Activation of the easter zymogen is regulated by five other genes to define dorsal-ventral polarity in the Drosophila embryo

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Summary

The product of the Drosophila easter gene, a member of the trypsin family of serine proteases, must be more active ventrally than dorsally to promote normal embryonic polarity. The majority of the easter protein in the embryo is present in the unprocessed zymogen form and appears to be evenly distributed in the extracellular space, indicating that the asymmetric activity of wild-type easter must arise post-translationally. A dominant mutant form of easter that does not require cleavage of the zymogen for activity (eaAN) is active both dorsally and ventrally. The eaAN mutant bypasses the requirement for five other maternal effect genes, indicating that these five genes exert their effects on dorsal-ventral patterning solely by controlling the activation of the easter zymogen. We propose that dorsal-ventral asymmetry is initiated by a ventrally-localized molecule in the vitelline membrane that nucleates an easter zymogen activation complex, leading to the production of ventrally active easter enzyme.

Key words: easter, dorsal-ventral patterning, Drosophila, serine protease, zymogen.

Introduction

The products of 11 maternally expressed genes required for the development of lateral and ventral structures of the Drosophila larva act by creating a ventral to dorsal gradient of the dorsal protein in nuclei of the blastoderm embryo (Steward et al., 1988; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). These dorsal group genes appear to encode components of a signal transduction pathway that relays information from an external asymmetric cue to the cytoplasm to promote the graded translocation of dorsal protein from the cytoplasm to the nucleus.

Genetic experiments have defined a flow of information among the dorsal group genes (Anderson et al., 1985; P. Hecht, D. Morisato, Y. J. and K. V. A., unpublished data). The Toll gene, which acts upstream of dorsal, encodes a transmembrane protein that is uniformly distributed in the plasma membrane of the early embryo (Hashimoto et al., 1988, 1991). Seven of the dorsal group genes act upstream of Toll. Recent data indicate that all seven of these genes are necessary for the asymmetric activation of the Toll protein, apparently by producing a ventrally localized ligand for Toll (Stein et al., 1991; Stein and Nüsslein-Volhard, 1992). Three of these genes, pipe, nudel, and windbeutel, are somatic-dependent (Stein et al., 1991; Manseau and Schüpbach, 1989); they are probably expressed during oogenesis in follicle cells, which secrete the eggshell. The other four genes, easter, snake, gastrulation defective and spätzle, are germline-dependent (Stein et al., 1991; Seifert et al., 1987; Konrad et al., 1988) and encode proteins that are apparently translated from maternal RNAs in the early embryo and secreted to the perivitelline space between the eggshell and the plasma membrane (Fig. 1; DeLotto and Spierer, 1986; Konrad and Marsh, 1990; D. Morisato and K. V. A., unpublished). Both easter and snake have significant structural similarity to extracellular serine proteases of the trypsin family (DeLotto and Spierer, 1986; Chasan and Anderson, 1989).

It is not known how the products of the seven genes upstream of Toll act together to generate a ventrally-localized Toll ligand, but asymmetric easter activity appears to be essential for localized ligand production. Females carrying EMS-induced dominant alleles of easter produce ventralized or lateralized embryos in which lateral structures are expanded at the expense of dorsal structures (Chasan and Anderson, 1989; Jin and Anderson, 1990). These dominant easter alleles, which are caused by mis-sense mutations in the catalytic domain (Jin and Anderson, 1990), exert their effects by causing a more uniform distribution of nuclear dorsal protein (Steward, 1989). From these results we have
of the role of zymogen cleavage by constructing a mutant form with any activity, which implies that zymogen cleavage requires a normal zymogen activation site to become spatially asymmetric. By analogy to other serine proteases (Chasan and Anderson, 1989), including a gene has all the features of the trypsin family of serine proteases (Chasan and Anderson, 1989). This precedent raises activity that would need to be proteolytically cleaved at this site to be catalytically active. The activities of many proteases, such as those of the blood coagulation cascade, are regulated by cleavage of the zymogen (Furie and Furie, 1988). This precedent raises the possibility that activity might be spatially regulated by asymmetric zymogen cleavage.

