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Specificity of Rel-Inhibitor Interactions in *Drosophila* Embryos

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The Rel family of transcription factors participate in a diverse array of processes, including acute responses to injury and infection, lymphocyte differentiation, and embryonic patterning. These proteins show homology in an extended region spanning about 300 amino acids (the Rel homology domain [RHD]). The RHD mediates both DNA binding and interactions with a family of inhibitor proteins, including IκBα and cactus. Previous studies have shown that an N-terminal region of the RHD (containing the sequence motif RXXRXRXXC) is important for DNA binding, while the C-terminal nuclear localization sequence is important for inhibitor interactions. Here we present a structure-function analysis of the *Drosophila* dorsal RHD. These studies identify another sequence within the RHD (region I) that is essential for inhibitor interactions. There is a tight correlation between the conservation of region I sequences and the specificity of Rel-inhibitor interactions in both flies and mammals. Point mutations in the region I sequence can uncouple DNA binding and inhibitor interactions in vitro. The phenotypes associated with the expression of a modified dorsal protein in transgenic *Drosophila* embryos suggest a similar uncoupling in vivo. Recent crystallographic studies suggest that the region I sequence and the nuclear localization sequence might form a composite surface which interacts with inhibitor proteins.

Rel-containing transcription factors have been implicated in a variety of developmental processes, including lymphocyte differentiation in the mammalian immune system (for a review, see references 1 and 26) and dorsoventral patterning in *Drosophila* embryos (12, 17, 43). Rel proteins also mediate acute responses to infection and injury in both insects and mammals (6, 15, 19, 30). Mounting evidence suggests that the regulation of Rel activity depends on a highly conserved signal transduction pathway, which includes conserved receptors at the cell surface and related target enhancers in the nucleus (10, 43). Rel proteins are ideally suited for responding to rapid changes in environmental stimuli since they are regulated at the level of nuclear transport (for a review, see references 1, 26, and 38). After the activation of related cytokine receptors such as the mammalian interleukin-1 receptor and the *Drosophila* Toll receptor (14, 27), an intracellular signaling cascade results in the translocation of Rel-containing proteins from the cytoplasm to the nucleus (1, 26, 38).

In *Drosophila* embryos, the dorsal (dl) morphogen is released from the cytoplasmic inhibitor cactus (cact) (7, 23), while NF-κB is released from IκB in mammalian liver and lymphoid tissues (for a review, see references 2 and 38). These inhibitors exhibit slightly different binding activities and show preferences for particular Rel-containing subunits. For example, IκBα preferentially interacts with c-Rel and the p65 subunit of NF-κB, while Bcl-3 shows stronger binding to the p50 subunit of NF-κB (for a review, see reference 2). Mammalian tissue culture assays suggest that the activation of the cytokine signal transduction pathway results in the phosphorylation and degradation of IκB, thereby releasing NF-κB for nuclear transport (3, 40). In *Drosophila melanogaster*, the Toll pathway is mediated, at least in part, by an intracellular kinase, pelle (37). Dissociation of dl from the cact inhibitor is correlated with the hyperphosphorylation of dl; it is currently unknown whether cact is also phosphorylated in a manner similar to that observed in mammalian systems (9, 44).

The cact and IκB inhibitors are related by virtue of a series of ankyrin repeats, which are essential for making direct protein-protein contacts with NF-κB and dl, respectively (2, 7, 23). There is considerable interest in the exact nature of Rel-inhibitor interactions since these interactions are key regulated processes in the interleukin-1 receptor–Toll signaling cascade. A combination of in vitro binding analyses and transient-transfection assays suggests that the primary point of contact between NF-κB (either the p50 or p65 subunit) and IκB corresponds to a C-terminal region of the Rel homology domain which contains the nuclear localization signal (NLS) (28, 45). These studies suggest that NF-κB–IκB interactions mask the NF-κB NLS so that it is unable to enter the nucleus. Upon modification of IκB, this interaction is disrupted so that the NLS is exposed and can mediate nuclear transport. Preliminary studies suggest a similar interaction between cact and the NLS region of the dl Rel homology domain (7, 23). Here we present a structure-function analysis aimed at identifying additional dl sequences that interact with cact.

Regulated nuclear transport generates a dl gradient that encompasses the entire ventral half of the early embryo (31, 34, 39). It has been proposed that this gradient is established through the differential activation of the Toll receptor pathway (29). The best candidate for a Toll receptor is spätzle (29), a large cytokine-like protein that is probably proteolytically processed in ventral regions of early embryos by one or more serine proteases, including easter (5). Peak levels of processed spätzle in ventral regions result in the full activation of the Toll pathway, causing all of the dl in these regions to enter nuclei. In contrast, lower levels of processed spätzle might lead to less robust activation of the Toll pathway in ventrolateral and lateral regions, thereby resulting in less nuclear protein. An implication of these genetic analyses is that the dl-cact interaction is subject to quantitative modulation in response to the extent of Toll activation.

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TABLE 1. Oligonucleotides used to create sequential deletions in the Rel homology domain

Amino acids deleted	Oligonucleotide sequence
47–66	AATGTGCGAAAGAAGGAGGGACGCTCG
67–86	TCGCTACGAGTGCCCGACAATCGAAAT
87–106	AGAAACAAGACCTATAAGGATACGCCAT
107–127	TCTCCTGCGTCACAGTCTGTACACTGG
128–139	GGCTGCAAGAAGGGCGCGGTGTTCAGT
140–150	GAGACAATGCGAAAAAAGAAGGACATT
151–167	ACTTGGGTATCCAGTGTGTCGATCCGTTTAAGACTGGCTT
168–188	GAGATCCGTGTGCGATTGTGCTTTCAA
189–198	ATCTGAATTCGGTGGAGCAGAAGGGTC
199–217	GTATTCATGGAGAGCGATAAAGAAGGCC
218–230	GGAGCCCATCTTCTGTAGCTGCTCGGC
231–245	TCATCTGCCGGCTGCTCTGCGAGAAGG
246–261	CCCAGATCATCCTGGAGAAGAATGGCC
262–282	TGCGATTCTTTGAGCAGACTGCCATTA
283–303	CGGATGTCCACAAGGTTTTTATTCAACTGCG
304–321	CAGAGCCCGCCAAGTTCGAGTACGTGC
322–341	GCGAGGCCCTGCCCGGCGGTGATCCCA

