The Maternal Nudel Protein of Drosophila Has Two Distinct Roles Important for Embryogenesis

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Manuscript received February 21, 1996
Accepted for publication April 24, 1996

ABSTRACT

The *nudel* gene is maternally required to define dorsoventral polarity of the Drosophila embryo. It encodes an unusual mosaic protein with a protease domain that may trigger the protease cascade required for ventral development. We describe phenotypic and molecular analyses of *nudel* mutations that provide further insight into nudel protein function. Surprisingly, *nudel* mutations primarily cause either dorsalized embryos in which dorsal cell fates are expanded over ventral and lateral cell fates or fragile eggs that fail to develop beyond early embryonic stages. The nudel protein is therefore required not only for embryonic dorsoventral polarity but also for structural integrity of the egg. Complementation and antagonistic interactions between *nudel* alleles suggest that the nudel protein is functionally modular and that protein-protein interactions are important for nudel protein function. Three *nudel* mutations that produce dorsalized embryos map to the protease domain of nudel, suggesting that this domain is specifically required for defining embryonic dorsoventral polarity. Finally, certain combinations of *nudel* alleles simultaneously produce completely dorsalized and normal embryos yet very few embryos of intermediate mutant phenotypes. The unusual biphasic distribution of phenotypes may indicate that *nudel* activity above a threshold is required to generate embryonic dorsoventral polarity.

THE 11 genes of the "dorsal group" are maternally active in establishing dorsoventral polarity of the Drosophila embryo (ANDERSON and NÜSSLEIN-VOLHARD 1986). They are required to specify ventral and lateral cell fates, as the loss in activity of any dorsal-group gene results in replacement of these fates by dorsal cell fates within a dorsalized embryo. The dorsal-group genes encode components of a signal transduction pathway that culminates in the formation of a morphogenic gradient (reviewed in MORISATO and ANDERSON 1995). At the beginning of the pathway, an extracellular signal is generated within the perivitelline space, the compartment between the embryonic plasma membrane and the surrounding eggshell. The extracellular signal acts as a ligand for the transmembrane protein Toll, ultimately triggering the nuclear localization of the dorsal protein (HASHIMOTO *et al.* 1988; STEIN *et al.* 1991; MORISATO and ANDERSON 1994; SCHNEIDER *et al.* 1994). While the Toll protein is uniformly distributed in the plasma membrane, the Toll ligand appears to be preferentially produced on the ventral side of the embryo (HASHIMOTO *et al.* 1991; STEIN *et al.* 1991). Ventral activation of Toll therefore leads to a ventral-to-dorsal concentration gradient of the dorsal protein in nuclei of the syncytial embryo (ROTH *et al.* 1989; RUSHLOW *et al.* 1989; STEWARD 1989). The dorsal protein functions as a morphogen, as its graded distribution determines the pattern of zygotic genes and cell fates expressed along the dorsoventral dimension of the embryo.

Genetic and molecular analyses of the dorsal-group genes that act genetically upstream of *Toll* have provided clues to how production of the Toll ligand could be ventrally localized. The Toll ligand is likely generated by proteolytic processing of the spätzle protein (MORISATO and ANDERSON 1994; SCHNEIDER *et al.* 1994). Processing of spätzle appears to occur at the end of a protease cascade involving the snake and easter proteins, which resemble serine protease zymogens that must be cleaved to become catalytically active (CHASAN *et al.* 1992; SMITH and DELOTTO 1994). Because the snake and easter zymogens and the spätzle protein seem to be freely diffusible within the perivitelline space, activation of the protease cascade must be spatially localized to limit production of the Toll ligand to the ventral side of the embryo (CHASAN *et al.* 1992; STEIN and NÜSSLEIN-VOLHARD 1992; MORISATO and ANDERSON 1994).

The products of three dorsal-group genes, *nudel, pipe,* and *windbeutel,* appear to play key roles in spatially regulating the protease cascade. Experiments with genetically mosaic females revealed that the activities of these genes are required in somatic tissue, presumably the follicle cells that secrete components of the eggshell around the oocyte during oogenesis (SCHÜPBACH *et al.,* 1991; STEIN *et al.* 1991). These and other experiments led to the idea that the *nudel, pipe,* and *windbeutel* gene products function to generate a positional cue in the eggshell that directs the local activation of the protease

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cascade producing the Toll ligand (STEIN *et al.* 1991; CHASAN *et al.* 1992).

We recently described evidence suggesting that the *nudel* gene product is an essential component of the positional cue (HONG and HASHIMOTO 1995). We cloned the *nudel* gene and found that it is expressed in follicle cells when these cells are secreting components of the vitelline envelope. [The eggshell consists of the inner vitelline envelope, which helps to maintain the shape of the egg, and the outer chorion, the major structural layer containing channels for gas exchange between the egg and the environment (SPRADLING 1993).] Although *nudel* is expressed during oogenesis, temperature-shift experiments suggest that *nudel* function is required after fertilization. The nudel protein resembles an unusual extracellular matrix protein containing several motifs that could bind protease zymogens, including a serine protease catalytic domain. These and other observations suggested that the nudel protein could be a component of the vitelline envelope that organizes the local activation of the protease cascade producing the Toll ligand.