In this report, we investigate the role of zymogen processing in the regulation of activity. Most of the protein in the embryonic extracellular space is in the unprocessed zymogen form. However both wild-type easter and the EMS-induced dominant easter allele products require a normal zymogen activation site to have any activity, which implies that zymogen cleavage is a necessary step in activation of both the wild type and mis-sense dominant proteases. We also studied the role of zymogen cleavage by constructing a mutant form of the easter protein that lacks the amino-terminal domain. This mutant, which should be active without zymogen cleavage, has a dominant phenotype like that of the EMS-induced dominant alleles: it promotes the development of lateral structures, but does not promote dorsal-ventral asymmetry. Using this mutant, we show that five of the six other genes upstream of Toll exert their effects on dorsal-ventral patterning solely through the activation of the easter zymogen. These results suggest that spatial regulation of easter's activity by localized zymogen activation is a key initial event in defining the polarity of the dorsal-ventral embryonic pattern.

Materials and methods

Stocks and mutant phenotypes

The EMS-induced dominant alleles of easter have been described (Chasan and Anderson, 1989; Jin and Anderson, 1990; Erdélyi and Szabó, 1989). Most recessive dorsal group alleles are described in Tearle and Nüsslein-Volhard (1987) or Schüpbach and Wieschaus (1989). The windbeutel alleles wbr98 and wbf46, the kind gift of T. Schüpbach, are strongly dorsalizing. Df (3R) ea^831~1253 and Df (3R) ea^5022~x1 are deficiencies including the easter locus, isolated as phenotypic revertants of dominant easter alleles (Chasan and Anderson, 1989; Erdélyi and Szabó, 1989; Chasan, 1991). These embryos were produced by ea^+/ea^5022~x1 females; these embryos have no detectable easter mRNA (Chasan, 1991). The phenotypes of mutant embryos were evaluated in the pattern of gastrulation and in the structures of the differentiated cuticle (Wieschaus and Nüsslein-Volhard, 1986).

Production of antibodies

Anti-easter antibodies were obtained by immunizing rats with
a trpE-easter fusion protein (Dieckman and Tzagoloff, 1985) containing the entire easter protein coding region except for the first four amino acids, which are part of the predicted signal peptide. The fusion protein was purified by electrophoresis from preparative SDS-PAGE gels. Rats were injected with 80-100 mg of fusion protein emulsified in synthetic adjuvant (MPL + TDM; RIBI Immunocore) for both initial injections and boosts. Antisera were affinity-purified (Harlow and Lane, 1988) on columns of the trpE-easter fusion protein bound to Affigel 10/15 (Drieve and Nüsslein-Volhard, 1988).

Preparation and analysis of Drosophila embryo extracts

Whole embryo extracts were prepared as described (Hashimoto et al., 1991). Perivitelline fluid was removed by micropipetting as described (Stein et al., 1991) from embryos laid by Toll females. Perivitelline extracts were prepared by vortexing embryos in buffer with silicone carbide particles, which preferentially releases the contents of the extracellular, perivitelline space (Jin, 1991). We have found that ~25% of the total easter protein is released into the perivitelline extracts and that easter is ~25-fold enriched in perivitelline compared to total embryonic extracts. Extracts were electrophoretically separated on SDS-PAGE gels under reducing conditions and transferred to nitrocellulose for western blot analysis (Towbin et al., 1979). Only an easter protein the size of the unprocessed zymogen was seen on western blots (Fig. 1 and data not shown) or by immunoprecipitation of in vivo labeled easter protein (Chasan, 1991).

Site-directed mutagenesis and assays for the activity of mutant alleles

Site-directed mutagenesis was performed as described (Jin and Anderson, 1990). All mutations were confirmed by sequencing (Sanger et al., 1977).