We present evidence that two distinct regions of the Rel domain are important for specific interactions with cact. First, as suggested in previous studies, the NLS plays a crucial role in Rel-inhibitor interactions (7, 23, 28, 45). dl binds cact with higher affinity than does Dif, and the conversion of the Dif NLS into the corresponding dl sequence increases the stability of Dif-cact complexes. A second region (designated region I), not identified in earlier studies, is also important for cact binding. Region I includes amino acid (aa) residues 218 to 245 and is located over 100 aa residues from the NLS. Region I mutations abolish cact binding. Moreover, the mammalian p65 protein binds only weakly to cact and contains a divergent region I sequence. The conversion of the dl region I sequence into the corresponding p65 sequence reduces cact binding in vitro and causes constitutive nuclear expression in transgenic embryos. Region I sequences are not required for DNA binding, and consequently, the mutant protein causes a dominant, ventralizing phenotype, due in part to the constitutive repression of the dl target gene, *zerknüllt* (*zen*) (20, 22, 24, 33), in both dorsal and ventral regions. There is a tight correlation between sequence homology in region I and the specificity of inhibitor interactions. The mammalian c-Rel and p65 proteins are closely related in region I and specifically interact with IκBα (for a review, see reference 2). In contrast, p50 contains a divergent region I sequence and preferentially interacts with Bcl-3. Recent crystallographic studies suggest that region I and NLS sequences may form a composite surface which interacts with inhibitor proteins (8).

MATERIALS AND METHODS

Plasmid constructions. Small deletions were introduced into the dl Rel homology domain by oligonucleotide-directed mutagenesis, as suggested by the manufacturer (Bio-Rad). Single-stranded DNA templates were prepared from *Escherichia coli* CJ236 cells after transformation with a pBluescript KS$^-$ recombinant plasmid containing a full-length dl cDNA cloned into a unique *Eco*RI site. The oligonucleotides shown in Table 1 were used to create sequential deletions.

Point mutations in region I (aa 231 to 237) and region II (the NLS) of the dl- and Dif-coding sequences were created with mutagenic oligonucleotides as follows: dlmC1, GTCATCTGCCGGGTTAACCGCAACTCGGGCTCCGTTTTCGGC; dlmNLS, GCGCACTTGAGCCAGAATGATCAGGAGACTGGCGG; dlmNLSp65, GATCCAGCGCACCTCGAGGAGAAACGTAAGAGGACTGGCGGTG; DifΔ(RLCSC), CTGACCATCACCGCGGCCACTGCG; DifmNLSdl, CACAATCGCCGGAAGAGGCAAAAAACAGTT; and DifmNLS, ACCAAACACAATAGCCAGAATGATCAAGAAACAGTTGAAAGC.

Preparation of glutathione *S*-transferase (GST) fusion proteins. Different dl-coding sequences were excised from a pBluescript recombinant plasmid containing a full-length dl cDNA by PCR (35). The following PCR primers were used to isolate dl678 (its full-length coding sequence), as well as the dl341, dl47–341, and dl47–244 truncated sequences: primer 1, CTCTCTAGAACA TATGTTTCCGAACCAGAACAAATGGA; primer 47, GGTCTAGAGCCC TACGTAAAGATCACCGAACAA; primer 244, CTCAAGCTTAGATGATC TGGGTGTTGCCGAAAAC; primer 341, CTCAAGCTTAAGTCTTCTGAC GTTTCCGCCTCAA; and primer 678, CTCAAGCTTTTACGTGGATATG GACAGGTTCGA.

The amplified fragments were cut with *Xba*I and *Hin*dIII and then inserted into the unique *Xba*I and *Hin*dIII sites of the pGEX-KG expression vector (13). The truncated dl403-coding sequence is contained on an *Nde*I-*Sac*I fragment; this was inserted into a modified pGEX vector containing a *Sac*I linker at a blunted *Hin*dIII site. The deletion series of GST-dl fusion proteins were prepared by inserting modified *Nde*I-*Sac*I dl DNAs into this vector. GST and GST-dl fusion proteins were prepared exactly as described in reference 18.

DNA binding assays. Aliquots containing 200 ng of a given GST-dl fusion protein were incubated with 100,000 cpm of a ^{32}P-labeled *zen* oligoB DNA sequence (16) in the presence of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.8)–50 mM NaCl–1 mg of bovine serum albumin (BSA) per ml–2.5 mM $MgCl_2$–10% glycerol–5.6 mM β-mercaptoethanol–10 mM EDTA–80 μg of poly(dI-dC) per ml at room temperature for 10 min. The reaction mixture was fractionated on a 4% polyacrylamide gel as described previously (19).

Protein binding assays. [^{35}S]methionine-labeled dl proteins were prepared with rabbit reticulocyte lysates, exactly as described by the manufacturer (Amersham). Aliquots containing 20,000 cpm of labeled protein were incubated at 30°C with 200 ng of either GST or a full-length GST-cact protein for 30 min in 20 mM HEPES-KOH (pH 7.5)–150 mM NaCl–0.1% Nonidet P-40–0.1% BSA–10% glycerol. Glutathione-agarose beads (Sigma) were subsequently added to the reaction mixture, which was then incubated for 1 h at 4°C. The beads were washed four times with the above-described buffer, eluted in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer, and fractionated on either a 10 or 7.5% polyacrylamide-SDS gel. Radiolabeled proteins were visualized by fluorography.

Transgenic flies. An *Ssp*I-*Spe*I DNA fragment containing either the full-length dl-coding sequence or the dlmC1 (region I mutant) sequence was ligated with a 1-kb *Kpn*I-*Hin*dIII genomic DNA fragment containing the *Drosophila* hsp83 promoter region (11). The recombinant hsp83-dl fragment was cloned into the unique *Kpn*I and *Spe*I sites of the pBluescript KSII vector and then excised by digestion with *Kpn*I and *Xba*I. This fragment was cloned into the unique *Kpn*I and *Xba*I sites of the pCaSpeRAUGbetagal P transformation vector (42). The resulting hsp83-dl P transposons were used to transform yw^{67}-yw^{67} embryos. Transgenic embryos were hybridized with a digoxigenin-labeled *zen* antisense RNA probe and stained as described previously (21, 41).