Here we describe genetic and molecular studies that provide additional insight into the functioning of the nudel protein. Although *nudel* was identified as a dorsal-group gene, we found that only some *nudel* mutations produce dorsalized embryos. Most *nudel* mutations, including putative null alleles, produce embryos that fail to develop beyond the syncytial blastoderm stage, apparently because of a defect in the vitelline envelope. The distinct phenotypes caused by the two different classes of *nudel* mutations suggest that the nudel protein has two roles important for embryonic development: to provide structural integrity to the egg and to establish dorsoventral polarity. Complementation between *nudel* alleles of the two classes suggest that the nudel protein is functionally modular. Negative interactions between *nudel* alleles indicate possible interactions at the protein level that may be important for nudel protein function. Molecular analyses of *nudel* mutations reveal that the central serine protease domain is specifically required for the dorsoventral patterning role of nudel. Finally, an unusual biphasic distribution of *nudel* phenotypes suggest that *nudel* activity above a threshold is required to induce embryonic dorsoventral polarity.

MATERIALS AND METHODS

Fly stocks: The mutant alleles and the deficiencies *l(3)3844* and *Df(3L)CH12* of the *nudel* (*ndl*) gene were isolated in mutagenesis screens described previously (HONG and HASHIMOTO 1995, and references therein). The wild-type stock was Oregon R.

Phenotypic analyses: Eggs were collected, embryos were examined under oil by light microscopy, and cuticles of embryos were prepared as described previously (WIESCHAUS and NÜSSLEIN-VOLHARD 1986). Morphological criteria were used to determine if embryos developed beyond the syncytial or cellular blastoderm stage, or displayed the dorsalized phenotype when examined at 3 days after egg laying. Dorsoventral pattern elements in the cuticles of embryos were scored to assess the degree of dorsalization (ANDERSON and NÜSSLEIN-VOLHARD 1986). Dorsalized phenotypes were classified according to ROTH *et al.* (1991) with the following modification. We distinguished the D2 and D3 phenotypes by the presence of an intact cephalopharyngeal skeleton (CS), rather than mesoderm, in D3 but not in D2 (Figure 1, B and C). Since most embryos in the D3 category show subtle cuticle defects, they may correspond to "cryptically abnormal embryos" produced by weak *gastrulation defective* alleles (KONRAD *et al.* 1988). Cuticles of at least 250 embryos that failed to hatch were examined for each experiment.

We considered eggs to be fragile if they collapsed spontaneously or upon simple manipulation with a sewing needle or paint brush. By these subjective criteria, eggs produced by class I alleles were clearly more fragile than those produced by class II alleles. A more objective criterion of egg fragility was the sensitivity of eggs to removal of the chorion with bleach. Greater than 50% of class I eggs, but less than 5% of class II eggs, disintegrated during this procedure. When placed under oil, class I eggs that did not immediately disintegrate gradually displayed bubbles of increasing size and number on their surfaces (see RESULTS). All of these eggs collapsed within 24 hr of chorion removal, the most after <6 hr. In contrast to eggs produced by the class I mutants, eggs produced by the class II alleles ndl^{46} and ndl^{111} remained intact for more than 1 week after chorion removal. However, eggs produced by the two other class II alleles, ndl^{9} and ndl^{260}, collapsed 1–2 days after chorion removal. Because these eggs did not collapse before or immediately after chorion removal, they were clearly less fragile than eggs produced by the class I mutants (Table 1).

DNA analyses: Genomic DNA, prepared as described previously (LEVIS *et al.* 1982), was used to isolate DNA encoding the serine protease catalytic domain by PCR. Both strands of the DNA were sequenced by the dideoxy chain termination method using an automated sequencing system (Applied Biosystems). The deletion in the ndl^{18} allele was localized by Southern analysis to the 3′ region of the *nudel* gene (HONG and HASHIMOTO 1995). The deletion was further localized by comparing the sizes of fragments generated from wild-type and ndl^{18} genomic DNA by PCR using primers in this region. DNA encompassing the deletion was isolated by PCR and sequenced as above.

RESULTS

Mutations in the *nudel* gene cause one of two distinct maternal-effect phenotypes: The *nudel* (*ndl*) gene was identified as a member of the dorsal-group genes that are maternally required to produce ventral and lateral cell fates in the embryo (ANDERSON and NÜSSLEIN-VOLHARD 1984, 1986). Females carrying loss-of-function mutations in a dorsal-group gene produce dorsalized embryos. The dorsalized phenotype is characterized by abnormal gastrulation and a larval cuticle having dorsal structures expanded at the expense of lateral and ventral structures (ANDERSON and NÜSSLEIN-VOLHARD 1986). When we examined the phenotype caused by *ndl* alleles in *trans* to a *ndl* deficiency (*Df*), we were surprised to find that only four out of 15 alleles produced the classic dorsalized phenotype. The majority of *ndl* alleles produced a clearly distinct phenotype, in

TABLE 1

Two classes of *nudel* mutations based on their maternal-effect embryonic phenotypes

Allele	Embryonic phenotype	Fragile egg
Class I		
ndl^{10}	Early developmental arrest	+
ndl^{11}	Early developmental arrest	+
ndl^{12}	Early developmental arrest	+
ndl^{13}	Early developmental arrest	+
ndl^{14}	Early developmental arrest	+
ndl^{15}	Early developmental arrest	+
ndl^{16}	Early developmental arrest	+
ndl^{17}	Early developmental arrest	+
ndl^{18}	Early developmental arrest	+
ndl^{133}	Early developmental arrest	+
ndl^{169}	Early developmental arrest	+
Class II		
ndl^{9}	Dorsalized	−/+[a]
ndl^{46}	Dorsalized	−
ndl^{111}	Dorsalized	−
ndl^{260}	Dorsalized	−/+[a]

Females hemizygous for each *nudel* allele over the deficiency *l(3)3844* were mated to OR males. Eggs were collected and incubated at 22°. The embryonic phenotypes and egg fragility were assayed as described in MATERIALS AND METHODS.

