lacZ Fusion Protein Affects Expression of Target Zygotic Genes. Since the dl–lacZ fusion protein is normally distributed, and does not interfere with the distribution of the endogenous protein, we assayed its effect on the expression of zygotic genes. Embryonic polarity is determined by the nuclear concentration of dl, which controls the precise pattern of zygotic gene expression along the dorsoventral axis (2, 4, 35). Zygotic genes such as twist and snail are activated by high levels of nuclear dl protein in the ventral-most cells (Fig. 2*B* and refs. 36–38). In contrast, zerknüllt, decapentaplegic, and tolloid are expressed only dorsally because they are repressed by dl in lateral and ventral cells (39–41). To evaluate dl protein activity in embryos from females expressing the wild-type dl and the dl–lacZ fusion proteins, we determined the expression of twist and zen proteins. The intensity of twist staining within the ventral domain at blastoderm stage is reduced to variable degrees in embryos expressing the fusion protein (compare Fig. 2*B* with 2*F*). The reduction in twist levels at gastrulation correlates with the observed dorsalized phenotype (Fig. 2 *G* and *H*). Consistent with weak dorsalization, the domain of zen protein expression is expanded laterally (data not shown). These results support the idea that dl–lacZ fusion protein interferes with the ability of wild-type dl to function as a transcriptional regulator.

Table 1. Additional dose of dorsal relieves dominant female sterility of *dl* dominant alleles

dl^{Dx} allele	$dl^{Dx*}/Df(2L)TW119$†		$dl^{Dx}/+$		$dl^{Dx}/+$; *hsdl2*‡	
	% hatch	Phenotype	% hatch	Phenotype	% hatch	Phenotype
dl^{D4}	0	D0	0	D3	72	D3
dl^{D5}	0	D0	0	D3	98	D3
dl^{D6}	0	D0	0	D3	89	D3
dl^{D7}	0	D0	0	D3	75	D3

*dl^{Dx} refers to the *dl* dominant alleles (D4–D7) isolated in a genetic screen for dominant female-sterile mutations on the second chromosome (18).

†83% of the embryos laid by $dl^{+}/Df(2L)TW119$ females hatched.

‡*hsdl2* is a P-element transformed line, containing one insertion of the complete *dl* cDNA, which is driven by the *hsp83* promoter on the third chromosome (S.G., unpublished data).

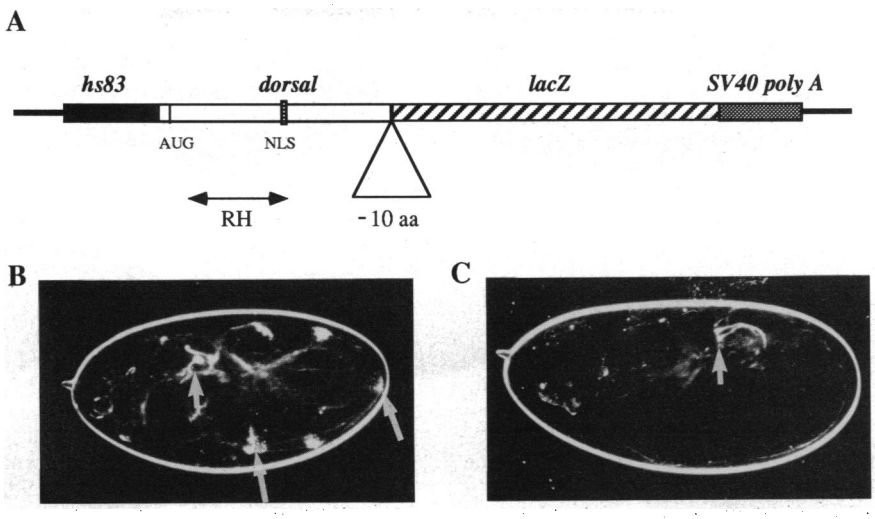

FIG. 1. Partial dorsalization of embryos expressing the *dl–lacZ* fusion gene. (*A*) *hsdl–lacZ* fusion construct. (*B* and *C*) Cuticle preparations of dorsalized embryos expressing dl–lacZ fusion protein in wild type (*B*) or *dl* minus background (*C*). These embryos are dorsalized as their ventral pattern elements—e.g., the ventral setae bands (long arrows) are reduced or absent compared to wild type. Instead, the dorsal epidermis with fine dorsal hair is expanded. The embryo in *B* shows the D2 phenotype, whereas the one in *C* shows the D1 phenotype. Ventral setae bands (long arrows) are reduced in D2 embryos when compared to wild-type but are absent in D1 embryos. D1 and D2 embryos have filzkörper (short arrows), structures derived from dorsolateral position.

