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Regulated Nuclear Import of the *Drosophila* Rel Protein Dorsal: Structure-Function Analysis

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The formation of a gradient of nuclear Dorsal protein in the early *Drosophila* embryo is the last step in a maternally encoded dorsal-ventral signal transduction pathway. This gradient is formed in response to a ventral signal, which leads to the dissociation of cytoplasmic Dorsal from the IκB homolog Cactus. Free Dorsal is then targeted to the nucleus. Dorsal is a Rel-family transcription factor. Signal-dependent nuclear localization characterizes the regulation of Rel proteins. In order to identify regions of Dorsal that are essential for its homodimerization, nuclear targeting, and interaction with Cactus, we have performed an in vivo structure-function analysis. Our results show that all these functions are carried out by regions within the conserved Rel-homology region of Dorsal. The C-terminal divergent half of Dorsal is dispensable for its selective nuclear import. A basic stretch of 6 amino acids at the C terminus of the Rel-homology region is necessary for nuclear localization. This nuclear localization signal is not required for Cactus binding. Removal of the N-terminal 40 amino acids abolished the nuclear import of Dorsal, uncovering a potentially novel function for this highly conserved region.

The *dorsal* (*dl*) gene encodes the morphogen essential for specification of the dorsal-ventral axis of the *Drosophila* embryo (37, 40, 42, 51, 53). Dorsal belongs to the Rel family of transcription factors, the members of which share a conserved 300-amino-acid (aa) region, the Rel-homology region (RHR) (50; reviewed in references 32 and 47). In addition to their structural resemblance, these transcription factors have similarities in their modes of action and regulation. Rel proteins are sequestered in the cytoplasm by their interaction with proteins belonging to the IκB family (reviewed in references 4 and 16). Signal-dependent dissociation of Rel from IκB proteins and their subsequent nuclear localization is a regulatory feature characteristic of Rel-family proteins.

The RHRs are located at the N termini of Rel proteins and have roughly 50% sequence identity (32, 47). The C termini of these proteins are divergent and function in transcriptional activation. Studies of vertebrate Rel proteins have revealed that information essential for their regulated nuclear import resides in the RHR. The RHR also mediates DNA binding, homodimer and heterodimer formation among family members, and interaction with the ankyrin repeats of IκB proteins (13, 16, 27, 34).

The vertebrate members of the Rel family include the two NF-κB subunits p50 and p65/RelA, the p105 precursor of p50, p52 and its precursor p100, c-Rel, and RelB (14, 32, 35, 41, 43, 47). These Rel proteins can form multiple interchangeable homodimers and heterodimers that function in cells of the vertebrate immune system. In contrast to the NF-κB heterodimer, Dorsal functions as a homodimer during *Drosophila* embryogenesis (20). Dorsal is also expressed in the larval fat body (30), as is the Dorsal-related immunity factor Dif (23). It is not known whether, in the fat body, Dorsal is a homodimer or whether it functions as a heterodimer with Dif.

During *Drosophila* embryogenesis, dorsal-ventral polarity is established by a maternally encoded signal transduction pathway. This pathway culminates in the graded nuclear translocation of Dorsal (40, 42, 51, 53). Dorsal is initially maintained in the cytoplasm through its interaction with Cactus, a member of the IκB family (12, 27, 56). The ventral-to-dorsal gradient of nuclear Dorsal becomes apparent at the syncytial blastoderm stage. It is formed as a result of a signal that originates ventrally in the extracellular perivitelline fluid (49). By differentially controlling the expression of specific zygotic target genes, different levels of nuclear Dorsal specify the pattern along the dorsal-ventral axis (25, 52).

Analysis of maternal-effect mutations that cause disruptions in the dorsal-ventral pattern of the larva has led to the identification of 12 components of this signal transduction pathway (reviewed in reference 7). Mutations that lead to the loss of function of *dl* as well as 10 other genes (the dorsal group genes) lead to the maternal-effect dorsalization of embryos. In contrast, loss-of-function mutations in the remaining component, *cactus* (*cact*), lead to maternal-effect ventralization (39). The 10 other dorsal group gene products positively regulate the nuclear import of Dorsal by controlling the dissociation of the cytoplasmic Dorsal-Cactus complex (27, 56). Two of the dorsal group genes, *pelle*, which encodes a protein kinase (46), and *tube*, which encodes a novel protein (31), function immediately upstream of Dorsal and Cactus, and Pelle may directly regulate the dissociation of the Dorsal-Cactus complex (2, 21).

Molecular and phenotypic analyses of *dl* alleles have revealed the importance of conserved amino acids that are required for *dl*'s proper function or distribution (24). To further define regions within the Dorsal protein important for (i) homodimerization, (ii) interaction with Cactus, (iii) nuclear localization, and (iv) response to the ventral signal, we have performed a structure-function analysis of Dorsal. By a combination of genetic and immunological techniques, the distribution and biochemical interactions of various Dorsal deletions were studied. Consistent with previous analyses of vertebrate Rel proteins, we find that the Dorsal RHR is suf-

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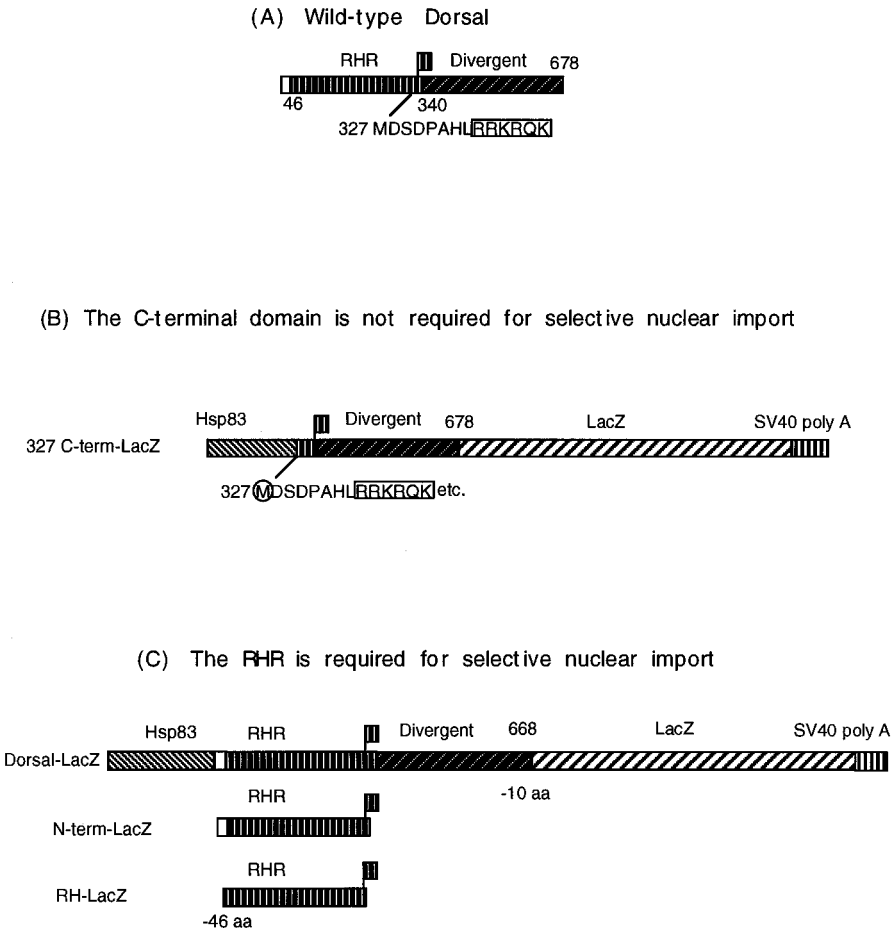