[a] Eggs produced by ndl^{9} or ndl^{260} were more sensitive to manipulation than normal eggs but were less fragile than eggs produced by class I mutants (see MATERIALS AND METHODS).

which most eggs failed to reach the gastrulation stage or to produce a cuticle. Based on the maternal-effect phenotypes that they produced, we have grouped *ndl* mutations into two major classes (Table 1).

Class I *nudel* mutations: early arrest of embryonic development: The majority or all (95–100%) of the eggs produced by a female hemizygous for a class I allele (*ndl/Df*) did not develop to the syncytial blastoderm stage. These eggs appeared to be fertilized, since they exhibited the cytoplasmic clearing that occurs at the egg periphery during initial stages of embryogenesis (SCHÜPBACH and WIESCHAUS 1989). In ~25% of these eggs, the plasma membrane separated away from the vitelline envelope (SCHÜPBACH and WIESCHAUS 1989). Of those eggs that reached the syncytial blastoderm stage (<5%), the majority stopped development at this stage or at subsequent stages through cellularization. Only a very small fraction (<1%) of the eggs produced by class I mutant females exhibited the dorsalized phenotype.

Eggs produced by the class I mutants were very fragile and sensitive to manipulation. Although a few eggs (5%) collapsed spontaneously, most (50–90%) appeared to rupture after removal of the chorion surrounding the vitelline envelope. When the eggs that survived chorion removal were placed under oil, bubbles appeared on their surfaces, as if their contents were slowly leaking out. These eggs eventually collapsed within a day. A similar "oozy eggshell" phenotype has been described for mutations that weaken the vitelline envelope (SAVANT and WARING 1989). A structural defect in the vitelline envelope may therefore be responsible for the fragility of eggs laid by the class I mutants. Among the class I mutations are the putative null alleles, ndl^{14} and ndl^{133}, which do not produce any detectable *nudel* RNA (HONG and HASHIMOTO 1995; Table 1). Thus, complete loss of maternal *nudel* function appears to cause early developmental arrest and the production of fragile eggs.

Class II *nudel* mutations: dorsalization of the embryo: The class II alleles ndl^{111} and ndl^{260} produced embryos that were completely dorsalized (D0; Figure 1) at all temperatures tested (18°, 22° and 29°; Table 2). The cuticles of these embryos showed only the structures normally restricted to the dorsal side. In contrast, the production of mutant phenotypes by the two other class II alleles, ndl^{9} and ndl^{46}, was dependent on temperature (Table 2; HONG and HASHIMOTO 1995). At 22° or 29°, all of the embryos produced by ndl^{46} homozygotes and ndl^{46}/Df hemizygotes were dorsalized and failed to hatch. Examination of their cuticles indicated that nearly all (98%) of the embryos were completely dorsalized, while the rest were strongly dorsalized, retaining some lateral structures (D1; Figure 1). At 18°, ~8% of embryos from ndl^{46} homozygotes as well as ndl^{46}/Df hemizygotes hatched. The activity of the ndl^{9} allele was more dramatically sensitive to temperature. At 22° or 29°, 100% of the embryos laid by ndl^{9}/Df females were completely dorsalized and failed to hatch, whereas at 18°, 24% of these embryos hatched (Table 2).

The eggs laid by class II mutants were not obviously fragile, and they remained intact following removal of the chorion. However, we observed gradual leakage, as described above, from eggs lacking their chorions laid by ndl^{9} or ndl^{260} mutants (Table 1; see MATERIALS AND METHODS). These eggs were also slightly more sensitive to manipulation than normal eggs. Interestingly, there was no correlation between structural integrity of the egg and mutant embryonic phenotype. For instance, ndl^{9} produced weakly dorsalized embryos at 18°, whereas ndl^{111}, which did not obviously perturb structural integrity of the egg, caused complete dorsalization of the embryo.

Intragenic complementation by *nudel* alleles: Because of the distinct phenotypes produced by the class I and II alleles, we wondered what phenotypes would be produced by a combination of alleles from each class. In all combinations tested, the fragile egg or the early developmental arrest phenotype associated with the class I alleles was never observed. Instead, most combinations produced the classic dorsalized phenotype, while several exhibited complementation and produced embryos that hatched (Table 3).

Whether the class I and II alleles complemented each other depended on which allelic combinations were tested (Table 3). For example, the two temperature-