The Antimorphic Effect of dl–lacZ Fusion Protein Can Be Alleviated by lacZ. The dominant female sterility and the dorsalized phenotype caused by the dl–lacZ fusion protein could be rescued by increasing the amount of the wild-type dl protein in a dose-dependent manner (Table 2). Approximately 2% of the embryos from *hsdl–lacZ22* females in wild-type background hatched. By increasing the dl concentration by either *hsdl38* or *hsdl2.51* transgenic lines, 14% or 40% of the embryos hatched (Table 2). The dorsalized phenotype of *dl–lacZ* embryos, therefore, seems to result from the ability of the fusion protein to lower the effective concentration of functional dl protein.

The β-galactosidase activity staining of embryos from *dl–lacZ* transgenic females (Fig. 2*A*) showed that the lacZ portion of the fusion protein forms tetramers (42). We suspected that the effective concentration of functional dl protein could therefore also be increased by introducing the unfused lacZ protein into *dl–lacZ* embryos. This would rescue the dorsalized phenotype caused by the fusion protein by lacZ forming a heterooligomeric complex with dl–lacZ fusion protein. Indeed, the dominant female sterility caused by the fusion protein was rescued by the presence of lacZ protein encoded by a *hslacZ* transgene. *hslacZ25* contains higher levels of lacZ activity than *hslacZ19* (results not shown). As seen in Table 2, the rescue of hsdl–lacZ phenotype was proportional to the dose of lacZ. Although the molecular mechanism resulting in increasing levels of functional dl protein is not clear, this result provides support for an interaction of dl with dl–lacZ protein.

An Anti-lacZ Antibody Immunoprecipitates dl–lacZ and Wild-Type dl Proteins. The antimorphic nature of *dl–lacZ* and *dl* alleles and the observed rescue of this phenotype by additional dl and lacZ suggests that the dl protein self-associates (34). As a direct test of the possible interaction between dl monomers, we took advantage of the transgenic flies, which express the endogenous dl and the dl–lacZ fusion proteins. Extracts from wild-type, *dl–lacZ* or *lacZ* embryos were immunoprecipitated with either an anti-dl or an anti-lacZ antibody. The resulting immunoprecipitates were analyzed by Western blots probed with either anti-dl (Fig. 3*A*) or anti-lacZ (Fig. 3*B*). The anti-dl antibody immunoprecipitated the wild-type dl (85 kDa) and dl–lacZ (200 kDa) proteins (Fig. 3*A*, lanes 1–3). In addition to the dl–lacZ fusion protein, the anti-lacZ antibody also immunoprecipitated the endogenous

Table 2. Dominant female sterility caused by *dl–lacZ* is exacerbated in hemizygous *dl* background but is relieved by additional dl or lacZ protein

Genotype	% hatch	Phenotype*	No. of *dl* copies
In(2L)dl^{r}/Df(2L)TW119; hsdl–lacZ7†	0	D1 (D2)	0
dl^{+}/In(2L)dl^{r}; hsdl–lacZ7	<1	D2 (D1)	1
dl^{+}/dl^{+}; hsdl–lacZ7	<1	D2 (D3)	2
hsdl–lacZ22†*dl^{+}/dl^{+}*	2	D2 (D3)	2
hsdl–lacZ22dl^{+}/dl^{+} hsdl38‡	14	D3	>2
hsdl–lacZ22dl^{+}/dl^{+}; hsdl2.51‡	40	D3	>2
hsdl–lacZ22dl^{+}/dl^{+}; hslacZ19§	11	D3	2
hsdl–lacZ22dl^{+}/dl^{+}; hslacZ25§	66	D3	2

*Parentheses indicate phenotypes of a minority of embryos.
†*hsdl–lacZ7* contains an insert on the third chromosome, whereas *hsdl–lacZ22* contains an insert on the second chromosome. Both lines exhibit similar degrees of female sterility.
‡Different *hsdl* lines were used to introduce variable amounts of dl protein. Line 38 has one insertion on the second chromosome, whereas line 2.51 has two independent insertions (line 2 and line 51) on the third chromosome that were recombined (S.G., unpublished data).
§*hslacZ* is a P-element transformed line, containing the *lacZ* gene, which is driven by the *hsp83* promoter. Line 19 contains less lacZ protein than line 25 (data not shown).