The primers used for easter zymogen activation site mutagenesis were: the Arg-127 to Gln: 5'TGTCGAATCA-3'; the Arg-127 to Leu: 5'CTTTCGAATCT-3'; and the Arg-127 to Gin: 5'TGTCGAATCA-3'. The cDNAs were subcloned into the 5' part of the wild-type genomic DNA (Jin and Anderson, 1990) in Bluescript (Stratagene) to replace the corresponding fragment of the cDNA carrying the zymogen site mutation with the corresponding fragment of the dominant allele genomic DNA. The serine-338 to alanine mutation was constructed previously (Jin and Anderson, 1990) including the 5'AGGCAGGATATCGGTCGCAC3' fragment of the dominant allele genomic DNA. The trpE-easter open reading frame (Chasan and Anderson, 1989) and the 5' part of the primer hybridize to the sequence encoding the first four amino acids of the catalytic domain (YGGY) and the 15 nucleotides in the 3' part of the primer hybridize to the sequence encoding the last five amino acids of the predicted signal peptide (KSSAG) (Chasan and Anderson, 1989). The template, which was cloned in the vector pGEM-7Zf(+)(Promega Biotech), had been isolated in a separate mutagenesis experiment and contained a deletion within the N-terminal region, which reduced the size of the region to be looped out. By analogy with other serine proteases, the cysteine at position 260 in the catalytic domain would form a disulfide bond with a cysteine residue in the N-terminal domain (Chasan and Anderson, 1989). To eliminate this unpaired cysteine, it was changed to a serine in a second mutagenesis. This mutagenic primer had the sequence 5'AGGCAGGGATATCGGTCGCAC3' .

For transcript injection assays, the templates were linearized and capped SP6 transcripts were made essentially as described (Krieg and Melton, 1987). Injections were as previously described (Chasan and Anderson, 1989).

Construction of stocks carrying dominant easter and recessive dorsal group mutations

Double mutants of dominant easter (ea<sup>D</sup>) alleles with dorsal, gastrulation defective and windbeutel were constructed by ordinary crosses. To make third chromosomes that carried both ea<sup>D</sup> and a recessive dorsal group mutation, heterozygous larvae were irradiated as described (Anderson et al., 1985) to produce X-ray induced mitotic recombinants in the male germ line. Recombinants were initially identified on the basis of the exchange of flanking markers and confirmed by testing for maternal effect phenotypes. The double mutants of ea<sup>D</sup> and ea<sup>R</sup> and recessive dorsalizing alleles were constructed using one of two dominant suppressors, TF<sup>26</sup> or a suppressor on the second chromosome (Y. J. and K. V. A., unpublished). TF<sup>26</sup> is an incompletely penetrant dominant dorsalizing allele of Toll that acts specifically as a dominant suppressor of dominant easter ventralizing mutations. From the progeny of females heterozygous for TF<sup>26</sup> and ea<sup>D</sup> or ea<sup>R</sup>, recombinants were recovered that carried TF<sup>26</sup> and the ea<sup>D</sup> allele on the same chromosome. To construct double mutant chromosomes of other third chromosomal dorsal group alleles with these ea<sup>D</sup> alleles, double recombinant progeny from ea<sup>D</sup> TF<sup>26</sup>/dorsal group allele females that retained the ea<sup>D</sup> allele, lost TF<sup>26</sup> and gained the other dorsal group allele were identified by marker exchange. Recombinant lines were tested for the presence of the desired maternal effect mutations in test crosses. The second chromosomal suppressor was used in a similar manner to construct some of the double mutants with ea<sup>R</sup>.

Results

The zymogen form of the easter protein is present in the perivitelline space

Although the phenotypes caused by the dominant alleles indicate that easter activity must be dorsoventrally asymmetric in the embryo, the easter protein appears to be uniformly synthesized in the early embryo, since both the easter transcript (Jin, 1991) and the newly synthesized easter protein (Chasan, 1991) appear to be uniformly distributed in the blastoderm embryo. The N-terminal signal sequence in the easter open reading frame (Chasan and Anderson, 1989) and the presence of easter-rescuing activity in the perivitelline fluid (Stein and Nüsslein-Volhard, 1992) suggested that the easter protein is secreted from the embryo into the extracellular perivitelline fluid that lies between the plasma membrane and the vitelline layer of the eggshell. Conventional techniques for antibody staining of Drosophila embryos require removal of the vitelline membrane and would cause the loss of proteins that are soluble in the perivitelline space. We were able, however, to confirm the presence of easter protein in
this compartment by micropipetting perivitelline fluid out of embryos (Stein et al., 1991), and assaying for easter protein on western blots (Fig. 1). These experiments showed that the easter protein was soluble and therefore presumably evenly distributed in the perivitelline fluid.