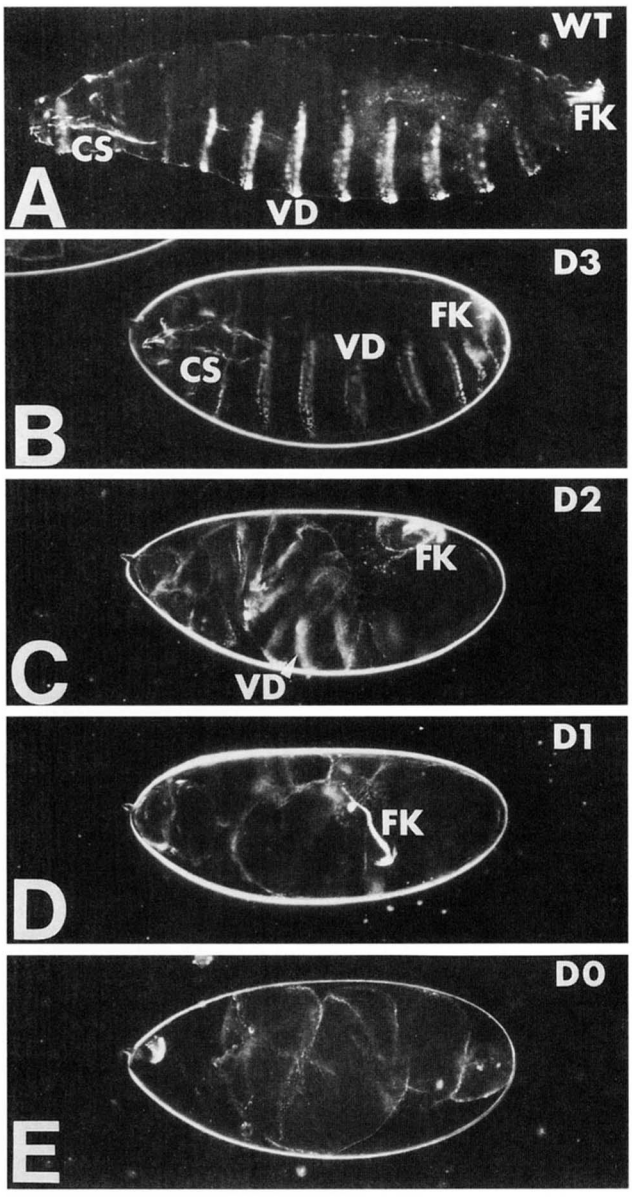

FIGURE 1.—Dorsalized phenotypes produced by *nudel* mutants. Dorsoventral pattern elements in the cuticles of embryos were used to classify the degree of dorsalization. (A) Embryo with wild-type dorsoventral pattern that hatched. (B) Weakly dorsalized D3 embryo, which failed to hatch, has all identifiable dorsoventral pattern elements, including ventrally derived denticles (VD) and dorsolaterally derived filzkörper (FK), but displays minor defects such as reduction in size of one or more ventral denticles. (C) Moderately dorsalized D2 embryo, in addition to the defects seen in the D3 embryo, has a severely disorganized cephalopharyngeal skeleton (CS) or is missing this structure entirely. (D) Strongly dorsalized D1 embryo lacks ventral denticles but has filzkörper. (E) Completely dorsalized D0 embryo displays only the dorsal epidermis. The dorsalized embryos shown here were produced by ndl^{46}/ndl^{46} females.

sensitive alleles, ndl^{9} and ndl^{46}, but not the completely dorsalizing alleles, ndl^{111} and ndl^{260}, complemented class I alleles. Complementation was observed at 22°, a restrictive temperature for both ndl^{9} and ndl^{46}. The ndl^{9} allele only complemented one class I allele, ndl^{15}, whereas ndl^{46} complemented most class I alleles. The extent of complementation with ndl^{46} varied greatly among the class I alleles (Table 3). For example, ndl^{15} was almost completely complemented by ndl^{46}, whereas the putative null alleles, ndl^{14} and ndl^{133}, failed to be complemented.

We also examined whether the class II alleles could complement each other (Table 2). When the two temperature-sensitive alleles, ndl^{9} and ndl^{46}, were placed in *trans*, a dramatic complementation was observed. At 18°, when ndl^{9} or ndl^{46} alone produced embryos that hatched at a frequency of 24 or 8%, respectively, the ndl^{9}/ndl^{46} combination produced a hatching frequency of 80%. At 22°, when either ndl^{9} or ndl^{46} alone produced completely dorsalized embryos (0% hatching),

TABLE 2

Temperature sensitivity and genetic interactions of class II *nudel* alleles

	ndl^{9}	ndl^{46}	ndl^{111}	ndl^{260}	*Df*
ndl^{9}	ND[a]	80 (50)	0 (2)	0 (0)	24 (0)
ndl^{46}		8 (0)	0 (0)	0 (0)	8 (0)
ndl^{111}			ND[a]	0 (0)	0 (0)
ndl^{260}				0 (0)	0 (0)

The percentages of embryos that hatched from eggs laid by females carrying the indicated combinations of class II *nudel* alleles were calculated. The results for embryos collected and incubated at 18° and at 22° (in parentheses) are shown. No hatchers were produced at 29° by any of the allelic combinations that were tested. The deficiency used was *l(3)3844*. Over 200 eggs from females of each genotype were analyzed.

[a] ND, not determined. Chromosomes carrying the ndl^{9} and ndl^{111} alleles are homozygous lethal.

TABLE 3

Complementation between class I and class II *nudel* alleles

	Class II allele			
Class I allele	ndl^{9}	ndl^{46}	ndl^{111}	ndl^{260}
ndl^{10}	0	1	0	0
ndl^{11}	0	28	0	0
ndl^{12}	0	5	0	0
ndl^{13}	0	1	0	0
ndl^{14}	0	0	0	0
ndl^{15}	52	90	0	0
ndl^{16}	0	22	0	0
ndl^{17}	0	34	0	0
ndl^{18}	0	55	0	0
ndl^{133}	0	0	0	0
ndl^{169}	0	3	0	0

The numbers refer to the percentages of embryos that hatched at 22° from eggs laid by females carrying the indicated combinations of class I and II *nudel* alleles. Since none of the class I and class II alleles produced hatchers when homozygous or when hemizygous over a deficiency at 22° (see RESULTS and Table 2), the percentages represent the extent of complementation between the class I and class II alleles.