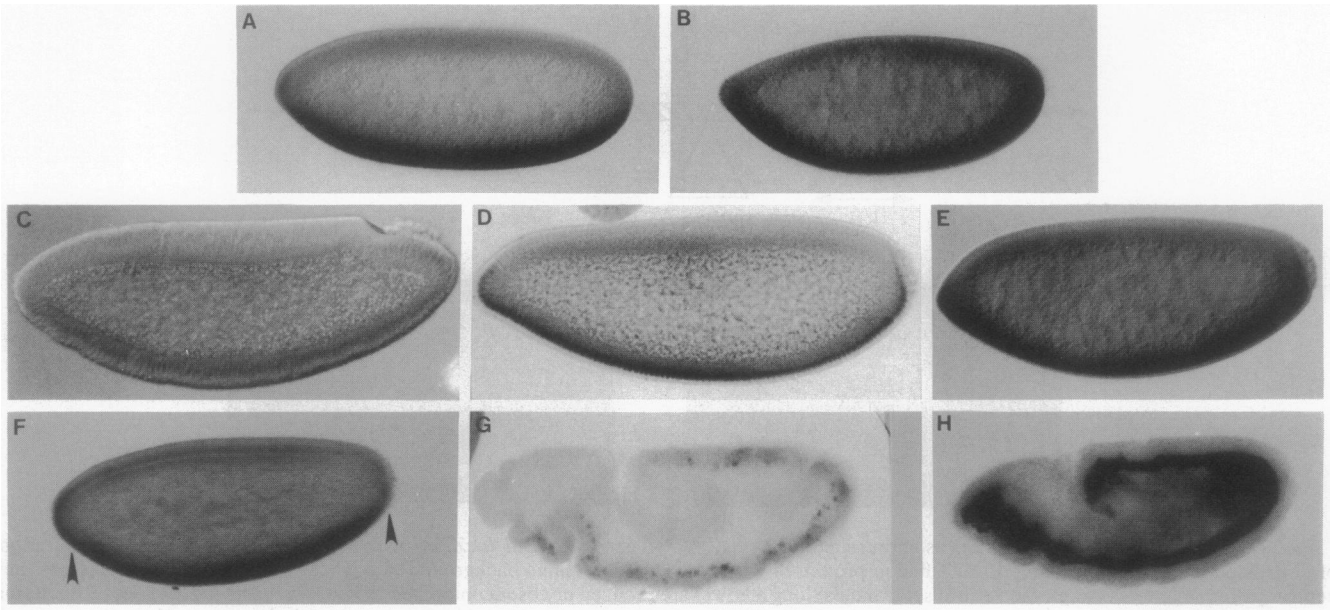

FIG. 2. Distribution and effect of the dl–lacZ protein. (*A* and *B*) Blastoderm embryos from homozygous tudor females, which serve as wild-type controls for dorsal and twist protein distribution. These embryos do not form pole cells and therefore are distinguishable from transformed embryos. Embryos in *A* and *B* are stained with anti-dl and anti-twi antibodies, respectively. (*C–E*) dl–lacZ fusion and endogenous dl proteins are distributed in a ventral-to-dorsal nuclear gradient in blastoderm embryos. Embryos laid by females transformed with the *dl–lacZ* insert were stained for β-galactosidase activity (*C*), stained with an anti-lacZ antibody (*D*), and stained with anti-dl antibody (*E*). (*F–H*) Expression of twist protein is reduced in embryos from females transformed with *dl–lacZ*. Arrowheads in *F* point to reduction of twist staining in a *dl–lacZ* transformed blastoderm stage embryo when compared with wild-type control (*B*). Reduction of twist expression correlates with partial dorsalization in germ-band extended stage embryos (compare *G* with *H*). All embryos are oriented such that their anterior end is on the left and the dorsal side is up.