FIG. 1. Schematic representation of P-element constructs used in transgenic experiments. (A) The wild-type Dorsal protein is made up of 678 aa. Most of the N-terminal RHR (aa 46 to 340) shows similarity to the vertebrate Rel proteins. The C-terminal half (341 to 678) is divergent and has several structural properties (Fig. 8 and reference 50). A short stretch of basic amino acids located at the C-terminus of the RHR represents the putative NLS (boxed amino acids), and its position is indicated by the flag. (B) Role of the C-terminal domain of Dorsal. The entire C-terminal half of Dorsal was fused to LacZ. Methionine at position 327 (circled) was used as the initiation codon (327 C-term-LacZ). This construct therefore preserves the putative NLS (flag). The fusion protein is no longer regulated by dorsal group genes or Cactus. It becomes uniformly nuclear all along the dorsal-ventral axis (Fig. 2). (C) Role of the N-terminal half of Dorsal. Constructs containing the entire N-terminal half of Dorsal (N-term-LacZ) and another containing just the RHR of Dorsal (RH-LacZ) are shown. Like the full-length Dorsal-LacZ construct (20), both of these constructs conferred dominant female sterility and resulted in an asymmetric distribution of the fusion protein (Fig. 3). (D) The conserved RRKRQK sequence was deleted by site-specific mutagenesis. The mutant protein was not fused to LacZ. This mutant protein remained cytoplasmic (Fig. 4). (E) Dorsal-LacZ constructs without the NLS. The NLS was deleted from two complementary constructs described above in which either the N-terminal (N-termΔNLS-LacZ) or the C-terminal (327ΔNLSC-term-LacZ) halves of Dorsal were intact. In a third construct (184ΔNLS-LacZ), only the first 184 amino acids were included. All of these fusion proteins remained in the cytoplasm of the embryo (Fig. 5). (F) Mapping of the dimerization and Cactus-binding sites. The RH-LacZ fusion was further deleted to remove the first 84, 138, or 222 amino acids of Dorsal. These fusions showed cytoplasmic staining even though their NLSs were intact (Fig. 6). All constructs except the ΔNLS clone (D) are in-frame fusions to the LacZ coding region. The *hsp83* promoter is used to express these mutant proteins. The simian virus 40 (SV40) poly(A) signal is used in all but the DorsalΔNLS construct (D), in which the endogenous *dl* poly(A) signal is utilized.

ficient for its selective nuclear import, that its nuclear localization signal (NLS) is present at the C terminus of the RHR, and that the overlapping dimerization and Cactus-binding regions map immediately N terminal to this NLS. In contrast to observations of vertebrate Rel proteins, we report that the Dorsal NLS is not required for its interaction with Cactus. We have also uncovered a potentially novel function for the N-terminal 38 aa of the RHR in the regulated nuclear import of Dorsal.

MATERIALS AND METHODS

Expression and analysis of Dorsal-LacZ proteins in the *Drosophila* embryo. Dorsal has 678 aa and consists of two parts (Fig. 1A); the N-terminal half is contained within aa 1 to 340, and the remaining amino acids make up the C-terminal half. Constructs consisting of either of these entire halves, with or without the putative NLS, fused in frame to LacZ, were expressed in transgenic animals. A series of N-terminal RHR deletions were also expressed as LacZ fusions in transgenic animals.

Because the *hsp83* promoter shows a constitutive expression pattern in the ovary similar to that of the *dl* promoter (19), it was used to drive the expression of all *dl* constructs. While the expression level of the transgenes is dependent on the site of insertion in the *Drosophila* genome, the majority of the *hsp83*-driven *dl* transgenes are poorly expressed (18, 19). Because of zygotic lethality, transgenic lines with expression levels high enough to saturate endogenous Cactus in the embryo are rarely obtained. Therefore, upon the expression of these mutant proteins, the distribution of endogenous Dorsal remains unaffected. The line that showed the highest intensity of embryo staining for each of the constructs was therefore chosen for further analysis. As estimated by antibody staining of embryos, the amounts of different mutant proteins in these transgenic lines were comparable. We analyzed the protein distribution of these Dorsal deletions in wild-type embryos, as well as in embryos with greatly reduced Cactus function. The distributions of some of these proteins were also studied in genetic backgrounds in which the dorsal group signal was either absent or present in abnormally high levels.

The interactions of the mutant proteins with endogenous wild-type Dorsal and Cactus were assayed genetically. The rationale was as follows. Both *dl* and *cact* genes exhibit haplo-insufficiency (37, 39). Because of this genetic property, the Dorsal-Cactus interaction is extremely dose sensitive (19). Thus, if the Dorsal-

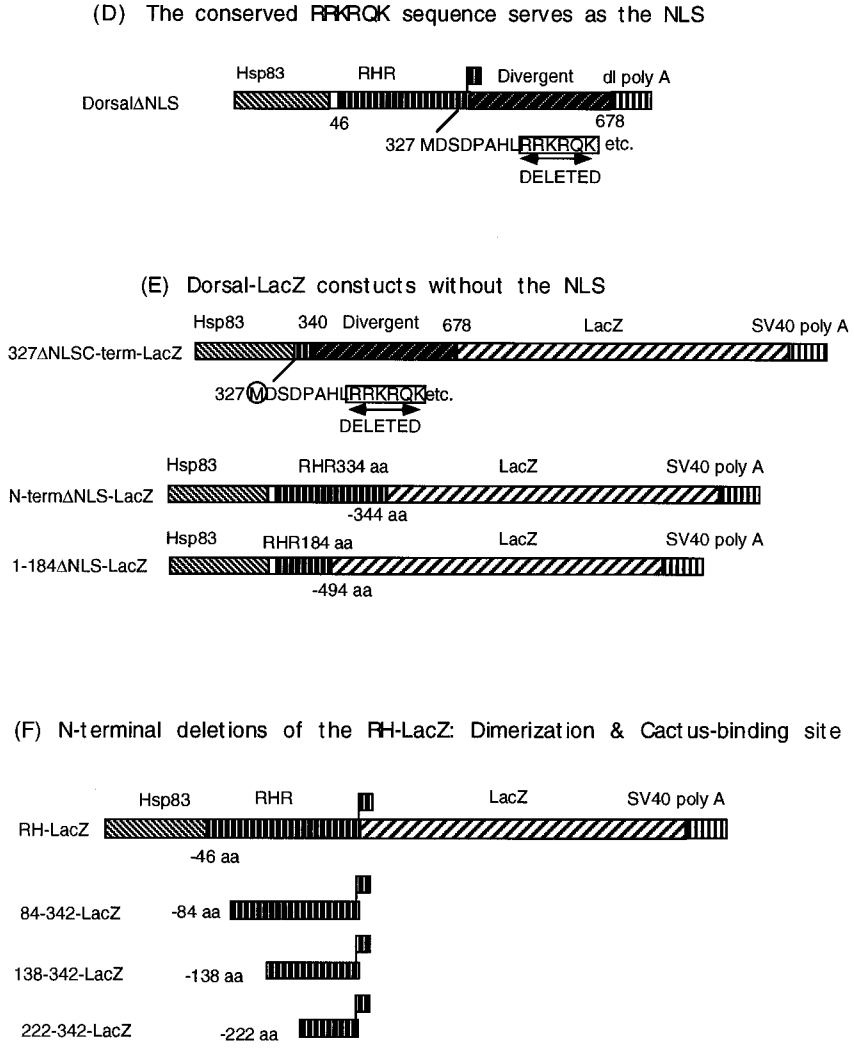

FIG. 1—*Continued.*

LacZ fusion protein interacts with either endogenous Dorsal or endogenous Cactus, an alteration of the embryonic phenotype is predicted upon reduction of the dose of either *dl* or *cact*. In general, dorsalization of a majority of embryos derived from a $dl^{-}/+$ maternal background reflects an interaction with endogenous Dorsal, whereas ventralization of a majority of embryos derived from a $cact^{4.2}/+$ maternal background reflects an interaction with endogenous Cactus.

We have previously demonstrated that a fusion between full-length Dorsal and LacZ does not disrupt the distribution of either the endogenous or transgene Dorsal and forms dimers with endogenous Dorsal (20). We also found that LacZ stabilizes the Dorsal RHR. A *dl* mutation which has a stop codon at glutamine 339, and therefore consists of only the RHR, is unstable (24). It is not detected by antibodies specific to the N-terminal half of Dorsal (57). The same region fused to LacZ results in a stable protein. The LacZ moiety also provides a convenient epitope to monitor subcellular localization of fusion proteins. Furthermore, in immunoprecipitation experiments, it allows us to biochemically differentiate between the Dorsal-LacZ–Cactus complex and the wild-type Dorsal-Cactus complex.

Plasmids and transformation of constructs. The construction and use of the *hsp83* promoter has previously been described in detail (19). Full-length wild-type *hsp83-dorsal* in BS^{+} (19) was used to synthesize the single-strand template for site-directed mutagenesis (28). A deletion primer (CCAGCGCACTTGACT GGCGGTGAT) designed to remove 6 aa, RRKRQK, was used in order to obtain the Dorsal construct with the NLS deleted (DorsalΔNLS) (Fig. 1D).