RESULTS

Delineation of the DNA binding domain. Sequences essential for DNA binding were defined by analyzing a series of small internal deletions within the dl Rel homology domain. In all cases, mutant forms of the dl-coding region were inserted into the pGEX-KG expression vector, and GST fusion proteins were tested in gel shift assays with a high-affinity dl binding site from the *zen* promoter (the oligoB sequence [16]).

A truncated GST-dl protein containing the N-terminal 403 aa residues binds the oligoB sequence about as efficiently as a

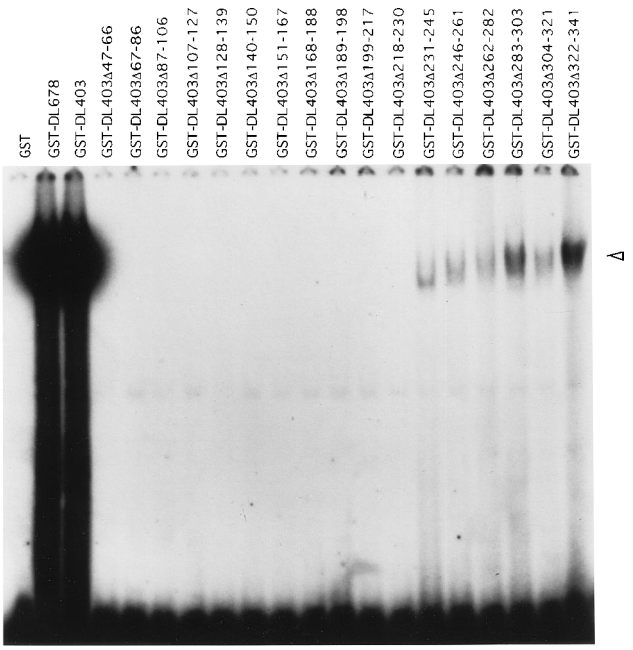

FIG. 1. DNA binding activities of mutant dl proteins. Gel mobility shift assays were done with various GST-dl fusion proteins containing a series of sequential deletions spanning the entire Rel homology domain. Comparable amounts of each protein were incubated with a ^{32}P-labeled DNA fragment containing a high-affinity dl binding site (oligoB [16]). The GST protein (prepared from the pGEX expression vector without the dl-coding sequence) lacks DNA binding activity (lane 1). The full-length dl-GST fusion protein (GST-DL678) and a truncated protein containing the first 403 aa residues (GST-DL403) generate similar amounts of a protein-DNA complex (indicated by the arrowhead at the right of the autoradiogram). Truncated dl403 proteins containing small deletions in the region spanning aa 47 to 230 lack detectable DNA binding activities. However, mutant proteins containing deletions between aa 231 and 341 (the C-terminal third of the dl Rel homology domain) retain some activity.

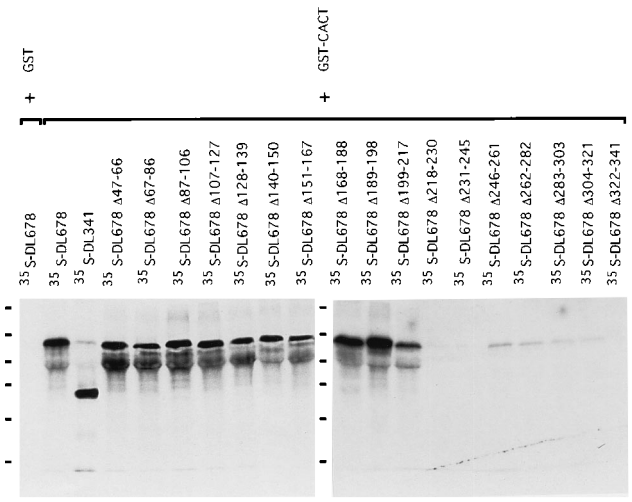

FIG. 2. dl-cact interactions. Mutant dl RNAs containing various internal deletions in the Rel homology domain were translated with a rabbit reticulocyte extract, and the resulting proteins were labeled with [^{35}S]methionine. Each labeled protein was incubated with a full-length GST-cact fusion protein, purified on glutathione-agarose beads, and fractionated on a polyacrylamide gel. Lane 1 shows results of a control experiment, whereby a full-length ^{35}S-labeled dl protein was incubated with nonchimeric bacterial GST lacking cact amino acids (there is no detectable background binding of the dl protein). In contrast, efficient binding was observed for the same protein after incubation with a chimeric GST-cact protein (lane 2). The numbers above each lane indicate the amino acid residues that were deleted within the Rel homology domain. Only three of the deletions (Δ218–230, Δ231–245, and Δ322–341, spanning the dl NLS) abolish binding. The lines to the left of the two autoradiograms are molecular mass markers (top to bottom, 200, 97, 68, 43, 29, 18.4, and 14.3 kDa). Most of the mutant dl proteins migrate with an apparent molecular mass of 73 kDa. The heavy staining below each dl band probably corresponds to breakdown products which migrate more rapidly. The wild-type dl protein migrates with an apparent molecular mass of 75 kDa; the truncated dl341 protein is 38 kDa.

full-length fusion protein (Fig. 1, lanes 1 to 3). Small sequential deletions, averaging about 15 to 20 aa, were created in the context of the GST-dl403 protein. All of the deletions from aa residues 47 to 230 abolish DNA binding (Fig. 1). In contrast, deletions spanning the region from aa residues 231 to 341 reduce but do not eliminate binding. The last deletion in the series removes the NLS (which spans aa residues 335 to 340) but does not abolish DNA binding.

Identification of sequences essential for cact binding. Mutant dl proteins were used for cact binding assays, as follows. The same sequential Rel deletions used for the DNA binding experiments (Fig. 1) were created in the context of a full-length dl cDNA. These cDNAs were transcribed in vitro from a pBluescript plasmid DNA template, and subsequently, the RNAs were used for in vitro translation reactions with [^{35}S]methionine and rabbit reticulocyte lysates. The RNAs were all translated with about equal efficiencies (data not shown). Equivalent amounts of labeled protein extracts were incubated with a full-length GST-cact fusion protein. After incubation, protein complexes were recovered with glutathione-agarose beads, washed, and then electrophoresed in an SDS-polyacrylamide gel (Fig. 2).