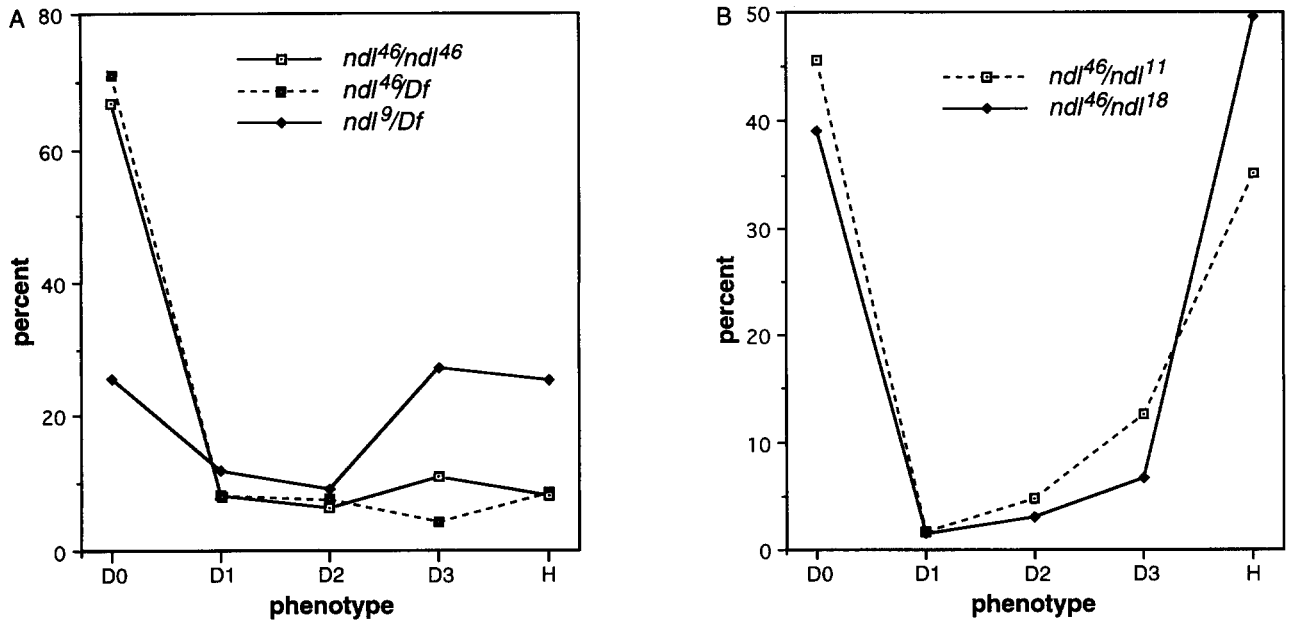

FIGURE 2.—Unusual distribution of phenotypes produced by *nudel* mutants. Graphs show the percentage of embryos produced by indicated *nudel* alleles at 18° that were completely dorsalized (D0), strongly dorsalized (D1), moderately dorsalized (D2), weakly dorsalized (D3), and phenotypically normal hatchers (H). (A) Embryos produced by ndl^{46}/ndl^{46}, ndl^{46}/Df and ndl^{9}/Df exhibited a broad range of phenotypes. Most notably, for ndl^{46}/ndl^{46} and ndl^{46}/Df mutants, even when some embryos were phenotypically normal, the majority were completely dorsalized rather than weakly dorsalized. (B) Embryos from ndl^{46}/ndl^{11} and ndl^{46}/ndl^{18} mutants exhibited a striking biphasic distribution of phenotypes in which phenotypically normal and completely dorsalized embryos predominated.

the ndl^{9}/ndl^{46} combination produced 50% normal embryos that hatched.

Combinations of the temperature-sensitive alleles and the completely dorsalizing alleles, ndl^{111} and ndl^{260}, exhibited very modest or no complementation (Table 2). In contrast, combinations of the temperature-sensitive alleles and a *ndl* deficiency produced significant number of embryos that hatched (Table 2). Since more dorsalized embryos were produced by ndl^{111} and ndl^{260} than the deficiency, when combined with the temperature-sensitive mutations, the ndl^{111} and ndl^{260} alleles displayed a negative effect on the activities of the temperature-sensitive alleles.

Unusual distribution of phenotypes produced by *nudel* mutations: For most dorsal-group genes, hypomorphic alleles exist that can readily be arranged in a phenotypic series, in which progressively stronger alleles produce greater expansion of dorsal structures at the expense of ventral and lateral structures (Figure 1; ANDERSON and NÜSSLEIN-VOLHARD 1986; KONRAD *et al.* 1988). Since 8% of embryos produced by females homozygous for ndl^{46} hatched at 18°, this allele could be categorized as a hypomorph for having a modest effect on dorsoventral patterning. The 92% of embryos that failed to hatch were therefore expected to have only mild dorsoventral pattern defects. However, these embryos displayed a surprisingly broad range of dorsalized phenotypes (Figure 2A). About 12% of the unhatched embryos appeared phenotypically wild type or were weakly dorsalized (D3), 7% were moderately dorsalized (D2), and 9% were strongly dorsalized (D1). Most remarkably, 72% of the unhatched embryos (66% of total) were completely dorsalized (D0). A similar distribution of phenotypes was produced by ndl^{46}/Df hemizygotes (Figure 2A). The ndl^{9} allele also produced the same range of phenotypes, but their distribution was more even (Figure 2A). Mutant alleles of one other dorsal-group gene, *tube,* have been observed to produce phenotypes ranging from normal to completely dorsalized embryos; however, the relative frequency of each phenotype has not been described (HECHT and ANDERSON 1993).

We observed a striking biphasic distribution of phenotypes when embryos from ndl^{46}/ndl^{11} and ndl^{46}/ndl^{18} mutants were examined (Figure 2B). The two phenotypes produced in greatest numbers by these mutants were embryos with a normal dorsoventral pattern and completely dorsalized embryos. Weakly or moderately dorsalized embryos represented only a small percentage of the phenotypes.