Different approaches were used to make the Dorsal-LacZ fusion proteins. The full-length Dorsal-LacZ fusion is described in reference 20. The construct containing the N-terminal half of Dorsal (N-term-LacZ) (Fig. 1C) was constructed as follows. With a mutagenesis primer (GACTGGCGGAGATCTCATGCAC CTG), a *Bgl*II site was created at aa 345, which is immediately after the NLS. The N-terminal region of the *hsp83dl* cDNA (19) was purified after a complete *Not*I digest and a partial *Bgl*II digest and cloned in frame with the *lacZ* gene in pCasPerβgal (55) at the unique *Bam*HI site. 184ΔNLS-LacZ was constructed by using the *Bgl*II site at aa position 184 to make an in-frame fusion with the *lacZ* gene in pCasperβGal at its unique *Bam*HI site.

The other Dorsal-LacZ fusions (46-342, 84-342, 138-342, 222-342, 327 C-term, N-termΔNLS, and 327ΔNLSC-term) were constructed by the following general approach. Restriction sites *Sma*I and *Bam*HI were engineered into primers (sequences are listed below) designed to amplify a specific region of the *dl* cDNA. Approximately 7 ng of linearized plasmid DNA with 200 ng of each primer was subjected to the amplification reaction in a 100-μl volume. The first amplification cycle consisted of steps of 95, 54, and 72°C for 45 s, 2 min, and 1 min, respectively, and it was repeated three times. The second amplification cycle consisted of a 95°C step for 45 s, a 70°C step for 2 min, and a 72°C step for 1 min. This cycle was repeated 25 times.

Amplified DNA was analyzed on an agarose gel, and either the *Sma*I or the *Bam*HI fragment was prepared to be cloned into the corresponding site of the *hsp83-lacZ* vector (44). In the absence of an insert carrying the AUG codon, this vector results in the growth of white colonies. However, when the insert provides the AUG codon that results in an in-frame LacZ protein fusion, transformed colonies appear deep blue when grown in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The different *hsp83-dorsal-lacZ* inserts were transferred to pCaSpeR as a *Not*I-*Kpn*I fragment. With primers along both strands, each insert was

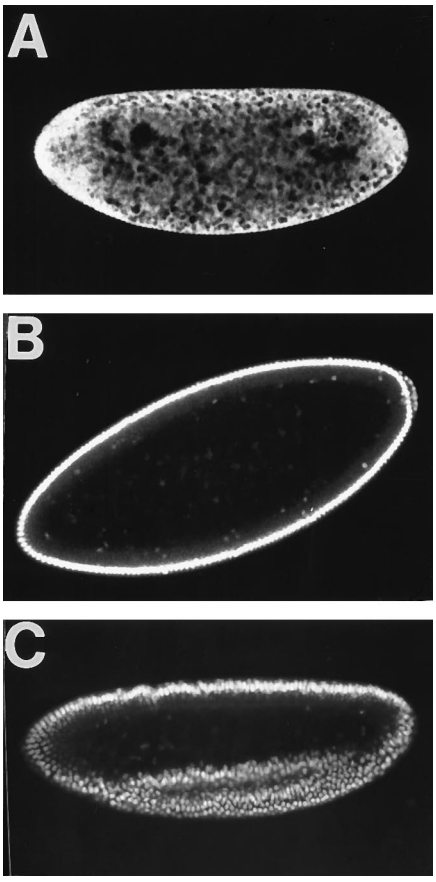

FIG. 2. The C-terminal half of Dorsal does not play a role in its selective nuclear import. The fusion construct described in the legend to Fig. 1B consists of the entire C-terminal half of Dorsal and uses the methionine at position 327 as the start codon. Like many other nuclear proteins in the early *Drosophila* embryo, this fusion protein remains cytoplasmic in cleavage-stage embryos (A). However, in the syncytial blastoderm, it becomes uniformly nuclear along the dorsal-ventral axis (B). The protein remains stable and is nuclear during gastrulation (C). Nuclear protein is visualized at these stages.

sequenced across the Dorsal-LacZ junction as well as across the amplified *dl* region (Sequenase; United States Biochemical Corporation). PCR primers were dlK46Sma, CG AAA CCC GGG CGA AAG ATG CCC; dlK84Sma, CT ACG CCC GGG AAC ATG ACC TAT C; dlM222Sma, CCC ATC CCC GGG AAG ACC ATG TCC GAC; dlD342SmaR, GGG ATC CCC GGG AGT CTT CTG ACG; dlM327Sma, TTC GAG CCC GGG ACA ATG GAC TCA G; dlS677SmaR, GTA TTA CCC GGG TAT GGA CAG GTT C; dlM1Sma, TTT CCC GGG ATA ATG TTT CCG AAC; and dlL334SmaR, CCT CCC GGG CGC TGG ATC TGA GTC.

To obtain flies carrying DNA inserts in their germ line (48), half-hour egg collections of homozygous w^1 or w^{1118} embryos were made. These were dechorionated and injected with different DNA constructs and helper plasmid pTurbo (38). Transformants were screened by eye color. *dl* stocks consisted of *al dp b Df(2L)TW119 cn bw/CyOb* (this deficiency removes *dl*) and *b pr cn sca In(2L)dl*^{1}*/CyOb* (this inversion breaks within *dl*) (19). *cact* stocks consisted of *b cact*^{A2}*/CyOb* and *cact*^{S1}*/CyO* (39). Gain-of-function dorsal group stocks consisted of *Tl*^{10b} or *Tl*^{9Q} *mwh e/TM3 Sb Ser/T(1:3)OR60* (45) and *ea*$^{5.13}$ *th st c p in n p*^{p}*/TM3/T(1:3)OR60* (6). Other stocks consisted of *tud*^{1}*/CyO* (5). Because embryos from homozygous *tud*^{1}*/tud*^{1} females lack pole cells, but are otherwise normal during early development, they serve as a positive control for anti-Dorsal antibody staining experiments.

Morphology and staining of embryos. Cuticle preparations of embryos were performed after dechorionation as described in reference 58. Embryos were fixed in paraformaldehyde and devitellinized for antibody staining. The monoclonal supernatant 7A4-39 was used. This monoclonal antibody reacts specifically against a C-terminus-specific epitope of Dorsal (56), which allowed distinction between endogenous Dorsal and Dorsal-LacZ proteins. Anti-LacZ antibody was purchased from Promega. Secondary anti-mouse antibodies were either fluorescently labeled (streptavidin-Cy 3; Jackson Laboratory) or conjugated with biotin (Vectastain). Detailed immunolocalization protocols for each visualization method have been described previously (51, 56). Stained and washed embryos were mounted in 90% glycerol (for fluorescence imaging) or Permount (for differential interference contrast imaging). A Bio-Rad model MRC600 confocal imaging head, equipped with an argon-krypton laser, mounted on a Nikon Optiphot II microscope was used for imaging stained embryos. The Nikon PlanApo 20× lens with a numerical aperture of 0.75 was used.

Immunoprecipitations and Western blots (immunoblots). Immunoprecipitations and Western blots were done as described previously (18, 56), using the C-terminus-specific 7A4-39 monoclonal anti-Dorsal antibody, which does not recognize the RHR of Dorsal. The anti-Cactus monoclonal antibody 3H12-29 (56) was used to visualize Cactus protein.

RESULTS

Using an in vivo approach, we have mapped regions within the Dorsal protein that are important for its dimerization and regulated nuclear import. To simultaneously stabilize small portions of Dorsal and monitor their distribution in genetically wild-type and mutant *Drosophila* backgrounds, we have used LacZ as a tag. The *hsp83* promoter was used to control the expression of all of the transgenes (reference 20 and Materials and Methods).

The C-terminal divergent half of Dorsal is not required for its selective nuclear import. The C termini of Rel proteins are completely divergent from each other in length and sequence. The C-terminal half of Dorsal contains several interesting structural features (50). It has stretches of glutamine, alanine, and asparagine repeats. Glutamine repeats are involved in protein-protein interactions and are implicated in transcriptional activation (8). To determine if this half of Dorsal has a

TABLE 1. Expression of Dorsal-LacZ constructs in wild-type and haploid *dl* or *cact* backgrounds[a]

Construct or line	% of embryos hatched from maternal genotype:		
	+/+ (phenotype)[b]	$dl^{-}/+$[c]	$cact^{A2}/+$[d]
No insert *w/w*	99 (wt)	83	70
Dorsal-LacZ	<1 (D2)	<1	<1
327 C-term-LacZ	86 (wt)	88	71
N-term-LacZ	<0.5 (D2)	0	<1
RH-LacZ	66 (D3)	<1	5
84-342-LacZ	98 (wt)	86	22
138-342-LacZ	99 (wt)	81	34
222-342-LacZ	99 (wt)	2	11
N-termΔNLS-LacZ	<1 (V3-V4)	0	0
184ΔNLS-LacZ	96 (wt)	83	73
327ΔNLSC-term-LacZ	96 (wt)	ND[e]	ND

[a] Maternal effects of the Dorsal-LacZ fusion proteins in different genetic backgrounds are shown. For each experiment, at least 300 fertilized eggs were counted and the numbers of embryos that hatched were established. The numbers of hatched embryos as percentages of total fertilized eggs are presented.