A full-length dl translation product fails to bind a nonfusion GST protein lacking cact sequences (Fig. 2, lane 1). Both the full-length protein and a truncated protein lacking the entire C-terminal half (dl341) bind quite efficiently to the GST-cact fusion protein (Fig. 2, lanes 2 and 3). Similarly, sequential deletions in the region spanning the N-terminal half of the Rel homology domain, from aa residues 47 to 217, do not impair binding to the GST-cact protein. In contrast, deletions in aa residues 218 to 245 (region I) completely abolish cact binding (Fig. 2). A similar loss in binding is also observed with a C-terminal deletion in the Rel domain (aa residues 322 to 341; region II) that removes the NLS. Deletions in the sequence located between regions I and II (aa residues 246 to 321) reduce but do not abolish cact binding. Evidence that region I might be a discrete cact binding domain stems from the observation that mutant proteins lacking neighboring sequences (199 to 217 and 246 to 261) retain substantial binding activity.

Because region I was not identified as an essential component of either cact or IκB binding in previous studies (for a review, see reference 2), this region was further characterized by creating site-directed mutations. Substitutions were made in a 7-aa sequence located in the center of region I. The 7-aa sequence spanning aa residues 231 to 237 (LCSCSAT) was selected for mutagenesis since it is highly conserved in the *Drosophila* Dif sequence but is quite divergent in mammalian Rel proteins such as p65. The mammalian p65 protein binds only very weakly to the GST-cact fusion protein (Fig. 3, lane 6), while the dl and Dif proteins bind quite well (Fig. 3, lanes 2 and 7, respectively). Converting the 7-aa region I sequence to the corresponding amino acid residues in mammalian p65 (LCSC SAT to VNRNSGS) nearly abolishes the binding of an otherwise-normal dl protein (Fig. 3, lane 3; compare with lane 2). A 5-aa deletion (RLCSC) that overlaps the 7-aa region I sequence abolishes Dif-cact interactions (Fig. 3, lane 8; compare with lane 7). These results establish the importance of region I

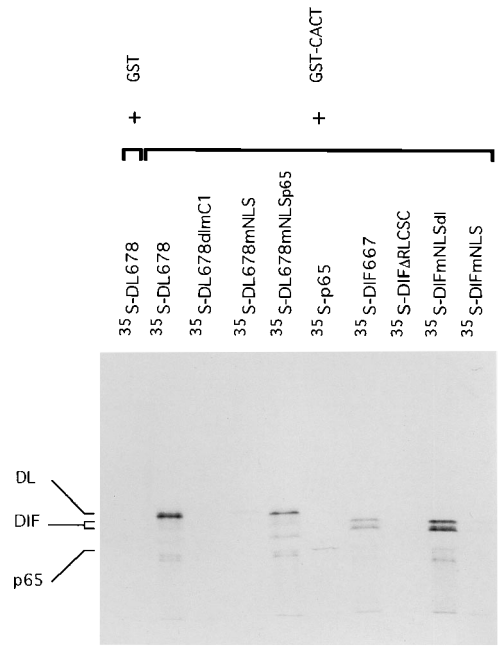

FIG. 3. Amino acid substitutions in the Rel homology domain. Binding assays were done as described in the legend to Fig. 2. The DL678dlmC1 mutant (lane 3) contains six amino acid substitutions in region I (aa 231 to 237) that convert the dl sequence into the corresponding mammalian p65 sequence. These substitutions were created in the context of an otherwise-normal full-length dl protein (678 aa). This results in a complete loss of binding to the full-length GST-cact fusion protein (compare lanes 2 and 3). The mNLS mutant contains substitutions in the dl NLS (aa 335 to 340) so that the sequence RRKRQK was replaced with SQNDQE. There is a severe reduction in binding to the GST-cact protein (lane 4; compare with lane 2). The mNLSp65 mutant contains substitutions in the NLS that convert it into the corresponding NLS of the p65 protein (EEKRKR). This results in a substantial reduction in binding to the GST-cact protein (lane 5), which is consistent with the observation that p65 binds poorly to cact (lane 6). A full-length ^{35}S-labeled Dif protein (DIF667) binds quite well to the GST-cact protein (lane 7). However, a 5-aa deletion in region I (removing the amino acid sequence RLCSC) abolishes this binding (lane 8). Binding is also lost when the Dif NLS (RRVAQK) is changed to the sequence SQNDQE (lane 10). However, the conversion of the Dif NLS to the corresponding dl NLS (DIFmNLSdl) enhances the Dif-cact interaction (lane 9).

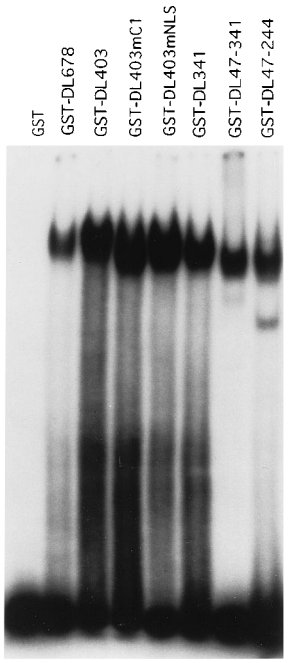

FIG. 4. dl mutations that uncouple DNA and cact binding activities. Gel shift assays were done as described in the legend to Fig. 1. GST-dl fusion proteins containing the N-terminal two-thirds of the Rel homology domain bind DNA [the *zen* oligoB sequence] quite efficiently, including the full-length fusion protein (DL678) as well as various truncated proteins (DL403 and DL341). In addition, fusion proteins containing just the Rel homology domain (DL47–341) or the N-terminal region of the Rel domain (DL47–244) also retain essentially normal binding activities. A truncated DL403 fusion protein containing point mutations in region I (mC1 [Fig. 3]) retains full binding activity (lane 4; compare with lane 3). Similarly, mutations in region II, the dl NLS, also have no discernible effect on DNA binding (lane 5). The control experiment, whose results are shown in lane 1, involved incubating the bacterial GST protein, which lacks dl sequences, with the ^{32}P-labeled oligoB DNA sequence.

in dl-cact binding. The divergence of this sequence in mammalian p65 might account, at least in part, for its poor binding to cact and its preference for other inhibitors.