Only a $50 \times 10^3 M_r$ protein, which corresponds to the full-length unprocessed zymogen form of the easter protein, was detected in whole embryo or perivitelline fluid extracts from wild type or dominant mutant females (Fig. 1 and data not shown). The size of the protein is somewhat larger than the predicted size ($41 \times 10^3 M_r$) of the easter zymogen (Chasan and Anderson, 1989), but is the same size as the protein made by in vitro translation and translocation using SP6 transcripts of the easter cDNA (Chasan, 1991). Cleavage of the zymogen at a defined site at the N-terminal end of the catalytic domain (Chasan and Anderson, 1989) would yield two smaller polypeptides, but two smaller bands that would correspond to the cleaved, activated easter protein were not detected on reducing and denaturing gels. We estimate that if a processed form of easter is present, it constitutes less than 10% of the steady-state amount of easter protein.

Wild-type easter requires a normal zymogen activation site for activity

Zymogen activation is a key step in allowing serine protease activity (Stroud et al., 1977). However, some serine proteases, such as human tissue plasminogen activator, have appreciable protease activity in the zymogen form (Tate et al., 1987). Because we were able to detect only the zymogen form of easter in embryos, it was important to test whether zymogen cleavage was required for wild-type easter activity.

Based on homology with other members of the trypsin family, activation of the easter zymogen should occur by a cleavage after the arginine-127 preceding the IYGG of the catalytic domain (Chasan and Anderson, 1989), suggesting that the enzyme that activates easter cleaves after basic residues. We used site-directed mutagenesis to change arginine-127 to glutamine or leucine, both of which are similar to arginine in size, but lack the basic group. Unlike mutants at other positions in the cleavage site, these mutants should not disrupt protease activity of the zymogen, because a similar mutant blocked normal processing but did not destroy protease activity of the zymogen form of tissue plasminogen activator (Tate et al., 1987). Transcripts of the wild-type easter cDNA fully rescue the dorsalized phenotype of embryos produced by easter females (Chasan and Anderson, 1989). In contrast, transcripts of an easter cDNA that encoded either glutamine or leucine at position 127 had no activity when injected into either wild-type embryos or easter embryos (Table 1). Thus, changing arginine-127 to glutamine or leucine abolished easter activity completely, suggesting that, even though we have not detected a cleaved form of easter biochemically, the wild-type easter is active only after zymogen cleavage.

The products of the EMS-induced dominant alleles act as processed proteases

The phenotypes of the dominant easter alleles indicate that the activities of these alleles are more spatially uniform than the activity of the wild-type easter. To understand how easter activity is normally spatially regulated, it is important to define the aspect of easter activity that is altered in the dominant alleles. We therefore carried out a series of genetic experiments to test whether the dominant alleles differ from the wild-type allele in their requirements for protease function, zymogen processing and the other dorsal group gene products for activity. Although the sequence of the easter protein suggests that it acts as a protease, it is possible that the easter

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>ea$^-$</th>
<th>wild-type</th>
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<tbody>
<tr>
<td></td>
<td>n$_g$, n$_c$</td>
<td>dorsalized at gastrulation</td>
</tr>
<tr>
<td>wild type (Arg$^{127}$)</td>
<td>23,23</td>
<td>0</td>
</tr>
<tr>
<td>Gln$^{127}$</td>
<td>88,17</td>
<td>88</td>
</tr>
<tr>
<td>Gln$^{127}$-ea$^{80}$</td>
<td>53,10</td>
<td>53</td>
</tr>
<tr>
<td>Gln$^{127}$-ea$^{13}$</td>
<td>49,7</td>
<td>49</td>
</tr>
<tr>
<td>Leu$^{127}$</td>
<td>73,12</td>
<td>73</td>
</tr>
<tr>
<td>Leu$^{127}$-ea$^{128}$</td>
<td>80,3</td>
<td>80</td>
</tr>
<tr>
<td>Leu$^{127}$-ea$^{13}$</td>
<td>76,11</td>
<td>76</td>
</tr>
</tbody>
</table>