[b] Transformed virgins carrying one (Dorsal-LacZ, N-term-LacZ, RH-LacZ, N-termΔNLS-LacZ) or two (the remaining constructs) copies of the insert were mated with wild-type males. Terminal phenotypes of the cuticles of unhatched embryos were observed and, where indicated, follow the classification of Roth et al. (39). Data on Dorsal-LacZ were reported previously (20). wt, wild type.

[c] To determine possible interactions between endogenous Dorsal and the Dorsal-LacZ fusion proteins, transgenic flies were crossed to carry a deficiency of *dl* [*Df(2L)TW119*; see Materials and Methods]. Virgin females, hemizygous for *dl* and expressing each of the fusion proteins, were mated to wild-type males. The percentages of hatching of embryos derived from these females were determined. In all experiments, unhatched embryos were weakly dorsalized. Unfertilized eggs were ignored.

[d] To determine possible interactions between endogenous Cactus and the Dorsal-LacZ fusion proteins, the transgenic flies were crossed to carry one copy of the strong maternal effect *cact*^{A2} allele. Virgin females, heterozygous for *cact*^{A2} and expressing each of the fusion proteins, were mated to wild-type males. The percentages of hatching of embryos derived from these females were determined. In all experiments, the phenotypes of the unhatched embryos were weakly ventralized. Unfertilized eggs were ignored.

[e] ND, not determined.

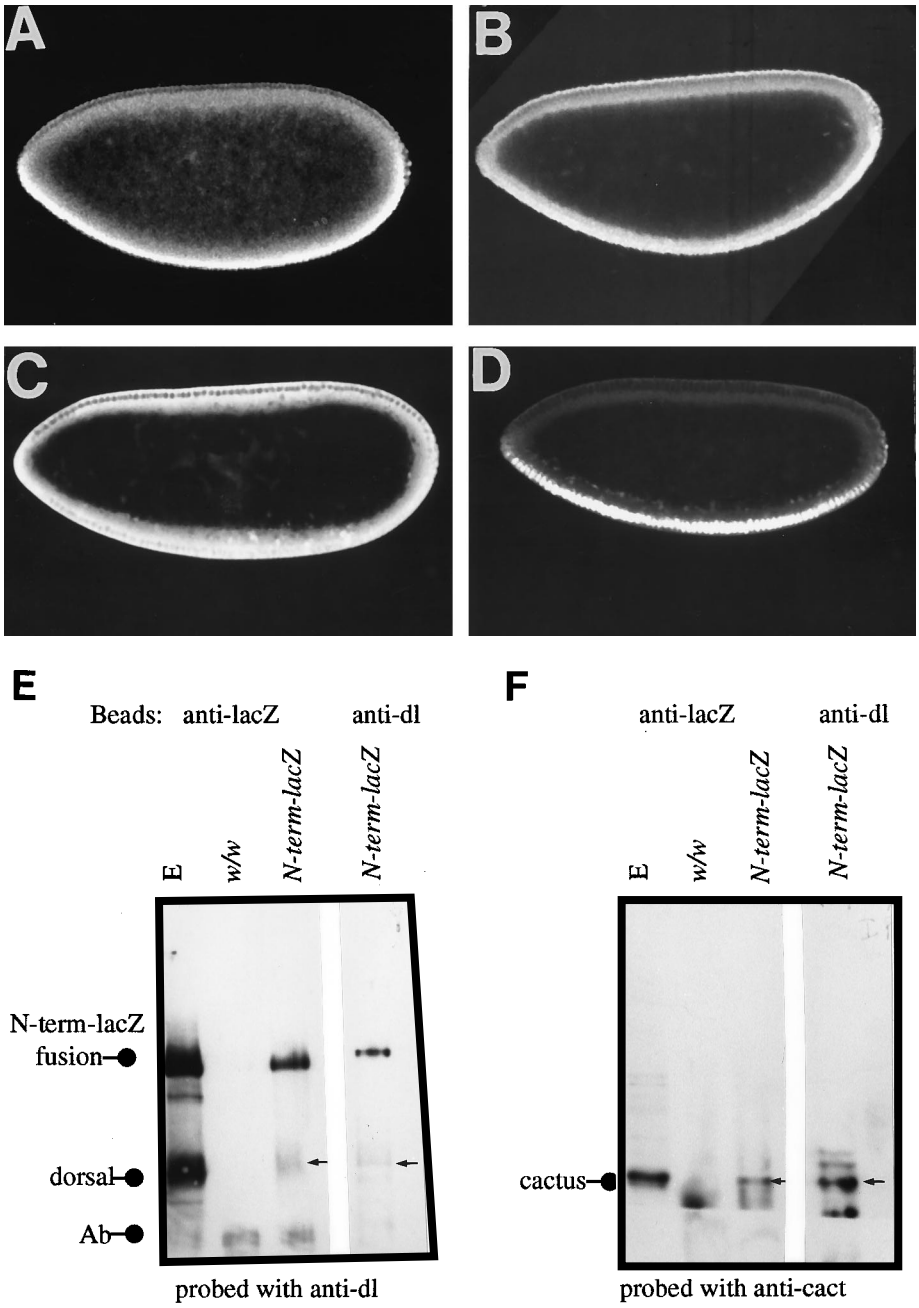

FIG. 3. (A to F) The N-terminal half of Dorsal is necessary for its asymmetric nuclear import. Embryos expressing full-length Dorsal-LacZ (A), N-term-LacZ (B), or RH-LacZ (C) were stained with anti-LacZ antibody. In all cases, nuclei on the ventral side have high levels of the fusion proteins, while nuclei on the dorsal side either did not stain or showed very little staining. Levels in the ventral nuclei are more pronounced when Dorsal is intact. (D) An RH-LacZ-expressing embryo stained with monoclonal anti-Dorsal antibody that specifically recognized a C-terminal epitope of endogenous Dorsal (absent from RH-LacZ) shows the wild-type staining pattern. Thus, expression of these fusion proteins does not affect the localization of wild-type Dorsal. A similar result has been described for embryos expressing the full-length Dorsal-LacZ fusion protein (20). (E to F) Dorsal dimerization and Cactus binding are mediated by the N-terminal RHR. Extracts were made from embryos expressing the N-term-LacZ fusion protein or *w/w* control embryos. Two Western blots of fractionated total extract (lanes E) or immunoprecipitates from experiments using anti-Dorsal or anti-LacZ antibody-conjugated beads (as shown above the lanes) were probed with either anti-Dorsal (E) or anti-Cactus (F) antibodies. Both anti-LacZ and anti-Dorsal antibodies immunoprecipitate endogenous Dorsal and Cactus (arrows) in addition to the fusion protein. Ab, antibody.

role in its selective nuclear uptake, a construct with the entire C-terminal half, initiating at methionine 327, just upstream from the putative NLS, was fused in frame to LacZ (327 C-term-LacZ [Fig. 1A and B]). Wild-type females that expressed this hybrid protein did not exhibit any noticeable dominant maternal-effect phenotype (Table 1). When stained with anti-LacZ antibody, pre-blastoderm-stage embryos showed uniform cytoplasmic distribution of the hybrid protein (Fig. 2A). In blastoderm-stage embryos, the hybrid protein was found to be predominantly nuclear all along the dorsal-ventral axis (Fig. 2B and C), although the presence of low levels of cytoplasmic protein cannot be ruled out. The stage at which the fusion protein appears in the nucleus suggests that a general mechanism that regulates the import of many other proteins in the