Region II, which contains the NLS at the C terminus of the Rel domain, also plays an important role in cact binding. It was previously shown that cact can block dl DNA binding by interacting with sequences that map in or near the dl NLS (2, 23). In order to determine whether the NLS might be directly involved in cact binding, point mutations were created within this 6-aa sequence (RRKRQK). The NLS spans aa residues 335 to 340, and when the NLS is converted to the sequence SQNDQE, an otherwise-normal dl protein completely fails to bind cact (Fig. 3, lane 4; compare with lane 2). The conversion of the dl NLS into the corresponding p65 NLS (EEKRKR) causes a reduction in cact binding (Fig. 3, lane 5). Further evidence that the NLS might make direct contact with cact is provided by the observation that converting the Dif NLS (RRVAQK) into the corresponding dl NLS enhances Dif-cact interactions (Fig. 3, lane 9; compare with lane 7). In summary, these results suggest that cact contacts at least two regions of the Rel homology domain, and both are important for the detailed specificity of Rel-inhibitor interactions.

One of the goals of this study was to identify a mutant form of the dl protein that uncouples DNA binding and dl-cact interactions. Analysis of sequential deletions suggests that the region I cact binding domain might partially overlap with sequences essential for DNA binding. The region spanning aa 47 to 230 within the Rel domain is required for DNA binding (Fig. 1), and a truncated GST-dl fusion protein containing aa 47 to 244 is able to bind the *zen* oligoB sequence (Fig. 4, lane 8). Additional tests were done to determine whether mutations in the region I and region II cact binding domains have any effect on DNA binding. As shown above (Fig. 1), a full-length GST-dl fusion protein (GST-DL678) and a truncated fusion protein (GST-DL403) bind equally well to the oligoB sequence. Mutations in region I that convert a 7-aa sequence into the corresponding p65 sequence (Fig. 3) have no effect on DNA binding (Fig. 4, lane 4; compare with lane 3). Similarly, inactivating the dl NLS by substituting 5 of its 6 aa residues has no effect on DNA binding (Fig. 4, lane 5). These results indicate that the DNA and cact binding activities of the dl Rel homology domain can be uncoupled. Point mutations in either region I or region II abolish cact binding (Fig. 3) but have no effect on DNA binding (Fig. 4).

It is highly unlikely that mutations in region I disrupt dl dimerization. Previous studies indicate that Rel-containing proteins bind as obligate dimers (for examples, see reference 8). In the unlikely event that the mutant protein can bind as a monomer, we would expect to observe an altered mobility for the protein-DNA complex. This is not seen (Fig. 4, lanes 3 and

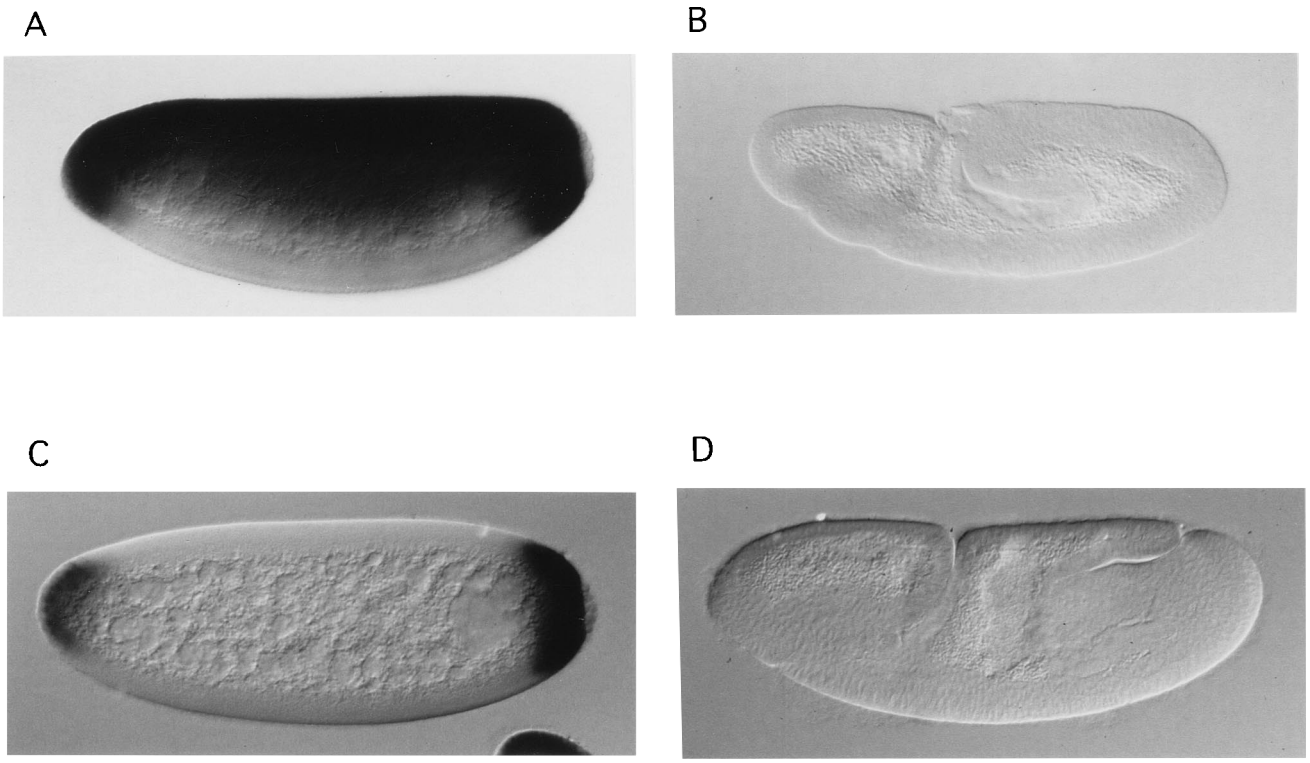

FIG. 5. Mutations in region I cause dominant, constitutive repression of the *zen* target gene. A full-length dl-coding sequence containing the mC1 mutation in region I (Fig. 3 and 4) was placed under the control of the maternal hsp83 promoter and expressed in transgenic embryos. One copy of this mutant hsp83-dlmC1 fusion gene causes dominant lethality in embryos derived from otherwise-wild-type females carrying two normal copies of the endogenous dl gene. This dominant female sterility was observed in 2 of 46 transformed lines, although a comparable number of transformants carrying the wild-type dl-coding sequence never exhibited this phenotype (data not shown). Embryos were hybridized with a digoxigenin-labeled anti-*zen* antisense RNA probe, and the staining patterns were visualized by immunohistochemical staining. Embryos are oriented with their anterior regions to the left. (A) Wild-type embryo at the nuclear cleavage cycle 14 stage. Staining is observed in dorsal regions and at the poles. Both high and low levels of dl repress *zen* in ventral and lateral regions. (B) Wild-type embryo that has completed germ band elongation (not stained). There is full extension of the posterior midgut invagination due to compaction of the amnioserosa. (C) Cycle 13 embryo containing the hsp83-dlmC1 transgene. Staining is restricted to the poles and lost in the dorsal regions. (D) Transgenic embryo comparable to the one shown in panel B. The amnioserosa is lost, and consequently, the posterior midgut fails to extend to anterior regions. This phenotype is similar to that observed in *zen* and *dpp* mutants.