Transcripts of cDNAs containing zymogen activation site mutations were assayed by injection into ea$^-$ or wild-type embryos. The wild-type transcript (Arg$^{127}$) always rescued ea$^-$ embryos both at gastrulation and in the pattern of the differentiated cuticle and had no dominant effect when injected into wild-type embryos. The zymogen site mutations destroyed the ability of transcripts to rescue the dorsalized phenotype when injected into ea$^-$ embryos and blocked the dominant activity of the dominant alleles when injected into wild-type embryos. Gln$^{127}$ and Leu$^{127}$ transcripts carry the zymogen site mutations in an otherwise wild-type cDNA. Leu$^{127}$-ea$^{128}$ and Leu$^{127}$-ea$^{13}$ transcripts contain the Arg to Gln zymogen activation site mutation and the nucleotide change in the lateralizing ea$^{13}$ or the ventralizing ea$^{128}$. Gln$^{127}$-ea$^{128}$ and Gln$^{127}$-ea$^{13}$ transcripts contain the Arg to Gln zymogen activation site mutation and the nucleotide change in the lateralizing ea$^{13}$ or the ventralizing ea$^{128}$. n$_g$: number of embryos scored at gastrulation. n$_c$: number of embryos scored in the pattern of the differentiated cuticle.
protein has additional functions and that the dominant mutations alter an aspect of easter function that is independent of protease activity. To test this hypothesis, we constructed an intragenic double mutant, ea831-ala338. This mutant contains both the point mutation in the dominant ventralizing allele ea831 and an alanine residue in place of the active site serine-338 (Jin and Anderson, 1990). The serine-338 to alanine change, which is a conservative amino acid replacement that should abolish protease activity without affecting protein structure, destroyed the activity of the wild-type easter (Jin and Anderson, 1990). When the ea831-ala338 genomic DNA was introduced into the genome by P-element mediated transformation, western blot analysis showed that it produced a stable full-length protein (data not shown). However, this protein was unable to promote the production of any ventral or lateral structures in an easter- background. The double mutant allele also had no dominant effect: females carrying the ea831-ala338 allele in the presence of one or two copies of ea+ produced 100% hatching embryos. Thus, all activity of ea831 absolutely requires the active site serine, and the dominant activity of the allele most probably reflects a change in the activity of the protease.

Since thezymogen form of easter is spatially uniform in its distribution, the more spatially uniform activity of the dominant easter alleles could be accounted for if the dominant proteins have some activity in the zymogen form. To test the activity of the zymogen form of the dominant alleles, we constructed cDNAs that contain both an amino acid change causing a dominant phenotype and a mutation at the zymogen activation site. Transcripts containing the point mutations that cause the dominant easter mutations produce ventralized or lateralized embryos when injected into young wild-type or ea- embryos (Jin and Anderson, 1990). Transcripts from cDNAs containing both the arg-127-to-leucine or arg-127-to-glutamine in addition to one of the mutations causing the dominant alleles ea1253 or ea1253 did not rescue any lateral or ventral structures when injected into easter+ embryos, nor did they alter the dorsal-ventral pattern of wild-type embryos (Table 1). Because all activity of the dominant easter alleles was lost when the zymogen activation site was mutated, we conclude that, like the wild-type allele, the dominant alleles require zymogen cleavage to have any activity.

To help understand how the EMS-induced dominant alleles escape normal spatial regulation, we examined the phenotypes of double mutants of dominant easter alleles with mutations in other dorsal group genes. Double mutants of dominant ventralizing or lateralizing easter alleles and recessive strongly dorsalizing alleles at the other ten dorsal group loci were all strongly dorsalized, indicating that the dominant alleles do not act by bypassing a regulatory step imposed by one of the other dorsal group genes (Table 2).

From the site-directed mutagenesis experiments and the analysis of double mutant phenotypes, we conclude that the EMS-induced dominant easter alleles are similar to wild-type easter in a number of respects. Both the wild-type and dominant alleles require the active site serine and a normal zymogen processing site for any activity. In addition, all activity of both wild type and dominant easter alleles depends absolutely on the activity of all other dorsal group genes.