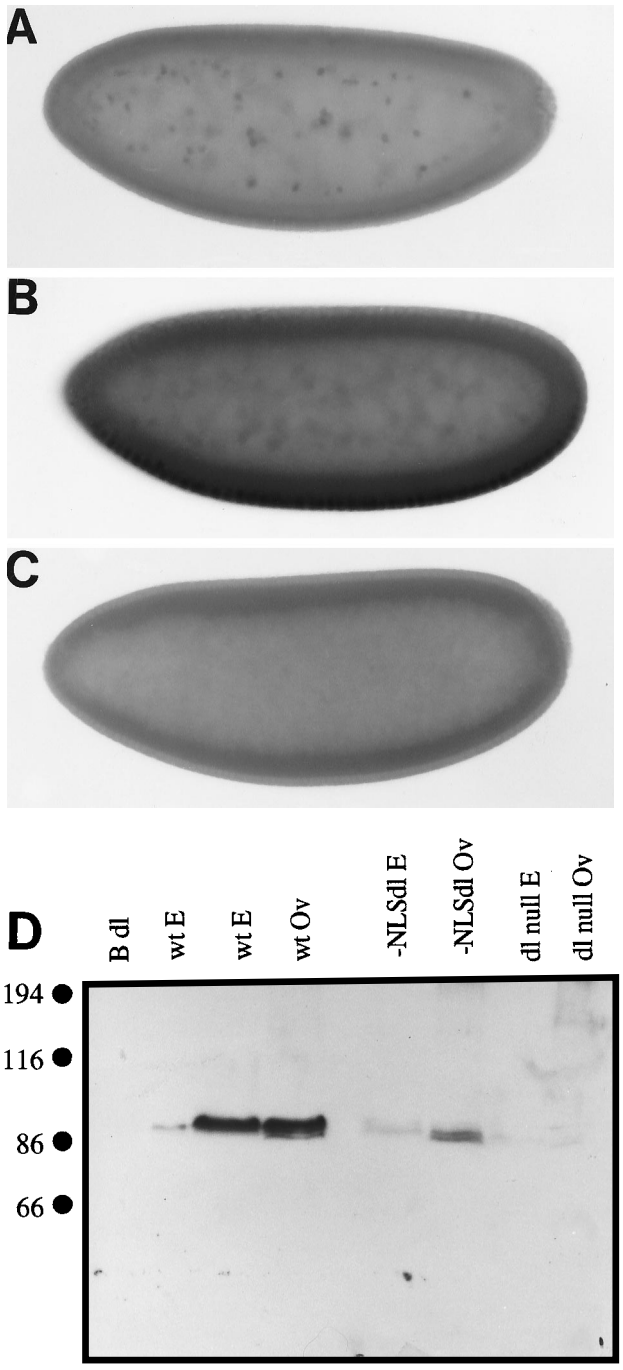

FIG. 4. The RHR of Dorsal responds to the presence of Cactus. (A) The RH-LacZ fusion protein in an embryo derived from a $cact^{A2}/cact^{S1}$ female. In this background, the fusion protein (anti-LacZ staining) is nuclear along the dorsal-ventral axis. (B) Embryos derived from *tud*/*tud* females lack pole cells but undergo normal embryogenesis. When stained with anti-Dorsal antibody, these embryos exhibit the wild-type gradient of nuclear Dorsal protein. (C) The conserved RRKRQK sequence serves as the NLS of Dorsal. This stretch of basic residues was deleted from the wild-type protein (Fig. 1D), and the mutant protein in a dl^- background was uniformly cytoplasmic. The level of the mutant protein in this transformed line is much lower than that of wild-type protein (B). (D) The NLS is not essential for Cactus stability. A Western blot was prepared with extracts from 0- to 3-h wild-type embryos (lanes wt E; 10 or 100 embryos were used), two pairs of ovaries from wild-type females (lane wt Ov), embryos and two pairs of ovaries expressing one insert of DorsalΔNLS (lanes -NLSdl E [100 embryos] and -NLSdl Ov [two pairs of ovaries]) but no endogenous protein, or embryos and two pairs of ovaries containing neither the wild-type nor the fusion protein (lane dl null E [100 embryos] or dl null Ov [two pairs of ovaries]). Lane B dl, bacterially expressed Dorsal. This blot was probed with anti-Cactus antibody. As expected, theCactus band was not visualized in *dl* null ovary or embryonic extracts. However, Cactus was clearly present in those animals that lacked endogenous Dorsal but contained the NLS-deficient protein, as was the case with extracts made from wild-type animals. Prestained molecular size markers (Sigma) were used.

early embryo (9) also controls the import of 327 C-term-LacZ. Thus, amino acids contained within this C-terminus of Dorsal contain information to direct nuclear localization of LacZ but lack information that is important for regulated nuclear import.

The Dorsal RHR is necessary for its dimerization and selective import. Since the information for selective nuclear localization of Dorsal does not reside within its C-terminal half, it was important to test whether the structural determinants that mediate its graded nuclear import are contained in the N-terminal half. We designed two LacZ fusions, one containing the entire N-terminal half of Dorsal (aa 1 to 345; N-term-LacZ) and the other containing only those amino acids (46 to 342) that are included in the RHR (RH-LacZ) (Fig. 1A and C). Similar results were obtained for both constructs. Wild-type females transformed with N-term-LacZ and RH-LacZ exhibited partial to strong dominant sterility as the embryos derived from these females were dorsalized (Table 1). This property of dominant female sterility and dorsalization of embryos is identical to that described previously for full-length Dorsal-LacZ (reference 20 and Table 1). At the blastoderm stage, embryos laid by these females showed a gradient of nuclear LacZ similar to that of wild-type Dorsal (Fig. 3A to C). However, the gradient differed from that of wild-type Dorsal in two respects. First, the ratio of nuclear to cytoplasmic fusion proteins was lower; second, cytoplasmic staining was more intense all along the dorsal-ventral axis. Much like the full-length Dorsal-LacZ (20), these fusions did not affect the localization of endogenous Dorsal (Fig. 3D).

To test if the nuclear import of the N-term-LacZ fusion protein is signal dependent, the transgene was crossed into a dorsal group null (gastrulation-defective) background. In this background, endogenous Dorsal protein remains in the cytoplasm (40, 51). When N-term-LacZ embryos derived from homozygous gastrulation-defective females were stained with anti-LacZ antibody, the fusion protein was found in the cytoplasm (data not shown). This result demonstrates that, like endogenous Dorsal, the N-term-LacZ fusion responds appropriately to the signal transduction pathway.

The interaction of RH-LacZ with Cactus was tested in embryos with drastically reduced Cactus function. The RH-LacZ transgene was crossed into the $cact^{A2}/cact^{S1}$ maternal background. When embryos derived from this ventralizing background were stained with anti-LacZ antibody, most of the fusion protein was nuclear along the entire dorsal-ventral axis (Fig. 4A).

Genetic experiments further support the idea that N-term-LacZ and RH-LacZ fusion proteins interact with both endogenous Dorsal and Cactus. Approximately 30% of the eggs laid by the control $cact^{A2}/+$ females do not hatch and are weakly ventralized (19). However, in the presence of one copy of RH-LacZ, 95% of the embryos remained unhatched and were weakly ventralized (Table 1). This genetic result is suggestive of interaction of RH-LacZ with endogenous Cactus. A clear genetic interaction was also observed when one copy of wild-type *dl* was removed. While 83% of the embryos from hemizygous *dl* females hatch, the presence of one copy of RH-LacZ reduced this number to less than 1% (Table 1). Perhaps because of the larger amounts of the N-term-LacZ protein in the

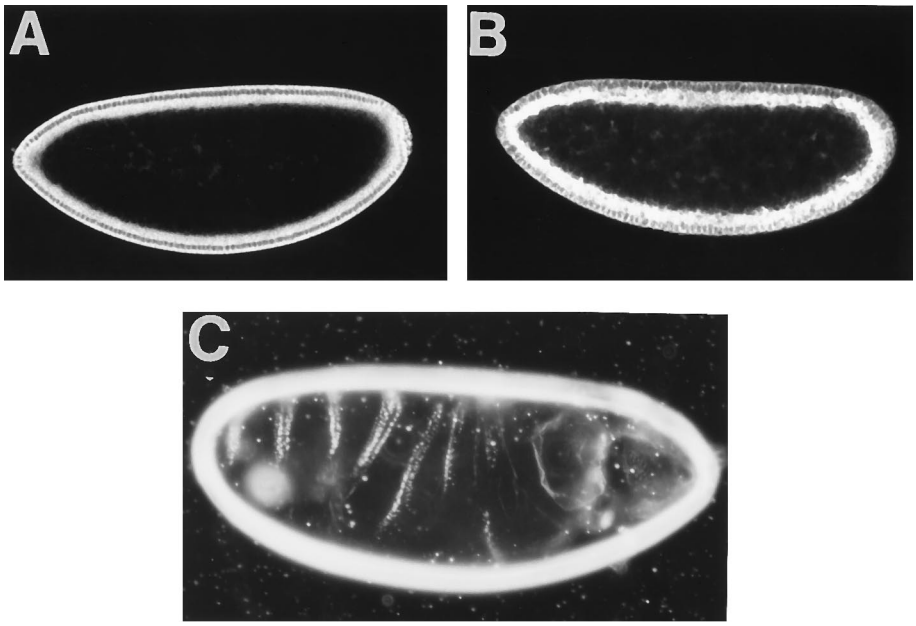

FIG. 5. (A to C) Fusion proteins without the NLS are cytoplasmic. (A) Embryo expressing the fusion protein 327ΔNLSC-term-LacZ (Fig. 1E) stained with anti-LacZ antibody. This protein initiates at Met-327 of Dorsal and contains all of the amino acids to the C-terminus. However, the NLS is deleted. (B) Embryo expressing a fusion protein consisting of Dorsal aa 1 to 334 (N-termΔNLS-LacZ). (C) Embryos of the type described for panel B show a ventralized phenotype (anterior to the right).

embryo, the dominant effects of N-term-LacZ transgenic lines were already strong in the wild-type background; therefore, the effects of lowering the endogenous dose of *cact* and *dl* were not as clear as they were with the RH-LacZ transgenic lines (Table 1).