4). Moreover, region I mutations do not impair binding to a FLAG-dl chimeric protein (data not shown).

Expression of region I mutants in transgenic embryos. P transformation experiments were done with a mutant form of the dl protein that contains amino acid substitutions in region I of the Rel homology domain. This is the same mutant protein used in the experiments whose results are shown in Fig. 3 and 4. As indicated above, this mutation contains six amino acid substitutions in a 7-aa sequence that is highly conserved in dl and Dif. The poor binding of mammalian p65 to cact correlates with the divergence of this sequence in p65, and the conversion of the dl or Dif sequence into the corresponding p65 sequence nearly abolishes dl-cact interactions (Fig. 3). Despite its poor binding to cact, this mutant protein retains essentially normal DNA binding activity (Fig. 4).

Transgenic lines that contain the mutant dl-p65 coding sequence under the control of the hsp83 promoter were established. Under noninduced conditions this promoter is strongly expressed in the female germ line but is only weakly expressed in somatic tissues. The hsp83 promoter was selected for these studies since it is essentially silent in males, so that any dominant lethal effects could be circumvented by maintaining the transgene through the male germ line. Previous studies suggested that the hsp83 promoter is about as active as the endogenous dl promoter (11). A total of 46 independent transgenic lines were obtained, and one copy of the transgene produced dominant female sterility in 2 of the lines. Embryos were obtained from the latter lines and hybridized with various digoxigenin-labeled antisense RNA probes.

These embryos exhibit a weak ventralizing phenotype similar to that observed for various cact alleles (31) (see below). The most substantial and consistent defect observed in these embryos is that the *zen* expression pattern is repressed in both dorsal and ventral regions. Normally, *zen* is activated by one or more ubiquitous factors but is silenced in ventral and lateral regions in direct response to both high and low levels of dl (Fig. 5A) (20, 22, 24). *zen* is expressed at the poles even though there are high levels of dl repressor present at these positions. Previous studies have established that dl repression activity is somehow blocked by the torso receptor tyrosine kinase signaling pathway, which is activated only at the poles (4, 32). Embryos containing the mutant dl-p65 protein constitutively repress *zen* in both dorsal and ventral regions (Fig. 5C). However, expression is retained at the poles, presumably because the repression activity of the mutant dl-p65 protein, like that of the normal dl protein, is blocked by the torso pathway at these positions. Constitutive repression of the *zen* pattern contributes to the ventralizing phenotype that is observed. For example, these embryos lack an amnioserosa, which is similar to the situation observed for *zen* mutants (Fig. 5D; compare with panel B). The simplest interpretation of this result is that mutations in region I of the Rel domain result in low, consti-

tutive nuclear transport and a corresponding block in *zen* expression (see Discussion).

DISCUSSION

This study provides evidence for multiple cact binding sites in the dl Rel domain. Both the NLS (region II) and a more central region of the Rel domain, region I, are essential for dl-cact interactions. There is a particularly tight correlation between region I sequences and the specificity of Rel-inhibitor interactions. For example, dl and Dif contain six matches in seven amino acids in region I and both bind cact. The mammalian p65 and c-Rel proteins contain six matches in seven amino acids in region I and preferentially bind IκBα. In contrast, p50 possesses a distinct region I sequence and selectively binds Bcl-3. Although region I overlaps the dl DNA binding domain, it was possible to uncouple cact and DNA binding. A modified dl protein containing point mutations in region I retains normal DNA binding activity but is greatly impaired in its ability to interact with cact. Transgenic embryos that express this protein exhibit a dominant ventralizing phenotype, which is consistent with the constitutive repression of *zen*. Recent crystallographic studies of p50 homodimers suggest that region I and NLS sequences might form a composite surface that interacts with inhibitors such as cact and IκB (8).

Both the NLS and region I mediate cact binding. Previous studies with both flies and mammals suggested that the NLS region of the Rel domain is important for interacting with cact and IκB inhibitors (for a review, see reference 38). This observation provides an appealing model for the regulated nuclear transport of Rel proteins, whereby the inhibitor blocks transport by masking the NLS. When modified by the interleukin-1 receptor–Toll signaling pathway, the inhibitor releases the Rel protein, which can now enter the nucleus because of the exposure of its NLS. This study provides additional evidence for the importance of the NLS in Rel-inhibitor interactions. Most notably, the *Drosophila* Dif protein, which is thought to participate in insect immunity, contains a divergent NLS that is distinct from the dl NLS (they share only one match in the 6-aa sequence). In vitro binding assays reveal that Dif binds less well to cact than does dl (Fig. 3). Point mutations that convert the 6-aa Dif NLS into the corresponding dl NLS are sufficient to enhance the cact binding activity of an otherwise-normal Dif protein. This result provides evidence for the notion that the NLS is in direct contact with cact. However, we cannot exclude the possibility that even subtle changes in the NLS cause an altered conformation of the Dif protein, which is responsible for enhanced binding.

It does not appear that the NLS is sufficient to account for the specificity of Rel-inhibitor interactions. Most notably, it has been shown that cact and IκB contain a series of six ankyrin repeats; mutations in any one of these reduce or abolish binding to Rel proteins (7, 23; for a review, see reference 2). The simplest interpretation of this observation is that the inhibitors contact multiple sites within Rel proteins. Our analysis of serial deletions indicates that an extended region of the dl Rel domain, spanning aa residues 218 to 341, might be important for interacting with cact. Deletions of aa residues 218 to 245 (region I) and 322 to 341 (region II) virtually abolish binding to cact. Deletions in the intervening region, aa residues 246 to 321, cause less severe reductions in binding. There are at least two possible explanations for these observations. First, perhaps cact specifically contacts only regions I and II, and deletions in the intervening sequence reduce binding because of conformational changes in the overall structure of the protein. For example, it is conceivable that the exact spacing between regions I and II is essential for interactions with sequential ankyrin repeats in cact. Alternatively, cact might make multiple contacts in the entire region from aa 218 to 341, but regions I and II possess the highest affinity. Either way, this study has demonstrated that two discrete sequences in the Rel homology domain, separated by about 100 aa, are both required for dl-cact interactions.