A form of easter that does not require zymogen cleavage is a dominant lateralizing allele

To investigate directly whether zymogen cleavage is a regulated step in the control of easter activity, we used site-directed mutagenesis to delete the amino-terminal domain in an easter cDNA (Fig. 2). In the N-terminal deletion mutant, which we term eaAN, the signal sequence is followed directly by the catalytic domain. This mutant form of easter should be secreted to the perivitelline space and cleavage by signal peptidase should release the active C-terminal catalytic domain, bypassing the normal requirement for zymogen cleavage. Similar mutant forms of trypsin and of tissue plasminogen activator retain protease activity (Vasquez et al., 1989; MacDonald et al., 1986).

To assay the activity of the eaAN mutant, we injected in vitro-synthesized transcripts into embryos from easter- females. As Fig. 3 shows, transcripts of the eaAN cDNA had biological activity, promoting the production of lateral structures never made in un.injected easter- embryos. The rescued pattern, however, lacked dorsal-ventral asymmetry. The pattern of gastrulation was like that of lateralized embryos: both ventral and dorsal pattern elements are absent and all cells behave like the normal lateral cells (Anderson et al., 1985). The cephalic furrow, which begins midlaterally in the wild-type embryo, initiated equally at all dorsal-ventral positions, and neither dorsal folds nor a ventral furrow formed. Also like lateralized embryos, the injected embryos differentiated laterally derived ven-

### Table 2. Phenotypes of dominant easter alleles in combination with strongly dorsalizing alleles at other loci

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>ndl</th>
<th>pip</th>
<th>wbl</th>
<th>gd</th>
<th>snk</th>
<th>spz</th>
<th>Tl</th>
<th>pfl</th>
<th>tub</th>
<th>dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ea213</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
</tr>
<tr>
<td>ea831</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
</tr>
<tr>
<td>ea1253</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
<td>D0</td>
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<td>D0</td>
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<td>D0</td>
<td>D0</td>
<td>D0</td>
</tr>
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</table>

All double mutant combinations tested were strongly dorsalized. The dominant alleles alone have a lateralizing (ea213), moderately ventralizing (ea831), or weakly ventralizing (ea1253) phenotype. Each combination of recessive alleles alone produces a strongly dorsalizing (D0) phenotype. The recessive alleles used were: TTR(s/b)SPReq (with ea213); TR(s/b)TT(s/b)SP (with ea831 and ea1253); spf(b)/spf(b); pfl/b/pfl/b; tub/til/tub/til; snk/b/snake (with ea213); ndll/ndll (with ea831); ndll/ndll (with ea1253); gd/gd; dl/dl; snk/b/3D; (3R)y/y and snk/b/3D; (3R)y/y (with ea213); snk/b/snake (with ea831); wbl/wbl/wbl/wbl; snk/b/y.
first amino acid of the catalytic domain (Chasan and Anderson, 1991). In addition, the cysteine in the catalytic domain that should form a disulfide bond with a cysteine in the N-terminal domain was changed to a serine to eliminate the unpaired cysteine.

...
it should be active in the absence of the gene products necessary for regulation. We therefore assayed eaΔN activity in embryos from females lacking the function of each of the six dorsal group genes besides easter that had been defined as genetically upstream of Toll. As shown in Table 3 and Fig. 4, eaΔN transcripts elicited the production of lateral structures in embryos from females mutant for pipe, nudel, windbeutel, gastrulation defective, and snake. Recipient embryos were lateralized and were indistinguishable from easter+ embryos injected with the same transcripts. Thus, unlike the EMS-induced dominant alleles, the eaΔN allele does not require these five dorsal group genes for activity. The epistasis of eaΔN over these genes shows that these five genes act genetically upstream of easter and are required for activation of the easter zymogen.

Among the genes upstream of Toll, the spätzle gene was unique in this epistasis assay. Embryos from spätzle+ females remained dorsalized when injected with the eaΔN transcript, indicating that even in the presence of the preactivated easter, the spätzle product is still needed for the activity of Toll and of the subsequent steps of the dorsal-ventral pathway.

**Discussion**

Although the easter protein appears to be uniformly distributed in the embryonic perivitelline space, the phenotypes of the previously described EMS-induced dominant alleles and of the eaΔN mutant described here argue strongly that a wild-type dorsal-ventral pattern can form only if easter activity is spatially asymmetric. Our studies on the two classes of dominant alleles allow us to infer how easter activity is confined to ventral regions of the wild-type embryo.