The genetic interaction of endogenous Dorsal and Cactus with N-term-LacZ was confirmed as a physical interaction in a coimmunoprecipitation experiment. Embryonic extracts from the N-term-LacZ transgenic line were incubated with anti-LacZ or anti-Dorsal antibody-coupled beads. Immunoprecipitates were probed on Western blots with monoclonal anti-Cactus or C-terminus-specific anti-Dorsal antibodies. As seen in Fig. 3E and F, bands of endogenous Dorsal and Cactus are clearly detected. From these biochemical, genetic, and staining results, we conclude that the Dorsal RHR has all the information required for homodimerization and interaction with Cactus. Furthermore, when fused with LacZ, it retains the essential properties that allow gradient formation.

A 6-aa motif is necessary for nuclear import of Dorsal. A 6-aa stretch of basic residues (RRKRQK) is present towards the C-terminal region of the Dorsal RHR within residues 335 to 340. Because of its conservation within Rel proteins, and because such short basic stretches have been shown to specify nuclear import of otherwise cytoplasmic proteins (26), it was assumed that this region constitutes the Dorsal NLS (24, 50). In tissue culture cells, this region has been found to be essential for the nuclear import of some vertebrate Rel proteins (1, 3, 11, 17).

To determine whether this 6-aa region constitutes the primary NLS of Dorsal, we deleted it from a full-length *dl* cDNA (Fig. 1D). To study the distribution of this mutant protein in the absence of endogenous Dorsal, the transgene was crossed into the *dl* null background. The DorsalΔNLS protein, expressed at low levels from the transgene, did not even partially rescue the maternal *dl* null phenotype. When the embryos were stained with anti-Dorsal antibody, DorsalΔNLS was found predominantly in the cytoplasm and nuclear protein was not detected (Fig. 4C).

The same six amino acids are also contained within 327 C-term-LacZ, and as described above, this fusion protein exhibits strong nuclear localization. When these six amino acids were deleted from 327 C-term-LacZ (termed 327ΔNLS-LacZ) (Fig. 1E), the fusion protein remained in the cytoplasm (Fig. 5A). In a complementary construct, the NLS was deleted from the N-term-LacZ construct (N-termΔNLS-LacZ) (Fig. 1E). When embryos expressing this fusion protein were stained with anti-LacZ antibody, predominantly cytoplasmic staining was observed again (Fig. 5B). In an additional construct, the first 184 amino acids of *dl* were linked to *lacZ* (184ΔNLS-LacZ; Fig. 1E). This fusion protein also remained in the cytoplasm and the staining was identical to that observed in Fig. 5A (data not shown). These experiments further support the finding that aa 335 to 340 are essential for the nuclear targeting of Dorsal.

The dimerization and Cactus-binding regions. To map regions within the RHR that are involved in dimerization and Cactus binding and to test whether these functions are separable, we conducted a deletion analysis. Three constructs were designed in which successively larger N-terminal deletions of the RHR were made, and the remainder of the polypeptide, including the endogenous NLS, was fused in frame to *lacZ* (Fig. 1F). The regions of Dorsal that remain in these constructs consist of aa 84 to 342, 138 to 342, and 222 to 342. When wild-type embryos with these fusion proteins were stained with anti-LacZ antibody, strong cytoplasmic staining, but no nuclear staining, was observed (Fig. 6A to C). Because of the presence of the NLS in these constructs, the cytoplasmic localization of these proteins was surprising.

None of the transgenic lines with the three deletion-fusion constructs caused dominant negative effects in the wild-type background. However, when the dose of either *dl* or *cact* was lowered, clear genetic effects were observed. In the hemizygous *dl* background, expression of 84-342-LacZ or 138-342-LacZ did

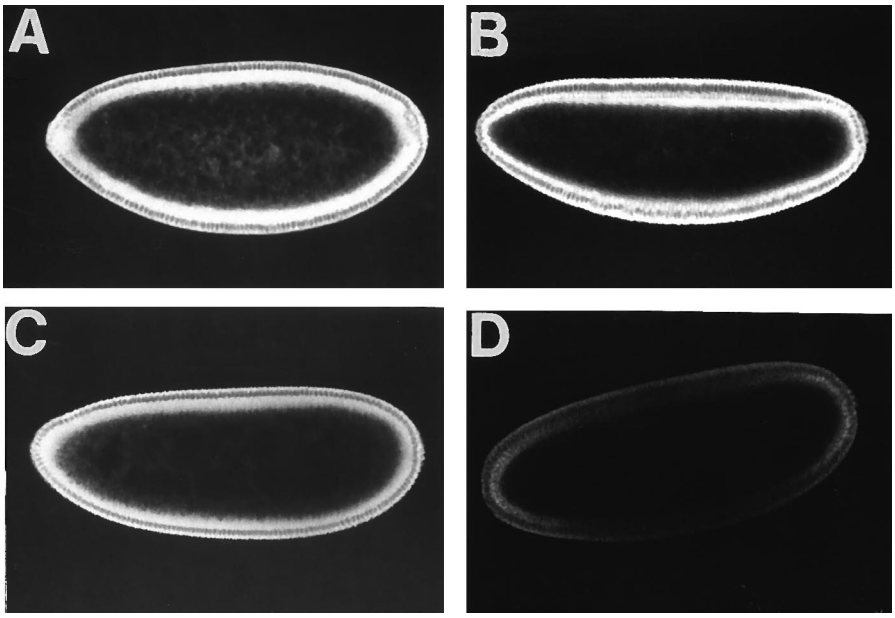

FIG. 6. (A to D) Mapping of the dimerization and the Cactus-binding sites. N-terminal deletions of the RHR of Dorsal (Fig. 1F). Embryos expressing the fusion proteins 84-342-LacZ (A), 138-342-LacZ (B), and 222-342-LacZ (C). (D) Control embryo expressing no fusion protein. All embryos were stained with anti-LacZ antibody. Cytoplasmic staining is observed.

not show strong effects. However, a dramatic effect was observed with the 222-342-LacZ construct. Compared with the control, in which no transgene is expressed and over 80% of the embryos hatch, the presence of one copy of the 222-342-LacZ construct lowered the number of embryos hatched to 2% (Table 1). Unhatched embryos showed a dorsalized phenotype. This dominant negative effect suggests that the region contained within aa 222 to 342 is important for dimerization.

When the dose of maternal *cact* was lowered for each of these deletion-fusion constructs ($cact^{A2}$/+ females with one copy of the transgene), the percentage of hatched embryos from these females was substantially reduced when compared with that of the control. Seventy percent of the embryos from the control $cact^{A2}$/+ females (no transgene) hatched. In the presence of one copy of each of the three transgenes (84-342, 138-342, and 222-342) only 22, 34, or 11% of the embryos hatched (Table 1). Unhatched embryos were weakly ventralized.

To confirm the interaction of the fusion proteins with endogenous Cactus, coimmunoprecipitation experiments were done. Anti-LacZ antibody-coupled beads were incubated with embryonic extracts carrying each of these fusion proteins. Western blots were probed with anti-Cactus antibody to detect endogenous Cactus. As shown in Fig. 7, lanes 4 to 6, all three fusion proteins were found in a complex with endogenous Cactus, demonstrating that, in vivo, the region between aa 222 and 334 is sufficient for Cactus binding. Lower levels of endogenous Cactus were detected in immunoprecipitates of 84-342-LacZ extracts in multiple experiments (Fig. 7, compare lane 4 with lanes 1 to 3, 5, and 6). The reason for this result is not clear; it is possible that the three-dimensional structure of this deletion-fusion protein is altered so that its in vivo interaction with Cactus is not as efficient.