dl protein (Fig. 3*A*, lane 4). This association of dl with the fusion protein must be through a specific interaction with its dl moiety, since anti-lacZ antibody did not immunoprecipitate dl from wild-type embryo extracts (lane 5) or from *hslacZ* embryo extracts, which contain wild-type dl protein and lacZ protein (lane 6). When a parallel blot was probed with anti-lacZ antibody (Fig. 3*B*), all extracts expressing either dl–lacZ fusion or lacZ alone showed the expected protein bands. This confirmed that the 85-kDa band in Fig. 3*A*, lane 4, is the endogenous dl protein and not a proteolytic product of the dl–lacZ fusion protein. The coimmunoprecipitation of wild-type dl protein with dl–lacZ fusion protein by anti-lacZ antibody clearly demonstrates that dl protein can self-associate in a protein complex.

Is dl Protein a Homodimer? We analyzed early embryonic extracts to further characterize this dl complex. Under nonreducing conditions, the dl protein was found in a large complex of ≈200 kDa (Fig. 3*C*, lane 2) when fractionated by the standard acrylamide gel electrophoresis in the presence of SDS. This complex was resolved into dl monomers upon treatment with DTT (lane 1) or with boiling (data not shown). The apparent molecular mass of this stable complex suggests that dl forms either a trimer or a dimer in association with other components. Morphological, genetic, and molecular studies predict that the dorsal protein interacts in the cytoplasm with the cactus gene product (2, 4, 6). If cactus inhibition of dorsal is by a mechanism similar to the direct inhibition of NF-κB by IκB, we expect the cactus protein to be a part of the dorsal complex. It is therefore possible that this complex consists of either a dl homodimer and cactus protein or a dl multimer.

DISCUSSION

On the basis of morphological, genetic, and molecular studies we had proposed a model in which dl interacts in the cytoplasm directly with the cactus gene product (6). In analogy with other transcription factors and because of the existence of dominant negative alleles of *dl*, it seemed likely that dl also functions as a dimer. Here, we show that expression of a dl–lacZ fusion protein causes a partial loss-of-function *dl* phenotype. This phenotype is not due to mislocalization of the fusion protein, as it is relocalized to the nuclei in a manner indistinguishable from the wild-type dl protein. Rather, the dl–lacZ fusion protein affects either the wild-type level or transcriptional function of dl protein, since the downstream zygotic genes are misregulated. The dl–lacZ fusion protein partially retains its function as a transcriptional regulator, since in a *dl* null background it results in partial rescue. It therefore seems that the last 10 amino acids are not essential for either proper localization or transcriptional regulation. This argument is strengthened by the observation that a truncated dl protein lacking these 10 amino acids rescues the *dl* null phenotype completely (R.S., unpublished work).

The phenotypic effect of dl–lacZ expression and the observed *in vitro* self-association of dl with dl–lacZ clearly suggest that dl must self-associate to function in the wild-type embryo. One explanation for the partial loss-of-function phenotype caused by the dl–lacZ fusion protein in wild-type background is steric hinderance provided by the lacZ moiety, which would, for instance, result in suboptimal DNA binding by the dl/dl–lacZ complex. Alternatively, the tetramerization of dl–lacZ may result in lowering the effective concentration of functional dl by sequestering wild-type dl protein.

Owing to their ability to form homo- or heterodimers with different binding and activation/repression potentials, transcription factors offer enormous capacity for regulation. Dimerization is only one form of protein–protein interaction used by transcription factors to carry out their precise functions (43). Our studies demonstrate that dl protein self-associates and that its dimerization seems to be critical for its function *in vivo*. An unlikely possibility is that dl protein exists in equilibrium between an active monomeric and an inactive multimeric form. In this scenario, the fusion protein