The point dominant mutations redistribute the activity of the processed protease

Each of the nine EMS-induced dominant mutations is caused by a single mis-sense mutation at a conserved site in the catalytic domain (Jin and Anderson, 1990).
would not require activation by zymogen cleavage of the protease by cleavage of the zymogen site, and we assume that only a small fraction of the total zymogen protein in the embryo is the only dorsal group gene that requires zymogen cleavage, abolishes normal spatial regulation of activity.

Unlike the EMS-induced dominant alleles, the eaAN mutant is epistatic to several dorsal group genes. This made it possible to order partially the function of the dorsal group genes upstream of Toll. Five of these dorsal group genes, the somatic-dependent genes pipe, nudel, and windbeutel and the germ line-dependent genes snake and gastrulation defective, are not required for the production of lateral structures in the presence of the eaAN protein, and therefore act upstream of wild-type easter to promote its activity. Since all five of these genes are required for activation of easter and are bypassed by a mutant form of easter that does not require zymogen activation, we infer that these five genes are required, directly or indirectly, for cleavage and activation of the easter zymogen.

Generating an asymmetric ligand for Toll
Recent data indicate that the products of the seven dorsal group genes upstream of Toll all act to produce a spatially localized ligand for the Toll protein, but these data do not order the activities of these genes or establish which of those genes could encode the Toll ligand (Stein et al., 1991). Our results show that pipe, nudel, windbeutel, snake and gastrulation defective exert their effects on dorsal-ventral patterning solely through the regulation of easter activity, and therefore none of these genes encodes a ligand that binds to and activates Toll. Because spaetzle is the only dorsal group gene upstream of Toll that is required for eaAN to exert its effect on the dorsal-ventral pattern, easter and spaetzle are required most directly to activate Toll. The easter protein and an activity that rescues the spaetzle phenotype both appear to be initially uniformly distributed in the perivitelline space (Fig. 1; Stein and Nüsslein-Volhard, 1992). One attractive hypothesis that would explain how a Toll ligand is asymmetrically produced is that asymmetrically active easter protease cleaves the spaetzle protein, and cleaved spaetzle protein then binds to and activates the Toll protein.

Models for localization of easter activity
Because asymmetric easter activity is crucial for generating a localized Toll ligand, and because the easter zymogen is uniformly distributed, it is crucial to understand how other gene products act post-translationally to confine easter activity to the ventral part of the embryo. The dominant easter alleles provide powerful tools to dissect that spatial regulation. Two very different kinds of mutations in easter disrupt the dorsal-ventral asymmetry of easter activity: the eaAN mutant lacks the amino-terminal domain and does not require activation of easter zymogen cleavage because the N-terminal domain was deleted. The eaAN mutant promotes the development of lateral structures, indicating that the mutant retains easter protease activity and normal substrate specificity. However, in contrast to wild-type easter, which appears to be active only ventrally, the eaAN mutant is equally active at all dorsal-ventral positions. Thus deletion of the amino-terminal domain, thereby bypassing the requirement for zymogen cleavage, abolishes normal spatial regulation of activity.

To help determine how these alleles lead to a change in the distribution of easter activity, we carried out experiments to test whether the products of the dominant alleles act as processed proteases.

We found that, like wild-type easter, the ventralizing eaAN allele absolutely requires the active site serine for activity. In addition, the dominant alleles eaAN and ea125.3 require a normal zymogen activation site. Like wild-type easter, these dominant alleles depend on the activity of all other dorsal group gene products to affect the dorsal-ventral pattern, indicating that these alleles do not bypass a regulatory step imposed by any of the known dorsal group genes. Our data suggest that both the wild-type and dominant forms of easter act as processed proteases and that the ventralizing and lateralizing effects of the dominant alleles are caused by a more uniform spatial distribution of the activity of the processed protease.

Cleavage of the easter zymogen is regulated by other dorsal group genes
The vast majority of the easter protein in the embryo is present as the unprocessed zymogen form. However, we have shown that the zymogen site, and we infer zymogen processing, is necessary for activity. Because we cannot detect processed easter, we assume that only a small fraction of the total easter protein in the embryo is in the catalytically active form. It seemed possible, therefore, that the production of the active easter protease by cleavage of the easter zymogen could be a rate-limiting, regulated step in dorsal-ventral patterning.