The Dorsal NLS is not essential for Cactus binding. Studies of vertebrate Rel proteins p50, p65, and c-Rel with IκB expressed in tissue culture cells have clearly shown that IκB directly contacts the NLS of these vertebrate Rel proteins (1). The various constructs with and without the Dorsal NLS described above provided an opportunity to determine whether Dorsal-Cactus interaction also requires the presence of the Dorsal NLS. Previous experiments have shown that Cactus is stabilized by Dorsal and that Cactus protein is not detectable in ovaries of *dl* null females (56). We tested extracts from wild-type Dorsal and DorsalΔNLS dl^{-}/dl^{-} ovaries and embryos to examine if, in these preparations, Cactus bands were detectable. As mentioned previously, DorsalΔNLS is expressed at a much lower level than wild-type Dorsal protein (Fig. 4C).

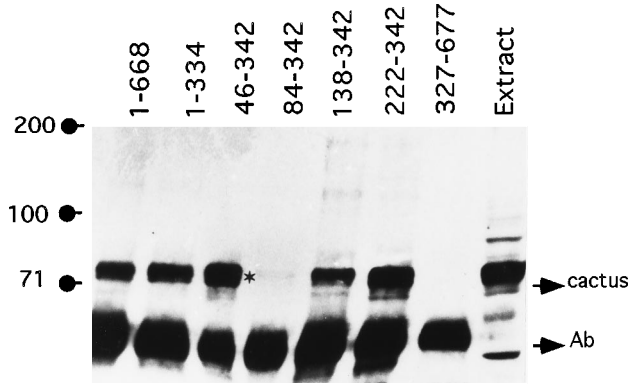

FIG. 7. In vivo Cactus binding is mediated by the region immediately N terminal to the Dorsal NLS. A Western blot with immunoprecipitates of embryonic extracts with RH-LacZ and deletions of this fusion protein is shown. The 327 C-term-LacZ construct was also used. Immunoprecipitations were done with anti-LacZ antibody. The blot was probed with anti-Cactus antibody. Lanes are labeled to indicate constructs that include the indicated amino acids (1-668 is the full-length Dorsal-LacZ fusion as shown in Fig. 1C; 1-334 is as shown in Fig. 1E; 46-342 is RH-LacZ as shown in Fig. 1C; 84-342, 138-342, and 222-342 are as depicted in Fig. 1F, and 327-677 is as shown in Fig. 1B). Molecular markers are given at the left. All the fusion proteins except 327-677-LacZ are able to immunoprecipitate endogenous Cactus. The fusion containing aa 84 to 342 interacts with Cactus inefficiently (asterisk). Ab, antibody.

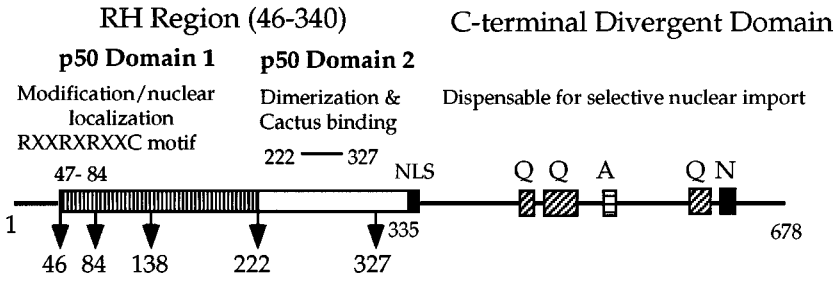

FIG. 8. Schematic of the primary structure of Dorsal, summarizing its functional organization on the basis of transgenic studies of *Drosophila melanogaster*, and analysis of the crystal structure of the mammalian p50 RHR. The RHR is composed of two distinct subdomains as shown. Regions required for both Cactus binding and Dorsal dimerization are contained within aa 222 to 327 of Dorsal, which overlaps with C-terminal domain 2 of p50 (13, 34). The nuclear localization signal of Dorsal maps to aa 335 to 340. Unlike p50, p65, and c-Rel proteins, this NLS is not essential for interaction with Cactus. Structural motifs in the C-terminal half are also indicated. This region is not essential for selective nuclear import of Dorsal.

Therefore, in this maternal background (DorsalΔNLS; dl^{-}/dl^{-}), the level of Cactus that can interact with DorsalΔNLS is expected to be substantially lower than wild-type Cactus levels. As evident in Fig. 4D, the Cactus band is clearly detected in both the ovarian and embryonic extracts. The amount of Cactus stabilized by DorsalΔNLS is, however, much lower than that found in wild-type extracts, and on the basis of embryo staining, it is roughly proportional to the amount of Dorsal expressed from the transgene. In the same experiment, Cactus is not detected in extracts made from control dl^{-} ovaries (Fig. 3D). This observation implies that Cactus can bind Dorsal even in the absence of the NLS.

This conclusion is further strengthened by the following results with fusion constructs. In blastoderm embryos, the distribution of the N-termΔNLS-LacZ fusion protein is restricted to the cytoplasm, whereas 327 C-term-LacZ is mainly nuclear. In an immunoprecipitation experiment, the interaction of these two proteins (one contains the Cactus-binding site but has no NLS, and the other contains the NLS but does not contain the Cactus-binding site) with endogenous Cactus was studied. Anti-LacZ immunoprecipitates from embryonic extracts of N-termΔNLS-LacZ contained Cactus, while those obtained from 327 C-term-LacZ extracts did not (Fig. 7, compare lanes 2 and 7). These results again show that Cactus does not require the Dorsal NLS for interaction. Furthermore, the LacZ moiety in the 327 C-term-LacZ fusion protein does not provide a fortuitous binding site for Cactus. Instead, the region within aa 222 to 334 contains the critical determinants for Dorsal-Cactus interaction.

Genetic results with N-termΔNLS-LacZ and 327 C-term-LacZ are consistent with the above-described biochemical observations. Lowering the dose of *cact* had no effect on the phenotype of 327 C-term-LacZ embryos; but when the N-termΔNLS-LacZ transgene was crossed into the $cact^{A2}$ heterozygous background, the phenotype of the N-termΔNLS-LacZ embryos became more severe (Table 1). In contrast to all other constructs, the embryos derived from N-termΔNLS-LacZ females were ventralized even in the wild-type background (Table 1 and Fig. 5C). This result may be due to the fact that the cytoplasmic N-termΔNLS-LacZ protein competes with endogenous Dorsal for Cactus binding. Alternatively, this phenotype may result from high levels of N-termΔNLS-LacZ protein. When the N-termΔNLS-LacZ embryos were stained with anti-Dorsal antibody, we found that the gradient of endogenous nuclear Dorsal was extended dorsally (data not shown).

A signal reception domain? A role for phosphorylation in the dorsal-ventral pathway was suggested by the cloning of *pelle*. This dorsal group gene encodes a protein kinase, and its kinase function is essential for the establishment of dorsal-ventral polarity (46). Furthermore, Dorsal has been shown to be multiply phosphorylated and this phosphorylation profile is governed by the Toll-dependent ventral signal (15, 56). Consistent with these observations is the finding that c-AMP-dependent protein kinase activates Dorsal nuclear localization in tissue-culture cells (36). These data suggest that Dorsal may be modified by the ventral signal. Since the distribution of nuclear RH-LacZ fusion is graded, the target amino acids that are modified as a result of the ventral signal are expected to reside within the RHR. Therefore, to investigate if the N-terminal deletions can react to a modified or enhanced ventral signal, we studied the distribution of the fusion proteins (84-342, 138-342, and 222-342) in two different gain-of-function dorsal group backgrounds. $easter^{5,13}$ is a dominant, lateralizing allele that causes intermediate levels of wild-type Dorsal to be translocated to the nuclei along the entire dorsal-ventral axis (6, 51). $Toll^{9Q}$ is a dominant allele and ventralizes embryos by causing high levels of Dorsal in all nuclei (45). In both of these mutant backgrounds, all three deletion fusion proteins remained in the cytoplasm (data not shown), indicating that they cannot respond to higher levels of the signal.