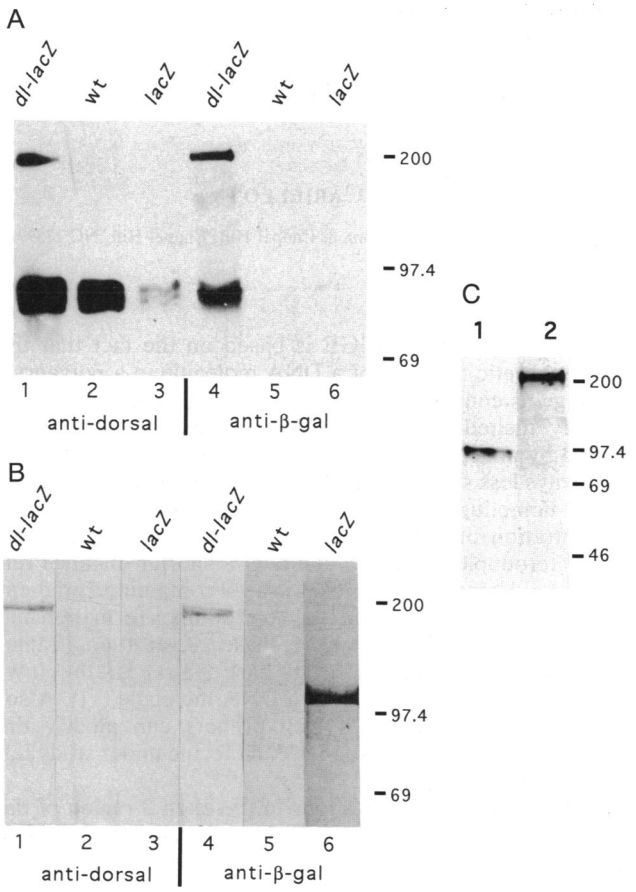

FIG. 3. Biochemical evidence for a dl complex. (*A* and *B*) Western analysis of coimmunoprecipitation of wild-type dl protein and dl–lacZ fusion protein by anti-lacZ antibody. The maternal genotypes of the embryos from which the extracts were made are indicated: dl–lacZ and lacZ are extracts made from embryos laid by *hsdl–lacZ22* and *hslacZ* mothers, respectively; wt represents an extract made from wild-type embryos. Extracts in lanes 1–3 were immunoprecipitated by anti-dl antibody; extracts in lanes 4–6 were immunoprecipitated by anti-lacZ antibody. Molecular size markers are indicated in kDa. (*A*) Blot probed with sheep anti-dl antibody followed by horseradish peroxidase-linked-secondary antibody (Jackson ImmunoResearch). Bound complexes were detected by enhanced chemiluminescence (Amersham). (*B*) Control blot probed with monoclonal anti-lacZ antibody (Promega) followed by alkaline phosphatase-linked secondary antibody. (*C*) Detection of DTT-sensitive dl complex in wild-type embryos. Whole embryo extracts (70 μg of protein) were either boiled in SDS-loading buffer with 100 mM DTT (lane 1) or resuspended in SDS-loading buffer without DTT and were not boiled (lane 2). Samples were separated on 10% SDS/polyacrylamide gel and electroblotted. The Western blot was probed with affinity-purified rabbit anti-dl antibody followed by alkaline phosphatase-linked anti-rabbit secondary antibody (Promega).

would stabilize the inactive complex, resulting in the dominant negative phenotype.

Our results are consistent with studies showing that rel proteins, like other transcription factors, can bind DNA as homo- or heterodimers (10–13). They do not exclude the possibility that other rel proteins exist and/or associate with dl. It is likely that the dimerization domain of dl resides in the rel homology region as is the case for p50 (44). Synthetic mutants of NF-κB/rel have been described that have a dominant negative effect (44), and one proposal for v-*rel*-induced oncogenic transformation relies on a direct interaction between v-rel and c-rel proteins (11). Such dominant negative effects may be due to interactions between mutant and wild-type subunits, similar to the interactions observed for antimorphic and wild-type dl proteins. Further genetic and biochemical experiments on dominant negative mutants will provide more insights into the mode of action of dl and of rel proteins in general.

We thank J. Horabin and J. Szabad for fly stocks, S. Roth and C. Rushlow for anti-twist and anti-zen antibodies, respectively, and G. Gray for fly food. We also thank P. Schedl for constant encouragement and our colleagues at Princeton for stimulating discussions and help with the manuscript. This work was supported by grants from National Institutes of Health, National Science Foundation, and the Horace W. Goldsmith Foundation. S.G. and A.M.W. were supported by the New Jersey Commission on Cancer Research.

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