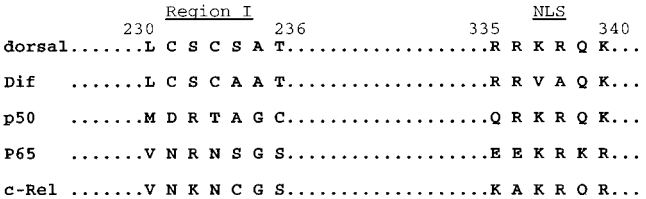

FIG. 6. Comparison of region I and NLS sequences. Alignment of the two inhibitor binding sites within different Rel homology domains, including the p50 and p65 subunits of NF-κB, dl, Dif, and c-Rel. The numbers above the dl sequences refer to the amino acid positions within the full-length dl sequence. There is a close correlation between the specificity of Rel-inhibitor interactions and region I sequences. For example, p65 and c-Rel share six of seven matches and bind IκBα, while p50 possesses a divergent region I sequence and preferentially binds Bcl-3.

There is a close correlation between the conservation of the region I sequence in different Rel proteins and the specificity of Rel-inhibitor interactions (Fig. 6). Dif and dl share six of seven matches in this sequence, and both bind the inhibitor cact. In vitro binding assays (Fig. 3) indicate that p65 only weakly interacts with cact; the p65 region I sequence is completely distinct from the dl and Dif sequences. Interestingly, there is also a correlation between region I conservation and the binding preferences of mammalian Rel proteins. Most notably, p65 and c-Rel preferentially interact with IκBα and share six of seven matches in region I. In contrast, p50 binds a distinct member of the inhibitor family, Bcl-3, and shares only two of seven matches with the p65 region I sequence and only one of seven matches with c-Rel (for a review, see reference 2).

The correlation between NLS (region II) sequence identity and inhibitor interactions is not as obvious. c-Rel has the same degree of homology (three of six matches) with both p65 and p50, even though it behaves like p65, and not p50, in inhibitor binding assays. Moreover, p50 and dl possess very similar NLSs (five of six matches) but bind distinct inhibitors. Thus, it appears that region I sequences may be predictive of specific Rel-inhibitor interactions, while NLS conservation is not. Nonetheless, as noted above, evidence that the NLS is important in this process stems from the observation that the conversion of the Dif NLS into the corresponding dl sequence results in enhanced Dif-cact binding (Fig. 3). It is conceivable that Rel-inhibitor interactions depend on the combinatorial summation of weak protein-protein contacts with distinct regions of the Rel homology domain.

Previous studies suggested that the "pseudofinger" DNA binding domain in the Rel homology domain (RXXRXR XXC) might be important for Rel-inhibitor interactions (see reference 25). This observation was taken as evidence that the inhibitor independently blocks Rel function through two distinct mechanisms, i.e., cytoplasmic retention (through interactions with the NLS) and inhibition of DNA binding. The RXXRXRXXC motif does appear to be an essential element of dl-cact interactions since mutant dl proteins lacking these sequences appear to retain full cact binding activity (Fig. 2, lanes 4 and 5).

Constitutive activation of the Toll pathway. The importance of region I was tested in vivo by examining the expression of a

mutant dl protein in transgenic embryos. Evidence that relatively conservative substitutions in a 7-aa region I sequence cause constitutive nuclear expression of the mutant dl protein was presented. It appears that only low levels of this protein enter nuclei, which is similar to the situation observed in cact hypomorphic mutations. First, only 2 of 46 transgenic lines exhibit completely penetrant female sterility resulting from lethal embryos with a dominant ventralizing phenotype. This might result from variable, weak expression of the mutant protein from the hsp83 promoter. We anticipate that homozygosing some of the other lines, thereby obtaining embryos with two copies of the hsp83-dl-p65 fusion gene, will result in additional examples of dominant lethality.

The phenotypes observed for the two lines that exhibit female sterility as heterozygotes is quite reminiscent of the situation observed for cact hypomorphs (31, 33). There is a slight expansion of the presumptive mesoderm in ventral regions, due to expanded limits of *twi* and *sna* expression (data not shown). Moreover, determinants of the dorsal ectoderm, including *zen*, are fully repressed in both dorsal and ventral regions. This constitutive repression causes a ventralizing phenotype, whereby the presumptive amnioserosa and portions of the dorsal epidermis follow a more lateral pathway of differentiation (Fig. 5). Previous studies have shown that *zen* exhibits the lowest threshold response to the dl morphogen gradient and is efficiently repressed even by low levels of dl in ventrolateral and lateral regions (22). Consequently, *zen* is the first target gene to respond (i.e., it is repressed) to even low levels of dl. The fact that the modified dl-p65 protein can activate and repress dl target genes such as *twi* and *zen*, respectively, suggests that mutations in region I do not impair in vivo DNA binding activities despite the tight linkage between the Rel sequences important for DNA binding and inhibitor interactions.

The chimeric dl-p65 protein appears to retain substantial cact binding activity. Complete constitutive nuclear transport of the protein should transform the entire dorsoventral axis into mesodermal derivatives, as is observed when the Toll signaling pathway is constitutively activated (as in $Toll^{10B}$ mutants [36]). In contrast, the dl-p65 protein causes only a slight expansion of the presumptive mesoderm, suggesting that just a small fraction of the total protein is constitutively expressed in nuclei. As discussed above, dl-cact interactions might involve several distinct sites of binding, including region I, the NLS, and possibly even sequences that map outside the Rel domain (34). More efficient constitutive nuclear transport might be obtained by creating double or triple mutants, for example, by simultaneously altering both region I and the NLS.