Initial molecular characterization of lesions in *nudel* alleles: The existence of a serine protease catalytic domain in the nudel protein suggests that nudel is directly involved in activating the protease cascade that produces the Toll ligand (HONG and HASHIMOTO 1995). We therefore examined by DNA sequencing whether this domain was altered in any of the four class II dorsalizing alleles. Unique amino acid changes in the protease domain were found in ndl^{46}, ndl^{111}, and ndl^{260}, but not in ndl^{9} (Figure 3A). Consistent for mutations

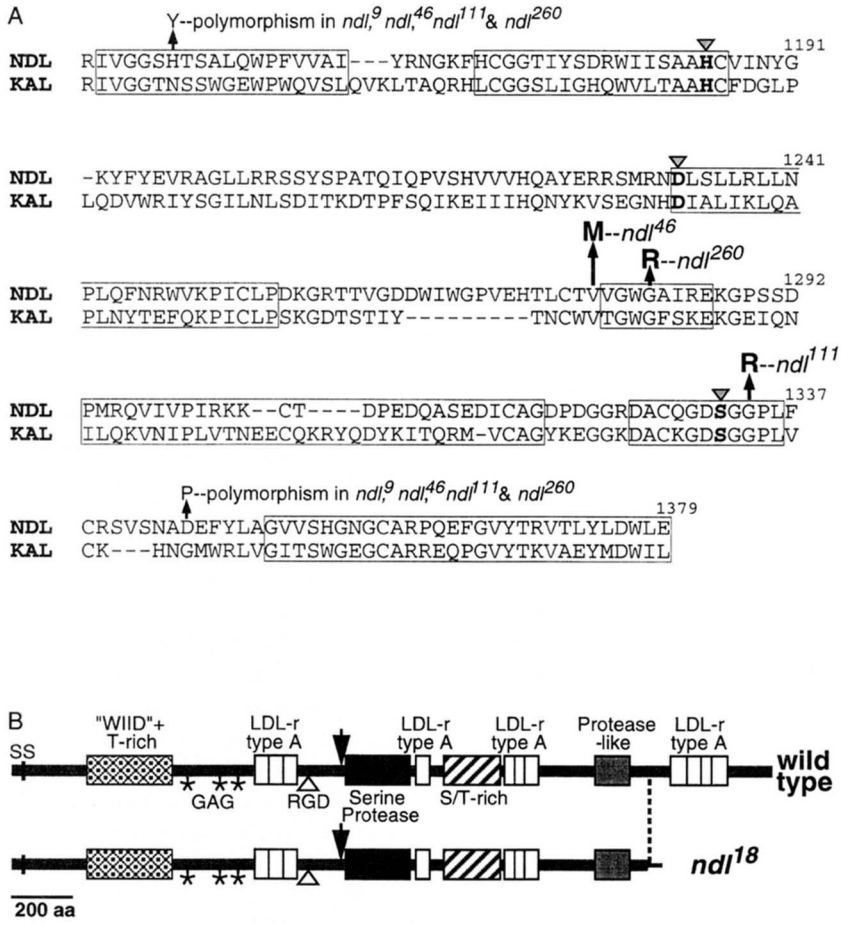

FIGURE 3.—Molecular lesions in *nudel* alleles. (A) The amino acid changes in the central serine protease domain (see B below) caused by class II *nudel* mutations. The serine protease domains of nudel (NDL) and human kallikrein (KAL), which have the highest degree of sequence similarity, are aligned (CHUNG *et al.* 1986; HONG and HASHIMOTO 1995). Numbers refer to the predicted amino acid sequence of nudel (HONG and HASHIMOTO 1995). Residues of the catalytic triad are shown in bold and marked by a triangle. Residues in the conserved regions of serine proteases, as defined by FURIE *et al.* (1982), are encased in boxes. Unique amino acid substitutions found in specific *nudel* alleles are indicated by bold letters above the affected residues. Changes found in all mutant *nudel* alleles sequenced to date, shown in plain letters above the sequences, are probably due to polymorphisms among the different stocks used to make the library from which *nudel* cDNAs were isolated (HAWLEY and WARING 1988) and to generate *nudel* mutations (ANDERSON and NÜSSLEIN-VOLHARD, 1984). (B) Truncated protein encoded by ndl^{18} allele. Shown at top is a schematic of the wild-type nudel protein, modified from HONG and HASHIMOTO (1995). The nudel protein contains a central serine protease domain, a possible proteolytic activation site (arrow), a putative N-terminal signal sequence (SS), six copies of the WIID or related sequence in a threonine-rich region (WIID + T-rich), three potential glycosaminoglycan attachment sites (GAG, stars), 11 LDL-receptor type A repeats (LDL-r type A), the RGD sequence (triangle), a serine/threonine-rich region (S/T-rich), and a protease-like domain. The class II mutations shown in A above are in the central serine protease domain. Shown below is a schematic of the truncated protein encoded by ndl^{18}. Due to a deletion in the 3′ end of the coding region that causes a shift in reading frame, the 402 amino acids normally at the C-terminus are replaced by 75 amino acids not in the wild-type protein.

generated by ethyl methanesulfonate mutagenesis, each change resulted from a G-to-A transition (LIM and SNYDER 1968). In ndl^{111}, a highly conserved glycine just two residues C-terminal to the active site serine was changed to arginine. In ndl^{260}, another highly conserved glycine was also changed to arginine. Since the residues mutated in ndl^{111} and ndl^{260} are in conserved regions thought to form the basic core structure of serine proteases, both drastic changes are likely to disrupt severely the protease activity of nudel (FURIE *et al.* 1982). In ndl^{46}, the valine adjacent to another conserved region was changed to methionine. Because this valine is conserved in serine proteases that are most similar in sequence to the protease domain of nudel, such as human kallikrein (Figure 3A; HONG and HASHIMOTO 1995), the change in ndl^{46} may cause a modest impairment of protease activity.