Not only were these proteins refractory to the increased intensity or altered distribution of the ventral signal, they did not shift their location even when Cactus function was drastically reduced. The transgenes were crossed into the trans-heterozygous *cact* background. Anti-LacZ antibody staining of embryos from mutant *cact* females ($cact^{S1}/cact^{A2}$ or $cact^{HE}/cact^{A2}$) showed that all three N-terminally truncated RH-LacZ fusion proteins remained in the cytoplasm and that their localization was unchanged from that observed in wild-type embryos (data not shown). In this background the endogenous Dorsal protein is mostly nuclear along the entire dorsal-ventral axis (40, 51). These data demonstrate that the deletion-fusion proteins are no longer regulated by the ventral signal and are unable to undergo nuclear translocation even when they are free from Cactus association. This inability of the N-terminal fusion proteins to translocate into the nucleus, even in the presence of the NLS, strongly suggests that the N-terminal 38 aa of the RHR harbor information that is critical for the regulated nuclear import of Dorsal.

DISCUSSION

Functional organization of the Dorsal protein. Our studies provide a general picture of the functional organization of the Dorsal RHR; they have broadly defined regions that are es-

sential for Cactus interaction, homodimerization and nuclear localization (Fig. 8). What we learn from these experiments is similar to information from experiments on vertebrate Rel proteins. However, two findings are novel: we find that the NLS is not essential for Cactus interaction and that the N-terminal stretch of about 40 aa of the RHR, which contains the highly conserved DNA-binding motif, is essential for the regulated nuclear import of Dorsal. Our findings do not implicate the C-terminal divergent half of Dorsal in its regulated nuclear import.

Findings presented in this study underline the importance of structural integrity for full function. In a deletion analysis similar to the one presented here, the functional importance of the integrity of the RHR in attaining the full transformation potential of v-Rel has been demonstrated in chicken embryo fibroblasts (33). Because the RHR functions in DNA binding, inhibitor binding, and dimerization and because several amino acid residues along the RHR are required for carrying them out (13, 32, 34, 47), an N-terminal or C-terminal deletion of the RHR results in a drastic loss of biological activity. The structural basis for this has become clearer with the solution of the crystal structure of mammalian p50 RHR.

The three-dimensional structural and functional units of the RHR. The three-dimensional crystal structure of the RHRs of the mouse and human p50 subunits of NF-κB bound to DNA have been solved recently (13, 34). This region consists of two β barrel domains, both of which contact the major groove of DNA. N-terminal specificity domain 1 (aa 40 to 237 of p50 and aa 47 to 216 of Dorsal) contains a recognition loop that interacts with DNA bases. C-terminal domain 2 (aa 248 to 350 of p50 and aa 225 to 327 of Dorsal), in addition to contacting DNA, has sites for dimerization and is predicted to interact with IκB. Significantly, our functional results fit well with the overall crystal structure of p50. Our fusion construct containing 222-342-LacZ neatly removes N-terminal domain 1 and essentially comprises C-terminal domain 2. Our genetic and biochemical assays establish that this domain 2 retains the ability to bind to Cactus and also contains the sequences essential for dimerization but surprisingly does not translocate to the nucleus.

The dimerization and Cactus-binding regions. Our in vivo studies have defined the overlapping dimerization and Cactus-binding regions as stretches of amino acids in the immediate vicinity of the Dorsal NLS (aa 222 to 326). In this analysis, it was not possible to distinguish between those amino acids that specifically mediate dimerization and those that mediate Cactus binding. The crystal structure of the p50 dimer shows that the dimer interface is contributed from 12 amino acids, all of which are contained within domain 2 (the 222-342-LacZ fusion). Six of these amino acids are completely conserved between p50 and Dorsal, and it is very likely that these amino acids also contribute to the Dorsal dimer interface.

In the absence of the inhibitor crystal structure, the molecular details of the Rel-inhibitor interaction are not known; however, it is proposed that the amino acids mediating this interaction are also located in domain 2 (13, 34). Located in domain 2 of Dorsal, aa 230 to 236 were found to be essential for Cactus interaction in in vitro assays and in embryos (54). Interestingly, two amino acids within this region (Cys-233 and Ser-234) were critical for Dorsal-Cactus interaction in a two-hybrid-interaction system in *Saccharomyces cerevisiae* (29). Whether these same amino acids are also essential for dimerization has not been investigated.

The association of Rel proteins (including Dorsal) with IκB proteins (including Cactus) has previously been tested in vitro, and in most instances, the NLS was found to be essential for IκB binding (1, 11, 22, 54). We were therefore surprised to find that in our experiments, the NLS was not essential for Cactus binding. Our conclusion was based on the following observations. (i) The 327 C-term-LacZ fusion protein is uniformly nuclear in blastoderm stage embryos. (ii) The N-terminal fusion, containing the entire RHR but lacking the NLS, genetically and biochemically interacts with Cactus (Fig. 7 and Table 1). (iii) Low levels of full-length DorsalΔNLS protein also clearly interact with and stabilize low levels of Cactus (Fig. 3). The difference between our in vivo results and the in vitro results may be explained by the differences in experimental systems. However, in an in vitro assay, Wulczyn et al. (59) have shown that the product of the IκB-like proto-oncogene *bcl-3*, which preferentially interacts with p50, interacts without requiring the presence of its NLS. While it is possible that Cactus/bcl-3 and IκB/MAD-3 proteins are sufficiently different in their specificities of interaction with the target Rel sequences, it is clear from the Dorsal-Cactus interaction studies that results from in vitro assays do not necessarily reflect the nature of interactions in vivo.

The direct association of IκB proteins is thought to mask the NLSs of Rel proteins. This observation provides an explanation for the inhibitory function of the IκB proteins, because the Rel-inhibitor interaction would prevent the NLS receptors from recognizing and transporting Rel proteins into the nucleus (1, 11, 22). Our data are consistent with this general model. Exactly how Cactus conceals the Dorsal NLS and how the general transport machinery controls Dorsal nuclear import in the absence of Cactus remains to be explored.

Requirements for regulated nuclear import of Dorsal. A short stretch of basic amino acids specifies the nuclear import of proteins (26). The 7-aa NLS of simian virus 40 large T antigen has been regarded as the prototype. A more complex NLS with the consensus bipartite motif has also been identified in some proteins. In this case, instead of a single cluster of basic residues, two separate clusters are required for efficient nuclear import (10). Deletion of one stretch of basic amino acids from Dorsal in a number of constructs clearly demonstrates that, like vertebrate Rel proteins (1, 3, 11, 17), this stretch of basic amino acids, conserved in both its location and composition, serves as the Dorsal NLS.

In addition to the NLS, which is a signal for unregulated import of Dorsal, the first 38 amino acids of the RHR (aa 46 to 84) appear to be required for regulated nuclear import of Dorsal. These N-terminal 38 amino acids are not an absolute requirement for nuclear localization since the 327 C-term-LacZ fusion protein is constitutively targeted to the nucleus. However, it is required for nuclear localization of fusion proteins containing the Cactus binding-dimerization region: deletion of these 38 amino acids interfered with nuclear targeting but not with the ability to bind Cactus. These results can be explained in different ways. The higher-order structure of the N-terminally deleted proteins is disrupted so that they cannot be imported to the nucleus, even though their interaction with Cactus is not affected by these deletions. Alternatively, Dorsal protein requires a signal for nuclear targeting and the target for this signal is absent in the N-terminal deletion constructs. This signal can be either Toll dependent or Toll independent; in the latter case, the signal would act to promote nuclear localization of free Dorsal after its dissociation from Cactus.

Amino acids between positions 46 and 84 include a fully conserved RXXRXRXXC DNA-binding motif. The crystal structure displays the conserved RXXRXRXXC motif of p50 in domain 1 as a loop contacting DNA (13, 34). As would be expected from the crystal structure, N-terminal deletions in Dorsal most likely have drastic effects on the higher-order

structure of domain 1 but do not seem to affect domain 2. Given that the conserved RXXRXRXXC loop contacting DNA is absent in the N-terminal deletions of Dorsal, one can imagine that the 84-342-LacZ fusion protein is in fact translocated to the nucleus but is unable to bind DNA and is therefore not retained there. This interpretation seems unlikely since the hypomorphic allele dl^2 maps very close to the DNA-binding RXXRXRXXC region, whereas the dominant negative mutation dl^{D7} maps within it; however, the Dorsal^2 and Dorsal^{D7} mutant proteins show normal ventral-to-dorsal graded nuclear distribution (24). These results imply that the initial stretch of 40 aa in domain 1 of Dorsal contains information in its primary or higher-order structure critical for nuclear localization of Dorsal. The mechanism by which this may occur remains to be elucidated.

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