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REFERENCES

1. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
2. **Beg, A. A., and A. S. Baldwin.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
3. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
4. **Casanova, J.** 1991. Interaction between torso and dorsal, two elements of different transduction pathways in the Drosophila embryo. Mech. Dev. **35:** 41–45.
5. **Chasan, R., and K. V. Anderson.** 1989. The role of *easter*, an apparent serine protease, in organizing the dorsal-ventral pattern of the Drosophila embryo. Cell **56:**391–400.
6. **Edbrooke, M. R., J. Folki, J. K. Cheshire, F. Li, D. J. Faulkes, and P. Woo.** 1991. Constitutive and NF-κB-like proteins in the regulation of the serum amyloid A gene by interleukin-1. Cytokine **3:**380–388.
7. **Geisler, R., A. Bergmann, Y. Hiromi, and C. Nusslein-Volhard.** 1992. *cactus*, a gene involved in dorsoventral pattern formation of Drosophila, is related to the IκB gene family of vertebrates. Cell **71:**613–621.
8. **Ghosh, G., G. Van Duyne, S. Ghosh, and P. B. Sigler.** The structure of NF-κB p50 homodimer bound to a κB site. Nature (London), in press.
9. **Gillespie, S. K. H., and S. A. Wasserman.** 1994. dorsal, a *Drosophila* Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. Mol. Cell. Biol. **14:**3559–3568.
10. **Gonzalez-Crespo, S., and M. Levine.** 1993. Interactions between dorsal and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in Drosophila. Genes Dev. **7:**1703–1713.
11. **Govind, S., L. Brennan, and R. Steward.** 1993. Homeostatic balance between dorsal and cactus proteins in the Drosophila embryo. Development **117:**135–148.
12. **Govind, S., and R. Steward.** 1991. Dorsoventral pattern formation in Drosophila. Trends Genet. **7:**119–125.
13. **Guan, K., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in E. coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:**262–267.
14. **Hashimoto, C., K. 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.
15. **Hultmark, D.** 1994. Ancient relationships. Nature (London) **367:**116–117.
16. **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.
17. **Ip, Y. T., and M. Levine.** 1992. The role of the dorsal morphogen gradient in Drosophila embryogenesis. Semin. Dev. Biol. **3:**15–23.
18. **Ip, Y. T., R. E. Park, D. Kosman, E. Bier, and M. Levine.** 1992. The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo. Genes Dev. **6:**1728–1739.
19. **Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei, and M. Levine.** 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell **75:**753–763.
20. **Jiang, J., H. Cai, Q. Zhou, and M. Levine.** 1993. Conversion of a dorsal-dependent silencer into an enhancer: evidence for dorsal corepressors. EMBO J. **12:**3201–3209.
21. **Jiang, J., D. Kosman, Y. T. Ip, and M. Levine.** 1991. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early Drosophila embryos. Genes Dev. **6:**1728–1739.
22. **Jiang, J., C. A. Rushlow, Q. Zhou, S. Small, and M. Levine.** 1992. Individual dorsal morphogen binding sites mediate activation and repression in the Drosophila embryo. EMBO J. **11:**3147–3154.
23. **Kidd, S.** 1992. Characterization of the Drosophila cactus locus and analysis of interactions between cactus and dorsal proteins. Cell **71:**623–635.
24. **Kirov, N., L. Zhelnin, J. Shah, and C. A. Rushlow.** 1993. Conversion of a silencer into an enhancer: evidence for a co-repressor in dorsal-mediated repression in Drosophila. EMBO J. **12:**3193–3199.
25. **Kumar, S., and C. Gelinas.** 1993. I kappa B alpha-mediated inhibition of v-Rel DNA binding requires direct interaction with the RXXRXRXXC Rel/kappa B DNA-binding motif. Proc. Natl. Acad. Sci. USA **90:**8962–8966.
26. **Liou, H. C., and D. Baltimore.** 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. **5:**477–487.
27. **Mathias, S., A. Younes, C. C. Kan, I. Orlow, and C. Joseph.** 1993. Activation of the spingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1beta. Science **259:**519–522.
28. **Matthews, J. R., E. Watson, S. Buckley, and R. T. Hay.** 1993. Interaction of the C-terminal region of p105 with the nuclear localization signal of p50 is required for inhibition of NF-κB DNA binding activity. Nucleic Acids Res. **21:**4516–4523.
29. **Morisato, D., and K. V. Anderson.** 1994. The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. Cell **76:**677–688.
30. **Reichart, J. M., P. Georgel, M. Meister, B. Lemaitre, C. Kappler, and J. A. Hoffmann.** 1993. Expression and nuclear translocation of the rel/NF-κB-related morphogen dorsal during the immune response of Drosophila. C. R. Acad. Sci. **316:**1218–1224.
31. **Roth, S., D. Stein, and C. Nusslein-Volhard.** 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell **59:**1189–1202.
32. **Rusch, J., and M. Levine.** 1994. Regulation of the dorsal morphogen by the Toll and torso signaling pathways: a receptor tyrosine kinase selectively masks transcriptional repression. Genes Dev. **8:**1247–1257.
33. **Rushlow, C. A., M. Frasch, H. Doyle, and M. Levine.** 1987. Maternal regulation of zerknullt: a homeobox gene controlling differentiation of dorsal tissues in Drosophila. Nature (London) **330:**583–586.
34. **Rushlow, C. A., K. Han, J. L. Manley, and M. Levine.** 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear trans-

port in Drosophila. Cell **59:**1165–1177.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Schneider, D. S., K. L. Hudson, T. Y. Lin, and K. V. Anderson.** 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo. Genes Dev. **5:**797–807.
37. **Shelton, C. A., and S. A. Wasserman.** 1993. Pelle encodes a protein kinase required to establish dorsoventral polarity in the Drosophila embryo. Cell **72:**515–525.
38. **Siebenlist, U., G. Franzoso, and K. Brown.** 1994. Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. **10:**405–455.
39. **Steward, R.** 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell **59:**1179–1188.
40. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBalpha: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
41. **Tautz, D., and C. Pfeifle.** 1989. A nonradioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98:**81–85.
42. **Thummel, C. S., A. M. Boulet, and H. D. Lipshitz.** 1988. Vectors for Drosophila P-element-mediated transformation and tissue culture transformation. Gene **74:**445–446.
43. **Wasserman, S. A.** 1993. A conserved signal transduction pathway regulating the activity of the Rel-like proteins dorsal and NF-κB. Mol. Biol. Cell **4:**767–771.
44. **Whalen, A. M., and R. Steward.** 1993. Dissociation of the dorsal-cactus complex and phosphorylation of the dorsal protein correlates with the nuclear localization of dorsal. J. Cell Biol. **123:**523–534.
45. **Zabel, U., T. Henkel, M. dos Santos-Silva, and P. Baeuerle.** 1993. Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. **12:**201–211.