The only class I allele that we have molecularly characterized is ndl^{18}. DNA sequencing of the protease domain of the ndl^{18} allele did not reveal any unique amino acid change from the wild-type sequence. However, both Northern and Southern analyses indicated that ndl^{18} encodes a truncated RNA and is missing DNA from the 3′ region of the *nudel* gene (HONG and HASHI-

MOTO 1995). Sequencing of this region of the ndl^{18} allele revealed the presence of a 569-bp deletion near the 3′-end of the coding sequence. Taking into account an intron of 53 nucleotides within the deleted region, the RNA encoded by ndl^{18} would be truncated by 516 nucleotides. As a result of this deletion, a frame shift would cause the production of a protein missing 402 C-terminal residues of the wild-type protein (Figure 3B).

DISCUSSION

We have carried out phenotypic and molecular analyses of maternal-effect mutations in the *nudel* gene, which encodes an unusual mosaic protein with a protease domain involved in defining dorsoventral polarity of the Drosophila embryo. Our results suggest that the maternal nudel protein has two roles important for embryonic development and is functionally modular. They also suggest that *nudel* activity above a threshold is required to generate embryonic dorsoventral polarity.

Two roles of the maternal nudel protein important for embryonic development: Mutations in *nudel* can be divided into two classes according to the distinct maternal-effect phenotypes that they produce. Class I mutations produce fragile eggs that arrest in development at early embryonic stages, whereas class II mutations produce dorsalized embryos (Table 1). The two separate phenotypes indicate that *nudel* is maternally required not only for defining embryonic dorsoventral polarity but also for viability of the embryo. The *nudel* gene is therefore unique among the dorsal-group genes that are required to establish embryonic dorsoventral polarity. Mothers completely lacking the activity of any of the other dorsal-group genes produce dorsalized embryos missing all ventral and lateral structures. In contrast, mothers carrying apparently null mutations in *nudel* produce fragile eggs that fail to develop beyond earliest embryonic stages. Only partial loss-of-function mutations in *nudel* produce the dorsalized phenotype. Interestingly, mutations in two genes involved in anteroposterior patterning of the embryo have been observed to produce fragile eggs, suggesting that the products of these genes are bifunctional like the nudel protein (DEGELMANN *et al.* 1990).

The egg fragility and developmental arrest caused by *nudel* mutations may result from a defect in the vitelline envelope, the innermost eggshell layer. This interpretation would be consistent with the observation that the eggs most frequently collapse when the chorion, the outer eggshell layer, is removed. Less fragile eggs that do not immediately collapse may stop developing at early embryonic stages because a defective vitelline envelope allows leakage of essential cytoplasmic material. Similar phenotypes of fragile egg and leaky vitelline envelope are also produced by mutations in the Sv23 gene, which encodes a major structural protein of the vitelline envelope (SAVANT and WARING 1989). The nudel protein could therefore be a component of the vitelline envelope. However, because the *nudel* RNA appears to be much less abundant than the Sv23 RNA, the nudel protein is not expected to be a major structural component (HONG and HASHIMOTO 1995). It may be a less abundant accessory factor that is required for proper assembly of the structural proteins.

Being physically associated with the vitelline envelope would also be important for the role of nudel in defining embryonic dorsoventral polarity. We proposed earlier that nudel functions in localizing production of the Toll ligand by acting as the scaffold of a zymogen activation complex (HONG and HASHIMOTO 1995). Within the complex, the protease domain of nudel would trigger the activation of the snake and easter proteases, which are required for processing of the spätzle protein to the Toll ligand. Having nudel immobilized by physical association with the vitelline envelope therefore provides a mechanism for spatially confining the protease activities of snake and easter, which appear to circulate as zymogens in the perivitelline space (CHASAN *et al.* 1992; STEIN and NÜSSLEIN-VOLHARD 1992).

Organization of nudel into functional domains: Intragenic complementation as we have observed between *nudel* alleles suggests that the nudel protein is organized into functional domains (Tables 2 and 3). For example, a class I allele may encode a protein with a defect in a region that is important for the role of nudel in assembling the vitelline envelope; whereas a class II allele could encode a protein with an alteration in a different region (see below) required for nudel to trigger the protease cascade that produces the Toll ligand. Because the mutant proteins retain different activities of nudel, they can compensate each other to provide full *nudel* function. The organization of nudel into functional domains is perhaps not surprising, since the protein is structurally modular.

Our preliminary molecular analyses of *nudel* mutations support the idea that nudel is a functionally modular protein. Three of the four class II alleles have point mutations in the central protease domain, which we earlier proposed functions to trigger the protease cascade producing the Toll ligand (Figure 3A; HONG and HASHIMOTO 1995). The changes caused by these point mutations are likely to result in proteins with either strongly or moderately reduced protease activity and therefore in embryos lacking normal dorsoventral polarity. For example, the ndl^{111} mutation, which is predicted to encode a protein with significantly impaired protease activity, causes the production of completely dorsalized embryos. Because the ndl^{111} mutation does not lead to obvious egg fragility, the protease activity of nudel appears to be primarily required for embryonic dorsoventral polarity and not for structural integrity of the egg.