To assess the role of easter zymogen cleavage in dorsal-ventral patterning, we used site-directed mutagenesis to create a mutant form of easter, eaAN, that would not require activation by zymogen cleavage...
which could incorporate the products of the model, this ventrally localized molecule nucleates the vitelline membrane, perhaps the product of the so-

zymogen cleavage, while the EMS-induced dominant alleles require the normal machinery of zymogen activation as well as an intact zymogen activation site for activity.

Two classes of models of how easter activity is spatially regulated can explain how the dominant mutations alter the dorsoventral pattern. In the first model, the EMS-induced dominant alleles and the eaAN mutant both disrupt a single regulatory step. For instance, there could be a spatially localized activator or receptor that requires both the amino-terminal domain and part of the catalytic domain for binding to the easter protein. In the absence of the amino-terminal domain or in the presence of mis-sense mutations in critical positions of the catalytic domain, easter would somehow be active, independent of this regulator. Because the activities of the EMS-induced dominant alleles are dependent on all the known dorsal group genes, this regulator would have to be the product of some uncharacterized gene.

We prefer a second model in which the two kinds of dominant mutations disrupt two different steps in easter protease function (Fig. 5). This model does not require invoking the existence of unknown genes and explains why several genes are required for activation of the easter zymogen. As in the model proposed by Stein et al. (1991), embryonic dorsal-ventral asymmetry is triggered by a molecule localized ventrally in the vitelline membrane, perhaps the product of the somatic-dependent pipe, nudel, or windbeutel genes. In our model, this ventrally localized molecule nucleates the assembly of an easter zymogen activation complex, which could incorporate the products of the snake and/or gastrulation defective genes, on the ventral side of the vitelline membrane (Fig. 5). This complex could be analogous to the membrane-localized prothrombinase complex, which accelerates the rate of prothrombin activation 10²-fold (Furie and Furie, 1988; Krishnaswamy, 1990). Because the activation complex is localized, the uniformly distributed easter zymogen is activated only ventrally. Once the zymogen is cleaved, the activity of the wild-type easter must still be confined to ventral regions. Localization of protease activity could occur if the processed easter has a very short half-life and decays before it diffuses to the dorsal side of the embryo. Alternatively, the processed easter protease could remain bound ventrally after proteolytic processing.

In the second model, the event which initiates ventral easter activity is the localized activation of the easter zymogen. The eaAN mutant does not require zymogen activation and is therefore active everywhere. The products of the EMS-induced dominant alleles, in contrast, would be activated ventrally by the normal zymogen activation machinery, but would be altered in that property of the processed enzyme that normally restricts its activity to the ventral side. Because of greater stability of the processed protease or lower affinity of the processed protease for a ventral binding site, the active products of the EMS-induced dominant alleles would diffuse within the perivitelline space after zymogen cleavage and cut the easter substrate dorsally as well as ventrally.

After the ventrally active easter protease cleaves its substrate, the product of that proteolytic reaction must also remain ventral. If easter's substrate is the ligand that activates Toll, diffusion of the processed ligand away from the ventral side could be prevented by immediate binding of the ligand to Toll. The easter substrate would therefore not need to be prelocalized. If the soluble perivitelline rescuing activity for spätzle (Stein and Nüsslein-Volhard, 1992) is the spätzle gene product and if spätzle is easter's substrate and Toll's ligand, then the rescuing activity would be the unprocessed spätzle, while processed spätzle would be tightly bound to Toll on the ventral side of the embryo.

Thus it seems likely that dorsal-ventral polarity depends on localized activation of a receptor, Toll, in which neither receptor nor ligand, nor even the protease that activates the ligand, is prelocalized. Instead, localized activation of a protease zymogen initiates a cascade of protein interactions that directs the spatially coordinated response of a group of target cells.

We thank Chip Ferguson for help with the figures. We thank Chip Ferguson, Peter Hecht, Donald Morisato, other members of the Anderson laboratory and Jasper Rine for comments on the manuscript. This work was supported by grants from the National Institutes of Health (GM 35437) and the National Science Foundation (DCB 8452030) to K. V. A.

References


(Accepted 24 February 1992)