The fourth class II allele, ndl^{9}, does not have a mutation in the protease domain. It may have a mutation in

one of the other motifs of nudel, the protease-like domain or the LDL-receptor type A repeat, that we have proposed is used to bind protease zymogens (HONG and HASHIMOTO 1995). A mutation in one of these motifs could prevent formation of the zymogen activation complex necessary for local activation of the protease cascade producing the Toll ligand. The *ndl*^{9} mutation primarily disrupts embryonic dorsoventral polarity and only mildly disturbs structural integrity of the egg. Thus, the observation that this mutation maps outside of the protease domain supports the idea that multiple domains of the nudel protein are required to activate the protease cascade producing the Toll ligand (HONG and HASHIMOTO 1995).

The absence of the wild-type C-terminal region in the protein encoded by the class I allele *ndl*^{18} suggests that this region is required for the role of nudel in providing structural integrity to the egg (Figure 3B). The C-terminal region could represent a domain required specifically for the structural function of nudel. The missing C-terminal region contains one of the four blocks of LDL-receptor type A repeats in nudel, but we do not yet know whether this block of repeats or one of the flanking regions is important. Alternative explanations are that truncation of the C-terminal region alters the biogenesis, stability, or physical properties of nudel.

We have not yet determined the locations of mutations in the other class I alleles. However, the difference in complementation behavior between the class I alleles and the two class II alleles *ndl*^{46} and *ndl*^{9} raises the possibility that not all class I alleles have changes in the same part of the nudel protein (Table 3). Most class I alleles are complemented to varying degrees by *ndl*^{46}, yet only one class I allele, *ndl*^{15}, is complemented by *ndl*^{9}. This difference may indicate that the *ndl*^{15} mutation alters a different part of the nudel protein than the other class I mutations, which may map to a region overlapping the site of the *ndl*^{9} mutation.

Possible interactions of the nudel protein: Some interactions between *nudel* alleles that we have observed may also reflect interactions between nudel polypeptides. An example is complementation between *ndl*^{9} and *ndl*^{46} (Table 2). Both are class II alleles that produce dorsalized embryos as the primary mutant phenotype, implying that the proteins encoded by these alleles cannot activate the protease cascade that produces the Toll ligand. The protein encoded by *ndl*^{9}, which has a wild-type protease domain, may retain protease activity yet be unable to initiate the protease cascade because it lacks the functioning of a distinct protein domain required to bind protease zymogens within an activation complex. In contrast, the protein encoded by *ndl*^{46}, having a mutant protease domain, could have the normal ability to bind protease zymogens but diminished ability to initiate the protease cascade. Normal *nudel* function could be reconstituted if the two mutant proteins dimerize. Dimerization could also be important for activating the protease activity of nudel, which is predicted to require proteolytic cleavage (HONG and HASHIMOTO 1995). Since zymogen forms of serine proteases have low intrinsic proteolytic activity, nudel has the potential to become auto-activated by cleavage at the N-terminus of the protease domain (GERTLER *et al.* 1974; HONG and HASHIMOTO 1995). Auto-activation could be achieved by dimerization, in which the protease domain of one nudel polypeptide cleaves another nudel polypeptide. Dimerization could alternatively be important for cell secretion of nudel, as appears to be the case for factor XI, a serine protease of human blood clotting (MEIJERS *et al.* 1992).

The possibility that the nudel protein dimerizes is also consistent with the negative or antimorphic effects of the class II alleles *ndl*^{111} and *ndl*^{260} on the two other class II alleles, *ndl*^{9} and *ndl*^{46} (Table 2). The protease domains of the proteins encoded by *ndl*^{111} and *ndl*^{260} have mutations that are likely to reduce proteolytic activity signficantly (Figure 3A). The *ndl*^{111} and *ndl*^{260} polypeptides could therefore complex with the *ndl*^{9} and *ndl*^{46} polypeptides to form dimers that fail to auto-activate the protease activity of nudel. An alternative possibility is that the *ndl*^{111} and *ndl*^{260} polypeptides form inactive complexes with the substrate of nudel.

Threshold of *nudel* activity: The allelic combinations *ndl*^{46}/*ndl*^{11} and *ndl*^{46}/*ndl*^{18} produce mostly wild-type and completely dorsalized embryos yet few weakly or moderately dorsalized embryos (Figure 2B). Our interpretation of this unusual biphasic distribution of phenotypes is that the level of *nudel* activity in the *ndl*^{46}/*ndl*^{11} and *ndl*^{46}/*ndl*^{18} mutants is often sufficient to overcome a threshold required for normal dorsoventral polarity. However, when *nudel* activity is below this threshold, embryos develop no polarity rather than diminished polarity. The threshold may be close to the level of *nudel* activity provided at permissive temperature by the *ndl*^{46}/*ndl*^{46} or *ndl*^{46}/*Df* mutant (Figure 2A), which produces a much lower percentage of embryos with a normal dorsoventral pattern than either the *ndl*^{46}/*ndl*^{11} or *ndl*^{46}/*ndl*^{18} mutant. Activation thresholds appear to be intrinsic to enzymatic cascades in which the enzymes are subject to inhibitory control (BELTRAMI and JESTY 1995). Thus, in mammalian blood clotting, the stimulus that initiates the protease cascade leading to clot formation must be at a sufficient level for protease activation to exceed inactivation. By analogy, the threshold of *nudel* activity that we have described may reflect the role of nudel as the trigger for the protease cascade involved in inducing embryonic dorsoventral polarity.

We thank LYNN COOLEY, ELLEN LEMOSY, and TIAN XU for helpful comments on the manuscript and HENRY TAN for excellent photography. This work was supported by grant GM-49370 from the National Institutes of Health.

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Communicating editor: T. SCHÜPBACH